Université de Montréal

The role of pericytes in the regulation of retinal microvasculature dynamics in health and disease

Par

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«The role of pericytes in the regulation of retinal microvasculature dynamics in health and disease»

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Résumé

Les péricytes sont des cellules contractiles qui s'enroulent le long des parois des capillaires. Dans le cerveau, les péricytes jouent un rôle crucial dans la régulation du diamètre capillaire et du débit sanguin vasculaire en réponse à la demande métabolique. Au cours de l'ischémie, il a été suggéré que les péricytes pourraient resserrer les capillaires, le restant même après la reperfusion et entraîner une insuffisance du reflux microcirculatoire. Malgré cela, on sait peu de choses sur le rôle des péricytes dans les maladies du cerveau et leur contribution à la dynamique capillaire et au débit sanguin local. Afin de mieux comprendre la réponse des capillaires et des péricytes lors d'une lésion ischémique de la rétine et d'identifier les mécanismes moléculaires de la constriction capillaire induite par les péricytes, nous avons caractérisé la réponse des péricytes au cours d'une ischémie rétinienne transitoire *ex vivo* et *in vivo* et nous élucidons les mécanismes de contractilité du péricyte ischémie. Nous avons démontré que l'ischémie entraînait des modifications vasculaires anormales telles qu'une réduction générale du diamètre capillaire et une augmentation du nombre de constrictions capillaires à l'emplacement du péricyte, ce qui suggère que l'ischémie favorise une constriction rapide des péricytes sur les capillaires rétiniens, entraînant un dysfonctionnement microvasculaire majeur.

Mots-clés: Système visuel, rétine, couplage neurovasculaire, péricyte, capillaires, circulation sanguine, ischémie, déficience vasculaire, imagerie en direct, microscopie à 2 photons.

Summary

Pericytes are contractile cells that wrap along the walls of capillaries. In the brain, pericytes play a crucial role in the regulation of capillary diameter and vascular blood flow in response to metabolic demand. It has been suggested that, during brain ischemia, pericytes constrict the capillaries, which remain constricted even after reperfusion resulting in impaired microcirculatory blood flow. Despite this, little is known about the role of pericytes in brain and retinal diseases and their contribution to capillary dynamics and local blood flow. To better understand the response of capillaries and pericytes during ischemic injury in the retina, and to identify the molecular mechanisms of pericyte-mediated capillary constriction, we characterized the response of pericytes during transient retinal ischemia *ex vivo* and *in vivo*. We demonstrated that ischemia leads to anomalous microvascular changes characterized by a marked reduction in capillary diameter. This response was accompanied by an increase in the number of capillary constrictions at pericyte locations suggesting that ischemia promotes rapid pericyte constriction on retinal capillaries leading to microvascular dysfunction. Lastly, we show that ischemia increases intracellular calcium in pericytes suggesting that pericyte contraction leading to capillary constriction is a calcium-dependent process.

Key words: Visual system, retina, neurovascular coupling, pericyte, capillaries, blood flow, ischemia, vascular impairment, live imaging, 2-photon microscopy.

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List of abbreviations

AC	Amacrine cells
BC	Bipolar cells
BRB	Blood-retinal barrier
C	Cone
Ca^{2+}	Calcium
CB	Cone Bipolar cell
CBF	Cerebral blood flow
CNS	Central nervous system
CRA	Central retinal artery
CRAO	Central retinal artery occlusion
DR	Diabetic Retinopathy
GABAergic	Gamma-aminobutyric acid neurotransmitter
GC	Ganglion cell
GLC	Ganglion cell layer
HC	Horizontal cell
INL	Inner plexiform layer
IOP	Intraocular pressure
IPL	Inner plexiform layer
IS	Inner segment
MC	Müller cell

NAION	Non-arteritic ischemic optic neuropathy
NFL	Nerve fiber layer
NMDAR	N-methyl-d-aspartate receptor
NVC	Neurovascular coupling
NVU	Neurovascular unit
O_2	Oxygen
OFF	Off sublamina
ON	On sublamina
ONL	Outer nuclear layer
OPL	Outer plexiform layer
OS	Outer segment
Ph	Photoreceptor
R	Rod
RB	Rod bipolar cell
RGC	Retinal ganglion cell
ROI	Region of interest
RPE	Retinal pigment epithelium
SMC	Smooth muscle cell
TPLS	Two-photon laser scanning microscopy

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Al amor de mi vida, por creer en mi y enseñarme lo que es el amor verdadero

A mi amigo y compañero, Luis, por ser familia

CHAPTER 1

1. INTRODUCTION

1.1. THE RETINA

1.1.1. RETINAL STRUCTURE

The retina is a translucent tissue in the back of the eye comprising a complex neural circuit that converts the graded electrical activity of light-stimulated photoreceptors into action potentials that travel to the brain via axons in the optic nerve. As Ramón y Cajal described over 100 years ago, the retina consists of three layers of neurons: the outer nuclear layer (ONL), the inner nuclear layer (INL), and the ganglion cell layer (GCL), separated by two layers of synaptic connections: the outer plexiform (OPL) and the inner plexiform layer (IPL). The ONL contains the cell bodies of photoreceptors, the INL contains bipolar, horizontal and amacrine cells, and the GCL contains the retinal ganglion cells (RGC) and displaced amacrine cells. The photoreceptors (rods and cones) are adjacent to the retinal pigment epithelium, a single layer of pigmented cells that are vital to maintaining the photoreceptor health. Rods and cones capture light stimuli through specialized external segments filled with visual pigments that allow the transmission of electrical impulses to bipolar cells. The signal is modulated at the level of the OPL by horizontal cells, and further processed in the IPL by bipolar and amacrine cells, which interact with RGC dendrites. RGCs then relay light signals from the retina to the brain trough their axons in the optic nerve (Figure 1).

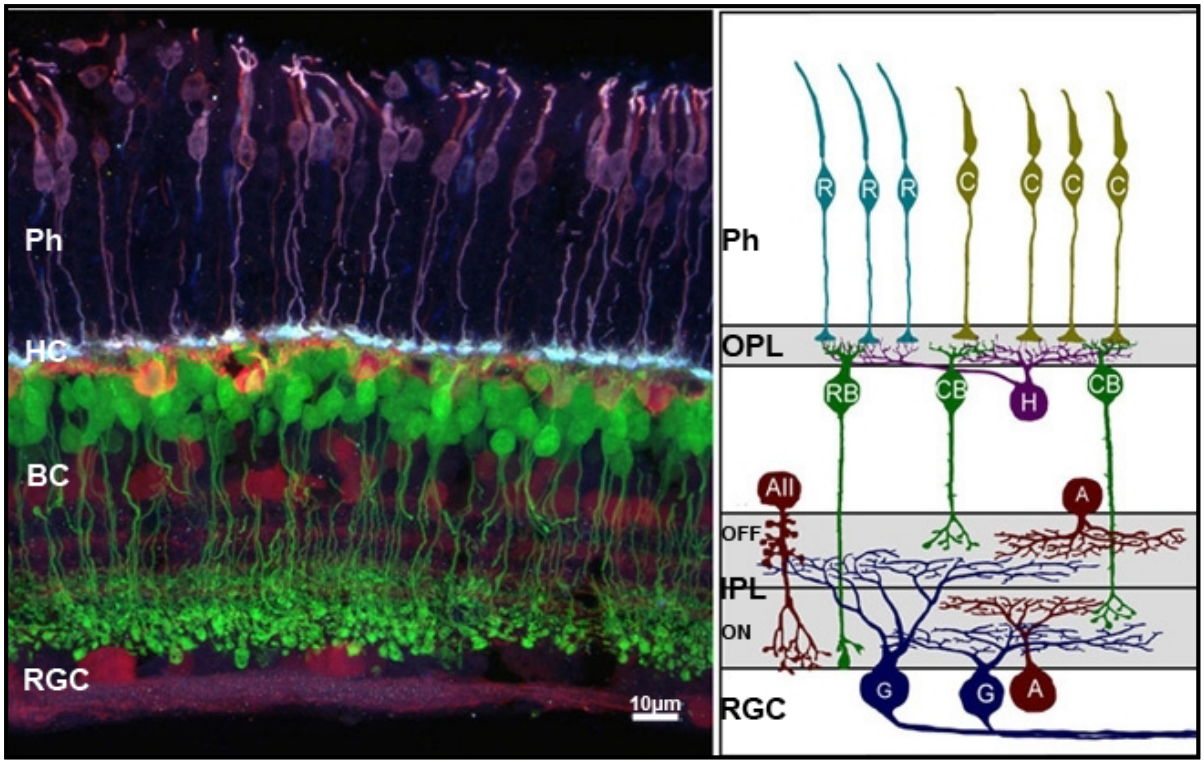

Figure 1. Retinal structure. (Left) Immunostaining of mouse retina showing the principal cell bodies and synaptic terminals. Photoreceptors (Ph) in purple/blue, bipolar cells (BC) in green, horizontal cells (HC) in yellow, amacrine cells (AC) and ganglion cells (RGC) in red. (Right) Schematic diagram showing the principal cells involved in retinal signaling. R, rod; C, cone; RB, rod bipolar cell; CB, cone bipolar cell; A, amacrine cell; G, ganglion cell; ON, ON sublamina; OFF, OFF sublamina; OPL, outer plexiform layer; IPL, inner plexiform layer. Modified from (Morgan and Wong, 2007).

1.1.2. CELL TYPES OF THE RETINA

Retinal Ganglion Cells (RGCs)

RGCs form one of the most complex information processing systems in the retina. Their soma is located in the ganglion cell layer while their dendrites extend into the IPL. These cells receive excitatory inputs (neurotransmitters) from bipolar cells, which integrate signals from photoreceptors and amacrine cells in the IPL. The output of visual processing in the retina, is projected to the brain, via the optic nerve, primarily to the lateral geniculate nucleus and also to secondary areas including the superior colliculus and hypothalamus. RGCs can be classified according to their morphology, synaptic connections, or light responses. For instance, RGCs depolarize and fire action potentials in response to either increased or reduced light intensity. Based on their responses, RGCs can be classified into ON, OFF and ON-OFF (Figure 1).

Amacrine Cells

Amacrine cell somata are located primarily in the INL, but displaced neurons are also present in the GCL (Jeon et al., 1998; Perry and Walker, 1980). Amacrine cells represent one of the most diverse cell types in the retina, with over 30 subtypes, and can be classified by their dendritic morphology, location, and neurotransmitter content (Masland, 2012; Vaney, 1990). Although the majority of amacrine cells are inhibitory interneurons, releasing the neurotransmitters GABA and glycine, some of them are cholinergic releasing the excitatory neurotransmitter acetylcholine, (Balasubramanian

and Gan, 2014; Taylor and Smith, 2012). Amacrine cells play diverse roles in visual integration, but their main role is to modify the output signals from bipolar cells before transmitting them to postsynaptic RGCs (Seung and Sümbül, 2014) (Figure 1).

Bipolar Cells

Bipolar cells are the only neurons that link the outer and inner retina and are, therefore, responsible for transmitting photoreceptor output to RGCs and amacrine cells. Bipolar cells are excitatory interneurons that establish synapses with photoreceptors and horizontal cells. Their axons contact RGCs and amacrine cells using glutamate as the main neurotransmitter system. Two distinct bipolar cell targets exist: rod bipolar cells and cone bipolar cells. In the mouse retina, many types of bipolar cells have been identified and can be readily characterized by their polarity (ON and OFF) and the time-course of their response (transient and sustained) (Euler et al., 2014; Seung and Sümbül, 2014). The ON and OFF pathways differ in response to changes in light stimulus and detect an increase or decrease in intensity, respectively (Figure 1).

Horizontal cells

Horizontal cell types vary depending on the species. Some animals have up to three cell subtypes, but most mammals have two types of horizontal cells (Masland, 2012). As a neuronal population, these cells appear to be relatively simple. Horizontal cells communicate laterally by gap junctions, generating a network across the OPL. The principal role of horizontal cells is to mediate communication between photoreceptors

and bipolar cells, while providing lateral inhibition to enhance the quality of the RGC signal (Herrmann et al., 2011) (Figure 1). Nonetheless, recent data demonstrate that horizontal cells can also play a role in global light adaptation in response to ON and OFF RGC diversity (Chaya et al., 2017).

Müller cells

Müller cells are the most abundant glial cell type in the retina and they play important functions due to their unique structure and position in this tissue. Müller cells extend apical processes to the outer limiting membrane, giving structural support, and basal processes (endfeet) surrounding RGCs that reach the inner limiting membrane. Müller cells regulate many aspects of retinal homeostasis including provision of trophic factors, communication between vessels and neurons, glucose retrieval from the circulation, glycogen processing and transfer of substrates such as lactate and pyruvate to neurons. Moreover, they are crucial for the maintenance and integrity of the blood–retinal barrier (BRB) (Forrester et al., 2016; Kolb, 2007) (Figure 1).

1.1.3. RETINAL VASCULATURE AND CHOROID

The human retina has the highest metabolic demand of all tissues in the body (Buttery et al., 1991). For this reason, the retina is highly vascularized and composed mainly of small vessels and capillaries, which are critical for oxygen and nutrient supply to neurons. The mammalian retina receives blood from two sources: the retinal and the

choroidal vasculature (i.e. choroid). The choroid derives from two different arteries, the long and the short posterior arteries. These vessels travel along the optic nerve, penetrate the sclera to enter the retinal space, and then ramify into arterioles and vessels, providing oxygen and nutrients to the retinal pigment epithelium and photoreceptors (Anand-Apte and Hollyfield, 2010). Drainage occurs via the vortex veins. On the other hand, the retinal vasculature derives from the central retinal artery (CRA). It ramifies into a capillary network, which supplies the inner retina and, therefore, connects with the venules and central vein artery (Grant and Lutty, 2015). The location of the CRA varies depending on the species. In humans, the CRA travels within the optic nerve. However, in rodents, it travels parallel to the optic nerve (Saint-Geniez and D'Amore, 2004). The interface between retinal blood vessels and their external milieu is commonly referred to as the blood-retinal-barrier (BRB). The BRB consists of interconnected endothelial cells covered with pericytes and glia, which allow the vessels to tightly regulate the passage of ions, molecules, and cells between the blood and the neural retina (Park et al., 2017). The BRB allows precise regulation of retinal hemostasis allowing proper retinal and choroidal function while protecting the neural tissue from toxins and pathogens. Pathological alterations of the BRB is an important component in the onset and progression of retinal diseases, including diabetic retinopathy (Díaz-Coránguez et al., 2017) (Figure 2A.).

1.1.4. VASCULAR PLEXUSES

In mammals, the retinal vasculature is constituted by three different plexuses classified according to the CRA's ramification and location. The vasculature in the ganglion cell layer (GCL) and the nerve fiber layer (NFL) is known as the superficial or inner plexus while the deeper plexus provides blood to the inner nuclear and the outer plexiform layer (Sun and Smith, 2018). The superficial plexus contains arterioles, venules and capillaries, whereas the deeper plexus is mainly formed by capillaries (Dorrell et al., 2007). This vascular organization is critical for proper retinal function, and its disruption leads to ocular diseases and visual loss (Kur et al., 2012) (Figure 2B).

1.1.5. NEUROVASCULAR UNIT

In the retina, the neurovascular unit (NVU) is a cellular complex formed by neurons, glial cells (astrocytes, Müller cells, microglia) and vascular cells (endothelial cells, vascular smooth muscle cells, pericytes). The neurovascular unit is essential for functional hyperemia, which is a vital process whereby the metabolic demand of active neurons is exquisitely matched by local blood flow to ensure availability of oxygen and nutrients (Winkler et al., 2011) (Figure 3&5). Vascular impairments play an important role in the etiology of many ocular diseases including ischemic optic neuropathy, age-related macular degeneration, diabetic retinopathy, glaucoma, and retinal venous occlusive disease. Despite the importance of the NVU, very little is known about the relationship between neuronal activity and blood flow in health and disease (Parkes et al., 2018).

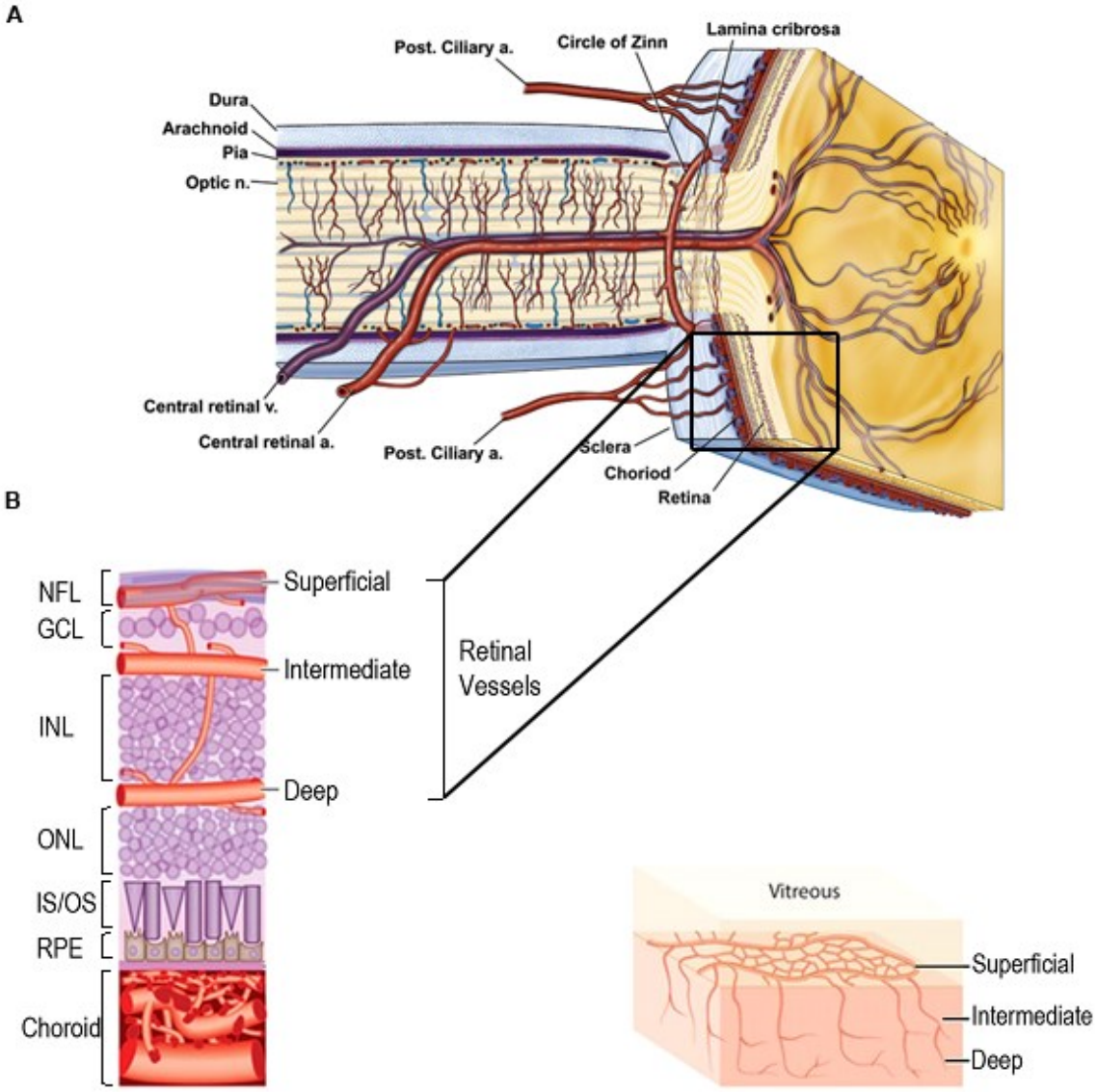
A
Post. Ciliary a.
Circle of Zinn
Lamina cribrosa
Dura
Arachnoid
Pia
Optic n.
Central retinal v.
Central retinal a.
Post. Ciliary a.
Sclera
Choriod
Retina
B
NFL
GCL
INL
ONL
IS/OS
RPE
Choroid
Superficial
Intermediate
Deep
Retinal Vessels
Vitreous
Superficial
Intermediate
Deep

Figure 2. Source of blood supply in the optic nerve and retina. (Superior) Drawing of ocular circulation showing vascular supply of the retina and choroid. The ophthalmic artery is divided into two branches, the central retina artery and the posterior ciliary artery. The central retina artery supplies the retina while the posterior ciliary artery supplies the choroid. Extracted from (Anand-Apte and Hollyfield, 2010). (Inferior) An enlarge view of the retinal and choroidal vasculature. On one hand, the retinal vessels are constituted by three interconnected layers enclosed between inner retinal neurons. The superficial retinal vasculature lies in the NFL; the intermediate rests in the INl and the deep retinal vascular plexus on the ONL. On the other hand, the photoreceptors in the ONL supplied by the choroid located below the RPE and the Bruch's membrane. NFL, nerve fiber layer; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; IS/OS, inner segment/ outer segment; RPE, retinal pigmented epithelium. Acquired from (Sun and Smith, 2018).

The important role of pericytes and smooth muscle cells (SMCs) in the regulation of vessel diameter, known as mural activity, has been recently recognized. Mural activity was first proposed in 1890 by Roy and Sherrigton (Roy and Sherrington, 1890). However, the past 15 years have seen an explosion of new data documenting the role of mural cells in the control of vascular hemodynamics in response to changes in retinal neural activity (Riva et al., 2005). Functional hyperemia is a vital process whereby the metabolic demand of active neurons is matched by local blood flow to ensure availability of oxygen (O_2) and nutrients (reviewed in Newman, 2013). Signaling between cells of the NVC: neurons, endothelial cells, astrocytes, smooth muscle cells, and pericytes - known as neurovascular coupling - is essential for functional hyperemia. The mechanisms mediating functional hyperemia are not fully understood. Initially, it was thought that alterations in vascular dynamics were caused only by changes in the metabolic rate of O_2, whereby a localized increase of O_2 consumption stimulated a vascular response. Substantial recent evidence has shown that retinal hemodynamics is not solely mediated by O_2, but also other factors including visual stimulation (Riva et al., 2005) and excitatory neurotransmitters (i.e. glutamate) (Mintun et al., 2001). Other molecules such as potassium (Karwoski et al., 1989) and nitric oxide (Donati et al., 1995) are important candidates of NVC regulation. Alterations in these factors during physiological and pathological conditions like diabetes (Nagel and Vilser, 2004) and open angle glaucoma (Resch et al., 2004) can disrupt NVC in the retina.

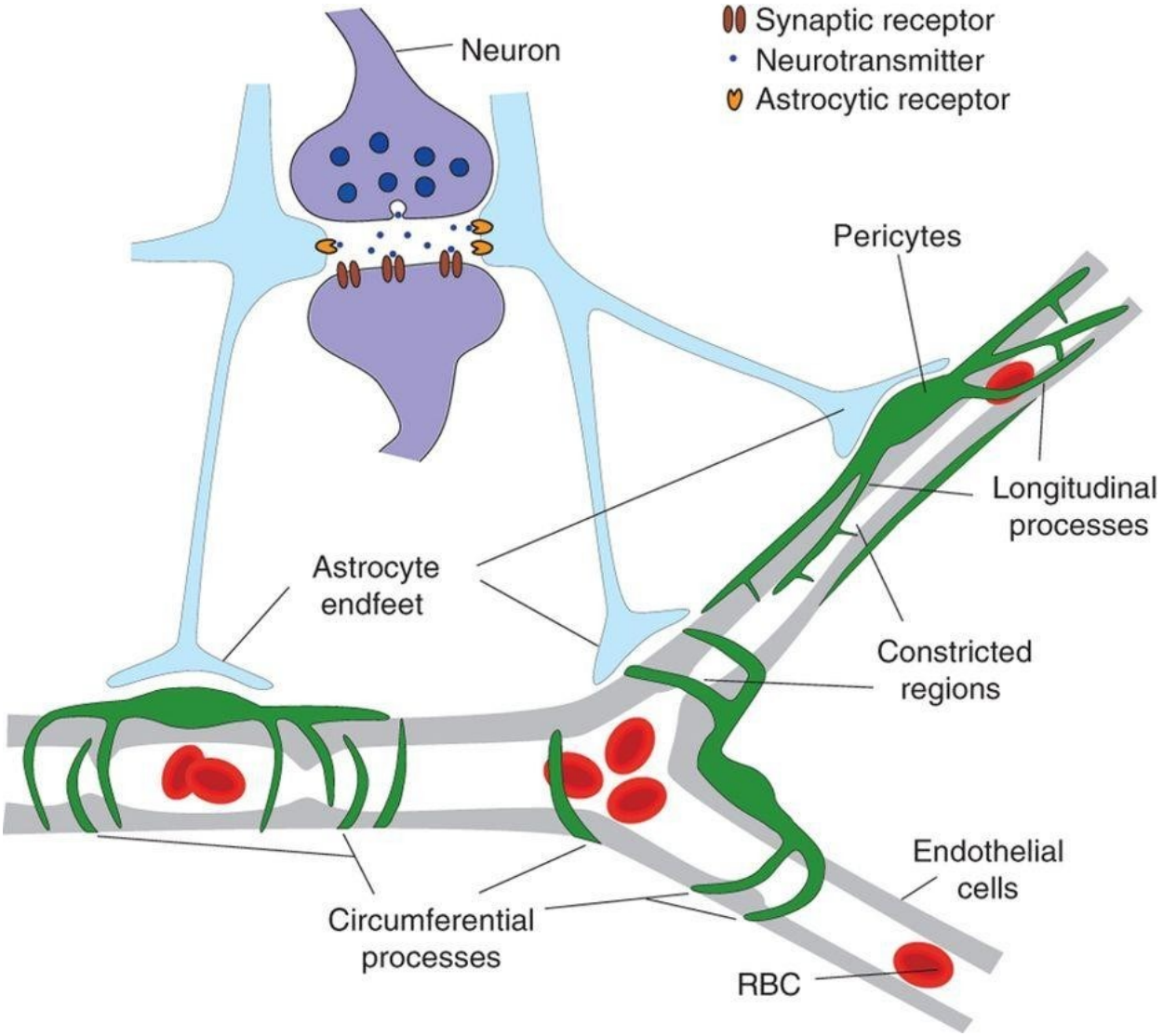

Figure 3. Schematic of neurovascular unit (NVU) and coupling. The NVU is comprised of endothelial cells, which are interconnected by tight junctions and neighboring cells such as astrocytes surrounding microvessels, microglia, neurons and pericytes wrapping the vessels regulating blood flow. Modified from (Mishra et al., 2014).

1.1.6. SMOOTH MUSCLE CELLS AND PERICYTES

Pericytes and SMCs are critical components of the NVU, maintaining BRB integrity and regulating cell-cell interactions. The role of these two mural cells in blood flow regulation is complex, thus a better understanding of their function is essential to elucidate pathological mechanisms in a number of neurodegenerative and neurovascular diseases (Iadecola and Nedergaard, 2007). The arterioles of mammals contain a layer of smooth muscle cells, the tunica media. In 1968, Somlyo found that SMCs are made up of contractile elements, actin filament rings wrapping around the arterioles (Somlyo and Somlyo, 1968). The distribution of SMCs along the vasculature becomes sparser in deeper plexuses, where capillaries become more abundant. Indeed, up to 90% of the capillary surface is covered by pericytes, which extend their arms longitudinally along the walls of microvessels (Figure 4&5).

The possibility that pericytes play an important role in the regulation of blood flow dynamics was initially proposed in 1871 when Eberth and collaborators reported the existence of “wrapping cells” along capillaries (reviewed in Attwell et al., 2016). In 1873, Rouget and colleagues identified these cells as pericytes and suggested that they could be responsible for the contraction of capillaries due to their unique location (Sims, 1986). Since then, pericytes have been proposed to play a role in the regulation of microvascular blood flow in response to local metabolic demand (Dalkara and Alarcon-Martinez, 2015). Hall et al. showed that the majority of blood flow regulation occurs at the capillary level (Hall et al., 2014a). Recently, it has been demonstrated that retinal

pericytes express alpha-smooth muscle actin, an important component of the contractile machinery (Alarcon-Martinez et al., 2018a), supporting the idea that pericytes play an important role in capillary constriction.

1.2. ISCHEMIC EVENTS IN THE RETINA

1.2.1. DEFINITION

Retinal ischemia is a common cause of visual impairment and blindness in the world (Osborne et al., 2004). The term ischemia refers to blood flow deficits in a specific area or tissue leading to insufficient energy and nutrient supply to affected cells (Osborne et al., 2004). All tissues need to be provided by O_2 and metabolic substrates that ensure cell survival and adequate function. During an ischemic event, nutrient and O_2 deprivation cause homeostasis dysregulation and, thereby, tissue injury. An important feature of ischemia is a post-injury response known as reperfusion, or the return of blood flow to the affected area, which has been shown to produce substantial tissue damage (Zheng et al., 2007). The lack of nutrients and O_2 generates reactive oxygen species leading to vasculature injury and neuronal degeneration, deficits that cannot be overcome by reperfusion (Zheng et al., 2007). Due to the wide variety of retinal diseases with an ischemic component, it is difficult to fully assess and estimate the contribution of retinal ischemia to vision loss (see below). The incidence of retinal ischemia increases with age due to a higher frequency of cardiovascular diseases.

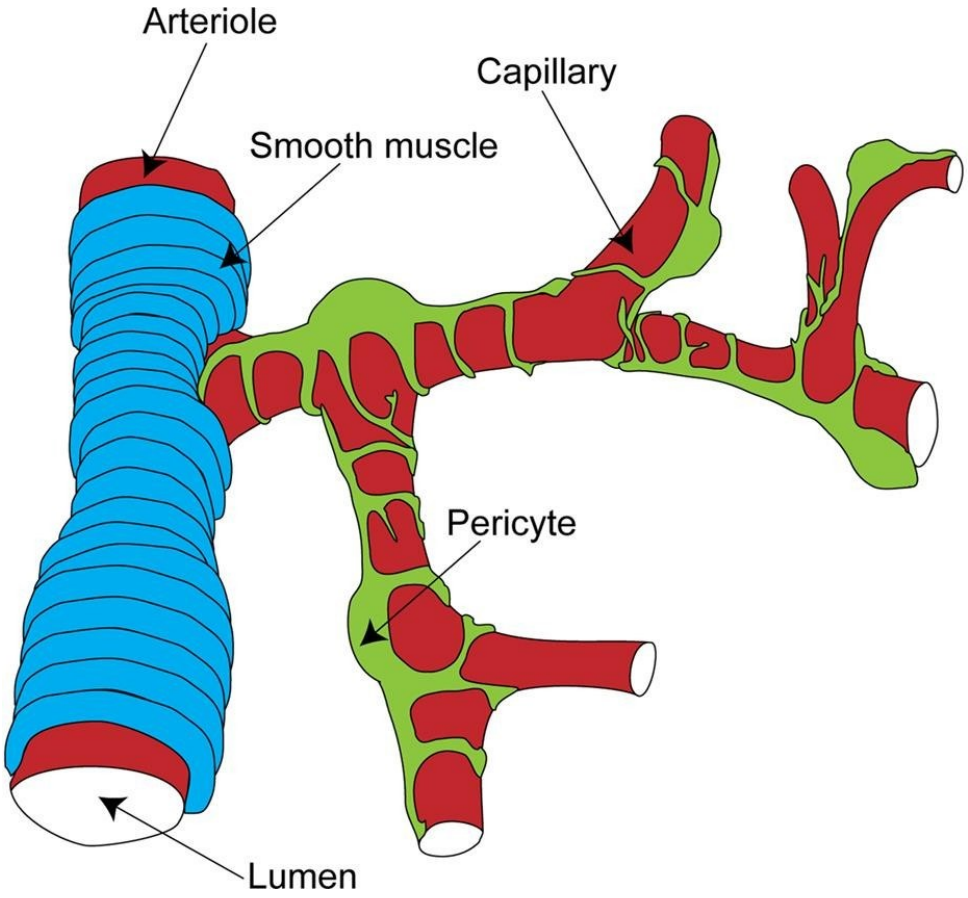

Figure 4. Schematic representation of the distribution of mural cells. Pericytes are located on small vessels, capillaries and smooth muscle cells surrounding large vessels, arterioles. Adapted from (Hamilton et al., 2010).

Therefore, a better understanding of cellular and molecular mechanisms underlying ischemia-induced retinal damage is necessary.

1.2.2. RETINAL ISCHEMIC DISEASES

Retinal vasculature diseases are a common cause of blindness in elderly people. Vascular diseases can result from systemic problems as in diabetic retinopathy (DR), or non-systemic deficits such as in non-arteritic ischemic optic neuropathy (NAION), central retinal artery occlusion (CRAO), or glaucoma (Lee and Ahn, 2017).

Diabetic retinopathy (DR)

DR is a consequence of diabetes affecting the retinal microvasculature and leading to visual impairment and eventual blindness (Klein et al., 1984). The major risk factor to develop DR is hyperglycemia, hypertension, and hyperlipidemia. It is estimated that around 100 million people worldwide are affected by DR (Leasher et al., 2016). During DR progression retinal blood vessels, particularly capillaries, become compromised and functional hyperemia is impaired. The microvascular permeability increases as the BRB is compromised, causing leakage of fluid and circulating proteins into the neural retina and deep retinal capillaries (Ben-nun et al., 2004). Since pericytes are located in deeper vascular plexus, and are important in maintaining BRB stability, they play a crucial role in DR (Dalkara and Alarcon-Martinez, 2015). For these reasons, much attention has been placed on identifying mechanisms of retinal pericyte failure in DR and the development of drug therapies to target these pathological changes.

Non-arteritic ischemic optic neuropathy (NAION): NAION is an important disease of the optic nerve caused by ischemia. NAION is mostly a disease of the middle -aged and elderly (Mizener et al., 1997) resulting in acute ischemia of the optic nerve head. NAION is an ischemic disorder affecting the posterior ciliary artery circulation in the optic nerve head leading to transient non-perfusion or hypoperfusion of the optic nerve head circulation (Hayreh, 2009). A whole host of systemic and local factors may disrupt optic nerve head circulation (Hayreh, 1996). The most important factor is caused by a transient fall of blood pressure in the optic nerve during sleep or due to embolic lesions of the arterioles that feed the optic nerve (reviewed in Hayreh, 2009).

Central retinal artery occlusion (CRAO): CRAO is the obstruction of the central retinal artery leading to infarction of the retina and vision loss. Blindness is caused by the interruption of blood supply to inner retinal layers. Risk factors for CRAO include diabetes, hypertension, smoking, and hypercholesterolemia (Schmidt et al., 2007). CRAO is an ocular emergency reminiscent of cerebral stroke, thus the timely management of CRAO is crucial to preserve vision (Cugati et al., 2013). During brain ischemia, pericytes were shown to constrict hence decreasing capillary blood flow (Yemisci et al., 2009).

Glaucoma: Glaucoma is the leading cause of irreversible blindness worldwide (Quigley and Broman, 2006). Glaucoma is characterized by the selective death of retinal ganglion cells (RGCs), the neurons that convey visual information from the retina to the brain (Almasieh et al., 2012). A major risk factor to develop glaucoma is elevated intraocular pressure, however, many patients continue to lose vision in spite of responding to therapies that regulate eye pressure (Leske et al., 2007). Recent studies have shown that glaucomatous patients with elevated eye pressure show substantial decrease in blood flow to the optic nerve head, retina and choroid (Wang et al., 2011). Our laboratory recently demonstrated pericyte dysfunction in a mouse model of ocular hypertension glaucoma (in preparation).

1.3. MECHANISMS OF VASCULAR DYSFUNCTION DURING ISCHEMIA

1.3.1. THE ISCHEMIC CASCADE: GLUTAMATE EXCITOTOXICITY AND CALCIUM RELEASE

During physiological conditions, the concentration of neurotransmitters in the extracellular milieu is typically low. Most of the energy is used to maintain the membrane potential of neurons and to recycle neurotransmitters released at synapses (Thoreson and Witkovsky, 1999). During an ischemic stroke, the brain tissue undergoes a reduction in blood flow leading to a lack of nutrients and O_2 necessary for the maintenance of ionic homeostasis, resulting in cell damage (Cai et al., 2017). Many

studies have reported release of glutamate and accumulation in the synaptic cleft after ischemia in both the retina and brain (Benveniste et al., 1984). Glutamate excitotoxicity, caused by hyperactivation of glutamate receptors, plays an essential role during ischemia-induced cell death (Dirnagl et al., 1999; Wahl et al., 1994). Indeed, massive release of glutamate into the extracellular space promotes an increase of intracellular Ca^{+2} in neurons by overactivation of the N-methyl-d-aspartate receptor (NMDAR) (Kalia et al., 2008). This high glutamate concentration generates a sustained depolarization and a loss of the membrane potential in neurons, an event known as anoxic depolarization (Dirnagl et al., 1999). Anoxic depolarization activates signaling cascades that promote neuronal death (Wang et al., 2007). The group of Murphy and collaborators showed that after two minutes of global ischemia, the levels of Ca^{2+} and subsequent neuronal membrane depolarization increase massively in neurons (Murphy et al., 2008). Of interest, this study reported that synapses and dendritic spine regrowth recovered after reperfusion (Murphy et al., 2008). During ischemia, NMDAR activation is partially mediated through the co-agonist glycine/D-serine, which contributes to membrane depolarization. A change in membrane permeability leads to Ca^{2+} influx causing mitochondria dysfunction and cell apoptosis (Wood, 1995). In this sense, the use of NMDAR antagonists was thought to be a promising pharmacological strategy to counter ischemic injury. However, clinical trials using this pharmacological approach have failed so far, suggesting that targeting excitotoxicity is not as simple as previously thought. Several hypotheses have been proposed as to why clinical trials using anti-NMDAR therapies for stroke, a disease with an important ischemic component, have

failed. First, although the penumbra zone is well-established in rodents, this area is not that clear in humans making it difficult to localize the site of drug action. Second, interfering with the function of the NMDAR generates significant adverse side effects that led to the termination of clinical trials. Third, anti-NMDAR clinical trials might have failed due to delays in diagnosis and the limited time window of intervention in which drugs are effective (reviewed in Lai et al., 2014).

1.3.2. THE RESPONSE OF PERICYTES DURING ISCHEMIA

It is well known that cerebral blood flow, at the level of capillaries, is disrupted during ischemia and remains reduced even after reperfusion (Leffler et al., 1989). In many models of retinal ischemia/reperfusion, the pathological response of pericytes has been associated with reduced blood flow due to oxidative and nitrative stress (Yemisci et al., 2009) leading to BRB breakdown (Díaz-Coránguez et al., 2017). Specifically, ischemia leads to pericyte constriction, which persists after reperfusion (Yemisci et al., 2009). In consequence, energy supply is limited due to constricted capillaries and reduced microcirculatory blood flow which can lead to neuronal damage (Hamilton et al., 2010). For this reason, a better understanding of the response of pericytes during retinal ischemia and the mechanisms underlying pericyte-mediated capillary constriction is important to develop better strategies that improve blood flow in ocular diseases with an ischemic component.

Calcium (Ca^{2+}) mediates the initiation and regulation of different responses after brain injury (Choi, 1988; Takano et al., 2009), and plays an important role in cell signaling

leading to contraction or dilation of blood vessels. For example, intracellular Ca^{2+} plays a critical role during physiological contraction of pericytes (Dehouck et al., 1997; Ramachandran et al., 1993). Borysova et al. demonstrated that Ca^{2+} signals in pericytes correlated with changes in capillary diameter suggesting a direct connection between Ca^{2+} dynamics in pericytes and capillary contractility (Borysova et al., 2013). Furthermore, Ca^{2+} plays a critical role in the regulation of neurovascular coupling (Mishra et al., 2014). Consistent with this, other cells of the NVU, such as astrocytes, are sensitive to ischemia-induce Ca^{2+} changes through voltage-dependent channels (Ding, 2014; Duffy and MacVicar, 1996). In spite of this, the role of Ca^{2+} in pericyte-mediated capillary constriction during retinal ischemia is poorly understood.

1.4. A MODEL OF ISCHEMIA IN THE RODENT RETINA: LIGATURE OF OPHTHALMIC VESSELS

In the laboratory, a number of animal models of retinal ischemia are routinely used to mimic ischemic diseases in humans (Table 1). An important criteria is that the model resembles as much as possible the clinical pathophysiology in human patients. A limitation of this approach is that there are important anatomical differences at the level of the vasculature among the most common species typically used (e.g. mouse, rat, guinea pig, rabbit). Despite important differences between the mouse and the human retinal vasculature, the mouse is an optimal species to induce inner retinal ischemia due to overall similar anatomy, equivalent responses to injury, and the convenience of using

mice including transgenic animals. One advantage is the location of the central retinal artery (CRA) in mice. In humans, the CRA travels within the optic nerve, but in mice it is located underneath the optic nerve hence it is easily accessible. This arrangement allows us to perform controlled retinal ischemia by compressing the CRA without damaging the optic nerve, which would cause neuronal death, and also reperfusion by releasing the source of compression. For these reasons, the mouse model of retinal ischemia used in this thesis is an excellent paradigm to test our hypotheses. Specifically, we used a model of retinal ischemia based on the exposure of the optic nerve and the underlying ophthalmic vessels. A longitudinal cut along the optic nerve sheath is made, and a very thin suture (10-0) is passed between the CRA and the nerve (see Methods). The CRA is compressed by tying a knot with the suture, a process called ligation, taking care not to damage the optic nerve. CRA ligation blocks the inflow and outflow of blood to the retina producing general ischemia. This is a well-characterized model that has been extensively used by a number of laboratories to generate useful data regarding mechanisms of ischemic damage in the retina (Vidal-Sanz et al., 2001).

Experimental method	Degree of ischemia	Human disease correlate
High intraocular pressure	Complete	CRAO, acute angle-closure glaucoma, ophthalmic artery occlusion
High intraocular pressure	Complete	CRAO, ophthalmic artery occlusion
High intraocular pressure	Complete	CRAO, ophthalmic artery occlusion
Bilateral occlusion of common carotid artery (2-vessel occlusion)	Incomplete	CRAO, ophthalmic artery occlusion, carotid insufficiency
Bilateral occlusion of vertebral and common carotid arteries (4-vessel occlusion)	Complete	Cardiac arrest/hypovolaemic shock
Photodynamic ablation	Incomplete	BRAO
Cardiac arrest	Complete	Cardiac arrest
Postmortem ischemia	Complete	—
Intravitreal injection of glutamate receptor agonists	Incomplete?	CRAO?

Table 1. Animal models of retinal ischemia. (Modified from: Osborne et al., 2004)

1.5. OBJECTIVES OF THE THESIS, HYPOTHESES AND EXPERIMENTAL APPROACHES

In this M.Sc. thesis project, I tested the **hypothesis** that pericytes play a crucial role in the regulation of microvasculature dynamics during retinal ischemia. My central goal was to better understand the response of capillaries and pericytes during ischemic injury in the retina and to identify the mechanisms of pericyte-mediated capillary constriction. To accomplish this, I proposed the following objectives:

1) Characterize the response of pericytes during transient retinal ischemia.

2) Elucidate mechanisms of pericyte contractility during retinal ischemia.

Our experimental design was based on:

A. In vivo and ex vivo visualization of pericytes during ischemia. For this purpose, I used a model of transient retinal ischemia induced by ligation of the central retinal artery (CRA), a procedure that interrupts blood flow to the retina. Ischemia was induced in transgenic mice carrying the pericyte-specific NG2 promoter driving red fluorescent protein (NG2:DsRed) or the genetically encoded calcium indicator GCaMP6 (NG2:GCaMP6).

B. Analysis of capillary dynamics. Changes in retinal capillary diameter and intracellular Ca^{2+} in pericytes were examined using two complementary quantitative approaches. First, I performed ex vivo measurements of capillary diameter at pericyte locations on flat-mounted retinas using an unbiased

stereological approach. Vessels and their associated pericytes were three-dimensionally (3D)-reconstructed using imaging software. Second, a novel and minimally invasive two-photon microscopy strategy was used for live imaging of microvasculature changes and Ca^{2+} dynamics in retinal pericytes in vivo.

CHAPTER 2

2. METHODS AND RESULTS

2.1. METHODS

Experimental animals

Animal procedures were approved by the University of Montreal Hospital Research Center and followed the Canadian Council on Animal Care guidelines. Experiments included adult female and male mice (2–6 months of age, 20-35 g) expressing: i) red fluorescent protein under control of the NG2 (Cspg4) promoter (NG2-DsRed) for selective visualization of retinal pericytes (008241, Jackson Laboratory, Bar Harbor, ME), or ii) the Ca^{2+} indicator GCaMP6 downstream of the NG2 promoter (NG2-GCaMP6), generated by crossing NG2-Cre mice (008533, Jackson Laboratory) with floxed GCaMP6 mice (024106, Jackson Laboratory). For multiphoton microscopy, albino NG2-DsRed and NG2-GCamp6 mice were generated by backcrossing with CD-1 mice. Animals were housed in 12 h light/12 h dark cyclic light conditions, with an average in-cage illumination level of 10 lux, and fed ad libitum. All procedures were performed under general anesthesia (20 mg/kg ketamine, 2 mg/kg xylazine, 0.4 mg/kg acepromazine). Data are shown as the average ± S.E.M. and individual values are shown in the graphs, and the number of animals used in each experiment is indicated in the figure legends.

Transient retinal ischemia/reperfusion

Transient retinal ischemia was performed by selective ligature of the ophthalmic vessels to block blood flow for up to 60 min, according to published methods (Vidal-Sanz et al.,

2001). Briefly, under general anesthesia, the left optic nerve was exposed and the optic nerve dural sheath was opened longitudinally. A fine 10-0 nylon suture was carefully introduced between the sheath and the optic nerve, and tied around the sheath. Because the optic nerve sheath contains the ophthalmic artery, this procedure interrupts retinal and choroidal blood flow without damaging the optic nerve itself. The ligature was released after different intervals of transient ischemia (10-60 min) to allow blood reperfusion to the retinal tissue. The number of pericytes remaining after ischemic damage was quantified from NG2-DsRed retinas using unbiased stereological sampling. We randomly took 3D-disectors (stacks) across the entire retinal whole-mount and quantified pericytes that came into the dissector frame. The total number of pericytes was calculated using the fractionator technique (Mouton, 2002).

Two-photon laser scanning microscopy (TPLSM)

In vivo TPLSM retinal imaging was performed as previously described[25] with modifications. Anesthetised mice were placed on a custom-made setup designed to accommodate light stimulation during live retinal imaging. Mice were kept on a homoeothermic blanket (Stoelting, Wood Dale, IL) to maintain body temperature (37°C) during imaging. The superior and inferior eyelids were opened and a 6.0 suture, attached to the ocular superior muscle, was used to rotate the eyeball to expose the sclera atop the medial superior and peripheral retina. The conjunctiva over the sclera was gently teased to place a 5-mm diameter coverslip (Harvard apparatus, Holliston, MA) and generate a flat plane for imaging (field of view: 400 × 400 μm) with a

multiphoton microscope (LSM780, Zeiss) controlled by Zen software (Zeiss). For excitation, a mode-locked Ti:sapphire laser (Chameleon Ultra, Coherent, Santa Clara, CA) was used through a water-immersion objective (20x, NA=1.0, Zeiss). Image acquisition was carried out using a wavelength of 820 nm to excite TRICT/DsRed protein and FITC-dextran, and the mean laser power at the sample plane was 15–50 mW. Imaging was performed through the entire thickness of the retina below the sclera (depth: 50 µm-300 µm). For vessel diameter analysis, multiple fields (25 x 25 µm, 90 x 90 pixels) were scanned at 12 Hz and acquired during light stimulation. Recordings with large-amplitude motion were discarded. TRITC-tagged lectin (Sigma) was injected intraocularly 1 hr prior to imaging for intravital visualization of vessel contour.

Analysis of capillary diameter

i) In vivo: Immediately prior to *in vivo* TPLSM imaging, tail vein injection of FITC-coupled dextran (70KDa, 1 mg/ml in 100 µl, Sigma) or intraperitoneal injection of fluorescein (5% in 100 µl, Novartis Pharma) was performed to label vessels. To visualize IP-TNTs, we intravitreally injected TRITC-lectin (*Bandeiraea simplicifolia*, 5 µg/ml in 2 µl, Sigma) 1 hr before imaging. Diameter measurements were performed by placing a linear probe at the desired location, perpendicular to the fluorescent plane of the filled vessel, using Image J (National Institute of Health) and a custom R routine freely available (www.r-project.org). The fluorescent pattern for each frame was exported to R and the vascular diameter computed. For each measurement, at least 2 trials were averaged.

ii) Ex vivo: For analysis of vessel diameter on flat-mounted NG2-DsRed retinas, we used a stereological sampling approach based on random placing of 3D-disectors (stacks) across the entire retina (6 slices with a field of view of 170 x 136 µm along the Z-axis). Images of all lectin-labeled microvessels within the 3D-disector frame were acquired with an ApoTome 2 optical sectioning microscope (40X objective, Carl Zeiss) and analyzed using ImageJ (National Institute of Health). The vessel diameter was measured at the selected site (e.g. pericyte location), as well as upstream and downstream of the same vessel segment, to estimate changes in diameter. We defined a constriction as a focal diameter reduction of at least 2 standard deviations (mean + 2SD) of the mean diameter in control animals (i.e. reduction >35%). The total number of microvessel constrictions was calculated using the fractionator method: total number of constrictions = quantified constrictions / ssf x asf x tsf, where ssf is a section sampling fraction of 1 (ssf = number of sections sampled / total sections), an area sampling fraction of 1/9 (asf = [a(frame)] / area x-y step between dissectors), and a thick sampling fraction of 1 (tsf = frame height / section thickness) (Mouton, 2002). This analysis yielded a representative value of the number of constrictions throughout each entire retina, which was then used to calculate the mean for experimental and control groups.

Quantification of calcium signals

NG2-GCamp6 mice were used to monitor Ca^{+2} rises in pericytes. At the end of each experiment, eyes were removed, fixed in 4% PFA, and retinas prepared as flat-mounts. Using an unbiased stereological sampling approach, images were acquired over the

entire retina with identical exposure time and gain settings for all experimental and control groups (40X objective, ApoTome 2, Zeiss). A region of interest (ROI) was manually drawn and used to measure fluorescence at the pericyte soma. Four measurements around the pericytes (background) and one measurement at the pericyte soma were taken. The average of the four values around the pericytes (Fo) where extracted from the fluorescence intensity of the pericyte soma (F) to normalize for background fluorescence.

Statistical analyses

All values are provided as the mean ± standard error of the mean (S.E.M.). We evaluated all cohorts with normality (Shapiro-Wilk test) and variance (F-test) tests. We compared values of response time, vessel diameter, number of constrictions, intracellular calcium, and stereological quantifications by means of two tailed Student's t-test or Mann-Whitney U test, where appropriate. For multiple comparisons, we used Analysis of Variance (ANOVA) followed by Dunnett's or Tukey's test, where appropriate. A p value ≤ 0.05 was considered significant. All regression lines of diameter change graphs were fit with the same order between experimental and control cohorts.

2.2. RESULTS

Ischemia reduces capillary diameter at pericyte locations ex vivo.

To establish whether ischemia induced changes in capillary diameter at pericyte locations, we used transgenic mice that selectively express red fluorescent protein (DsRed) in pericytes under control of the neural/glial antigen 2 (NG2) promoter (i.e. NG2:DsRed) (Figure 5B). In this transgenic line, pericyte-specific NG2 expression is detected in a large number of pericytes (>80%) (He et al., 2016), thus allowing visualization of individual pericytes and their processes on capillaries. A key question in the field is whether capillary constriction is a consequence of pericyte contraction during retinal ischemia. For this purpose, retinal ischemia was induced in anesthetized mice by ligation of the CRA with a 10-0 suture placed between the optic nerve and the ophthalmic vessels to produce blood flow blockage for 1 hour (Figure 6A). The right eyes were never operated on and intact mice were used as a control. To visualize retinal microvessels, operated eyes were removed, fixed in 4% paraformaldehyde (PFA), and incubated overnight with isolectin, which labels both endothelial cells and pericytes. NG2:DsRed positive cells colabeled with isolectin were 3D-reconstructed and analyzed. A detailed analysis of capillary diameter changes at pericyte locations was carried out by systematic uniform random sampling and ImageJ (National Institute of Health) (Figure 6B-D).

Following ischemia, capillaries were visibly reduced compared to those in non-injured retinas (Figure 7A-D). Our data show a generalized reduction in capillary diameter in ischemic retinas relative to sham-operated controls in all vascular plexuses

(ischemia: 4.7 ± 0.2 µm, sham-operated control: 5.2 ± 0.2 µm, Student's t-test, $p<0.001$, n= 1374-1971 capillaries/group, N= 4-6 mice/group) (Figure 7E-F). The analysis of the number of capillary constrictions at pericyte locations, demonstrated a substantial increase in ischemic retinas relative to the physiological capillary diameter reductions observed in controls (ischemia: 1,038 ± 277 constrictions at pericyte locations, control: 60 ± 36 constrictions at pericyte locations, Student's t-test, $p<0.01$). These changes could not be attributed to pericyte death because no significant loss was detected in ischemic retinas relative to non-injured controls (N=4-5 mice/group, Student's t-test, n.s.: not significant, p=0.46) (Figure 7G). Our results demonstrate that capillary diameter markedly decreases at pericyte locations during retinal ischemia.

Capillaries constrict at pericyte locations after ischemic injury in vivo.

To assess whether ischemia regulates capillary diameter at pericyte locations in vivo, we used a novel and non-invasive strategy based on two-photon microscopy for live imaging of the vasculature that yields high-quality images from the choroid to the inner retina (Takihara et al., 2015). To monitor the retina in vivo, a specific albino line was created by crossing NG2:DsRed mice with CD-1 mice. Vessels were labeled by intravenous injection of FITC-dextran and intravitreal injection of isolectin GS-IB4 to monitor pericyte contraction (Figure 8). Retinal ischemia was induced in anaesthetized mice (1 hour), as described above, followed by in vivo retinal visualization. The microvasculature was monitored during 60 min following ischemia induction (Figure 9A-D). Consistent with our ex vivo experiments, a marked reduction in capillary diameter

was observed after 30 min of ischemia (Figure 9C) (ischemia: 27% ± 15% capillary diameter changes, sham-operated control: 0% ± 0.2% capillary diameter changes, Student's t-test, $p<0.001$, n= 30-76 capillaries/group, N= 6-8 mice/group). Live imaging data using two-photon microscopy confirmed robust capillary constriction at the level of pericytes on retinal capillaries during ischemia in vivo. Collectively, our data demonstrate that ischemia promotes rapid pericyte constriction on retinal capillaries causing major microvascular dysfunction.

Intracellular calcium in pericytes increases in response to ischemia.

It has been previously shown that Ca^{2+} is important during physiological contraction of pericytes (Dehouck et al., 1997; Ramachandran et al., 1993). Borysova et al. demonstrated that an increase in intracellular Ca^{2+} in pericytes, correlated with changes in capillary diameter, suggesting a connection between Ca^{2+} increase in pericytes and contractility (Borysova et al., 2013). Therefore, we asked whether Ca^{2+} increase in pericytes influenced capillary constriction after ischemia induction. Genetically-encoded calcium indicators facilitate imaging of genetically defined pericyte population ex vivo and in vivo. Therefore, we generated a new transgenic line expressing the calcium indicator GCaMP6 under the control of the pericyte-specific NG2 promoter, allowing visualization of Ca^{2+} changes only in pericytes (Figure 10).

Intravitreal injection of isolectin prior to ischemia induction was used to label pericytes. After 1 hour of ischemia, eyes were rapidly collected and fixed in 4% PFA for 1 hour at room temperature. The retinas were removed and placed as whole-mounts

allowing pericyte imaging using an Apotome fluorescent microscope (Apotome 2, Zeiss). The intensity of Ca^{2+} signals in individual pericytes was measured using ImageJ software as described above. Under ischemic conditions, intracellular Ca^{2+} in pericytes was significantly increased in the soma and along the arms of the pericyte relative to sham-operated control (Ischemia: 12.56 ± 2 a.u., control: 5.33 ± 2 a.u., Student's t-test, $p<0.001$, N= 6 mice/group) (Figure 11). These data indicate that intracellular Ca^{2+} increases in pericytes during ischemia.

Increased calcium in pericytes after ischemia in vivo.

To determine whether ischemia promotes Ca^{2+} increase in pericytes, we crossed NG2cre:GCaMP6 mice with CD-1 albino mice to visualize the expression of Ca^{2+} dynamics in pericytes using two-photon microscopy of live retinas is only possible in albino mice. We used two-photon imaging to measure intracellular Ca^{2+} increase in pericytes in the retina of anesthetized mice. In vivo time-lapse imaging in these mice revealed that, after ischemia, pericytes had a substantial increase in intracellular Ca^{2+} in pericytes, consistent with our previous findings ex vivo (Student's t-test, $p<0,001$, N= 6 mice/group) (Figure 12). These data provide strong evidence that ischemia promotes Ca^{2+} increase in pericytes potentially leading to their contraction hindering blood supply and damaging the retina irreversibly.

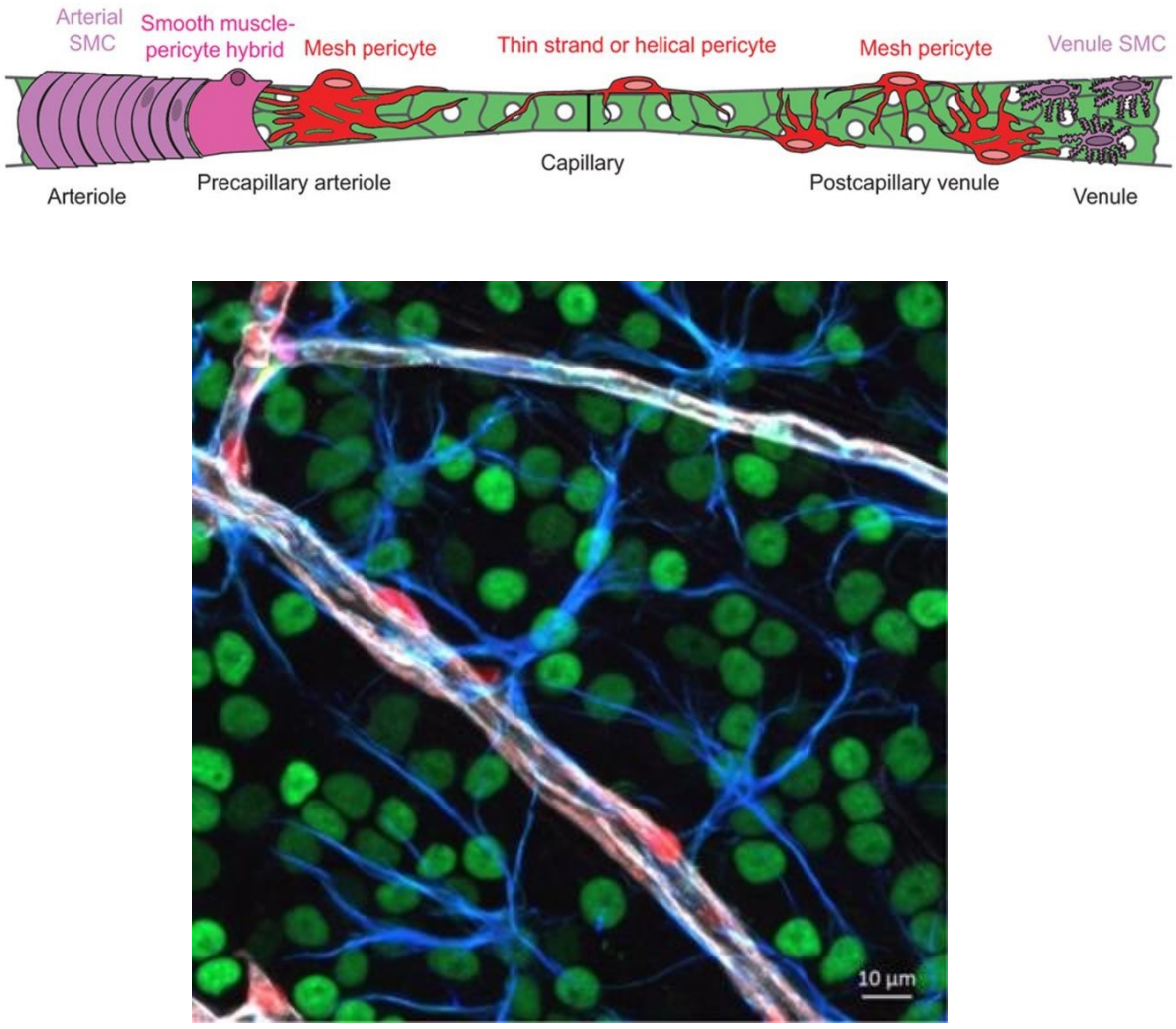

Figure 5. (A) A schematic representation of the different morphologies of brain pericytes. Vascular tree composed by a heterogenous cell population determined by morphology, location and function on the vasculature. Pericytes at the capillary level, transitional pericytes on pre-capillaries and post-capillaries and arterioles surrounded by smooth muscle cells. (B) Ex vivo immunohistochemistry of the neurovascular unit representing different cell types, including retinal ganglion cells (RBPMS, green), astrocytes (GFAP, blue), vascular endothelial cells (lectin, white) and pericytes (DsRed, red). Scale bars = 10 µm (Adapted from Hartmann et al., 2015).

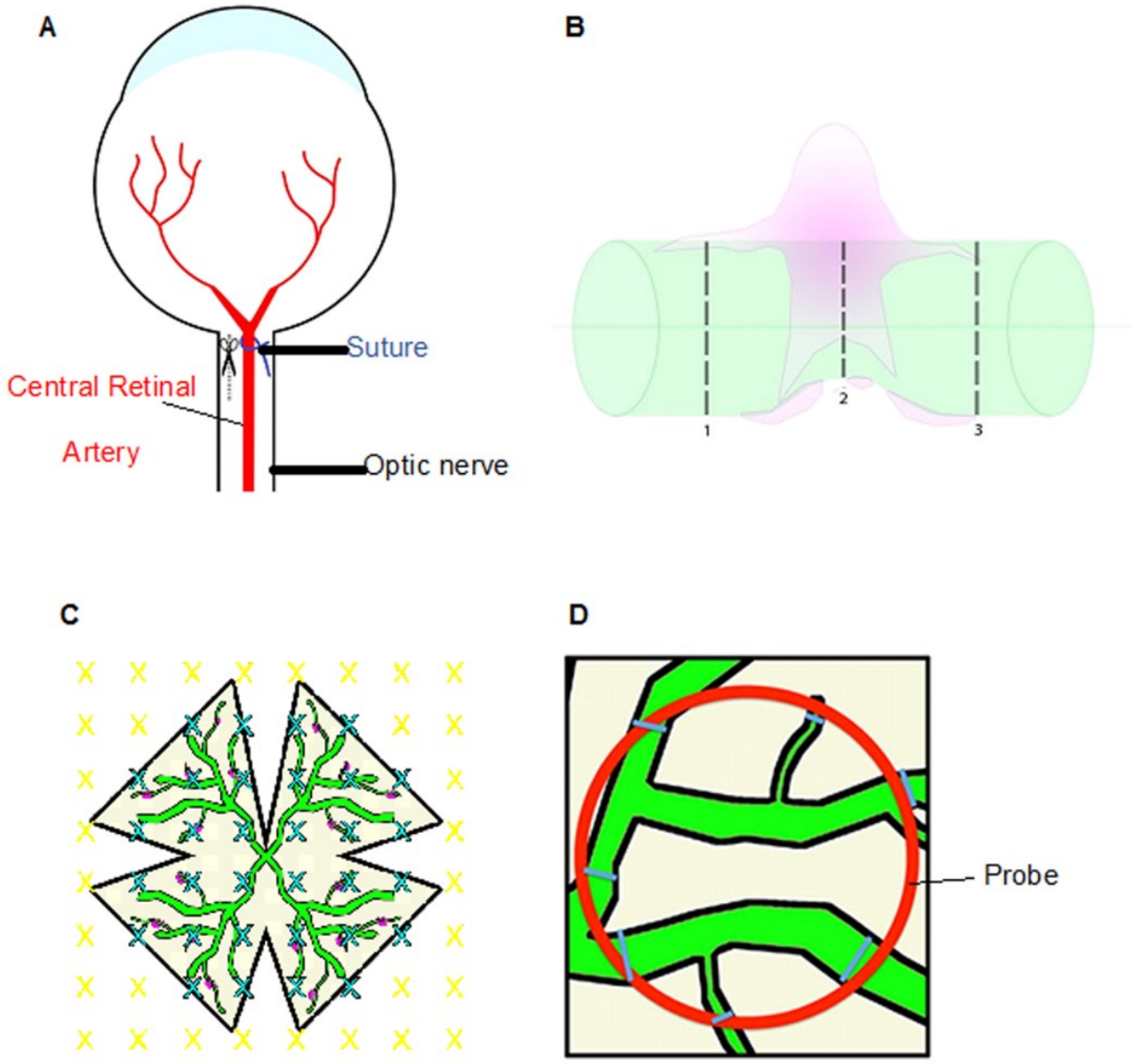

Figure 6. (A) The illustration depicts how ischemia was induced in the retina. Using a suture, a knot was made in the posterior ciliary short arteries and the central retinal artery to disrupt the blood supply to the retina for 1 hour. (C) Retinas were sampled using a systematic uniform random method (C), a circular probe was positioned (D) and the diameter of capillaries touching the probe were measured (B).

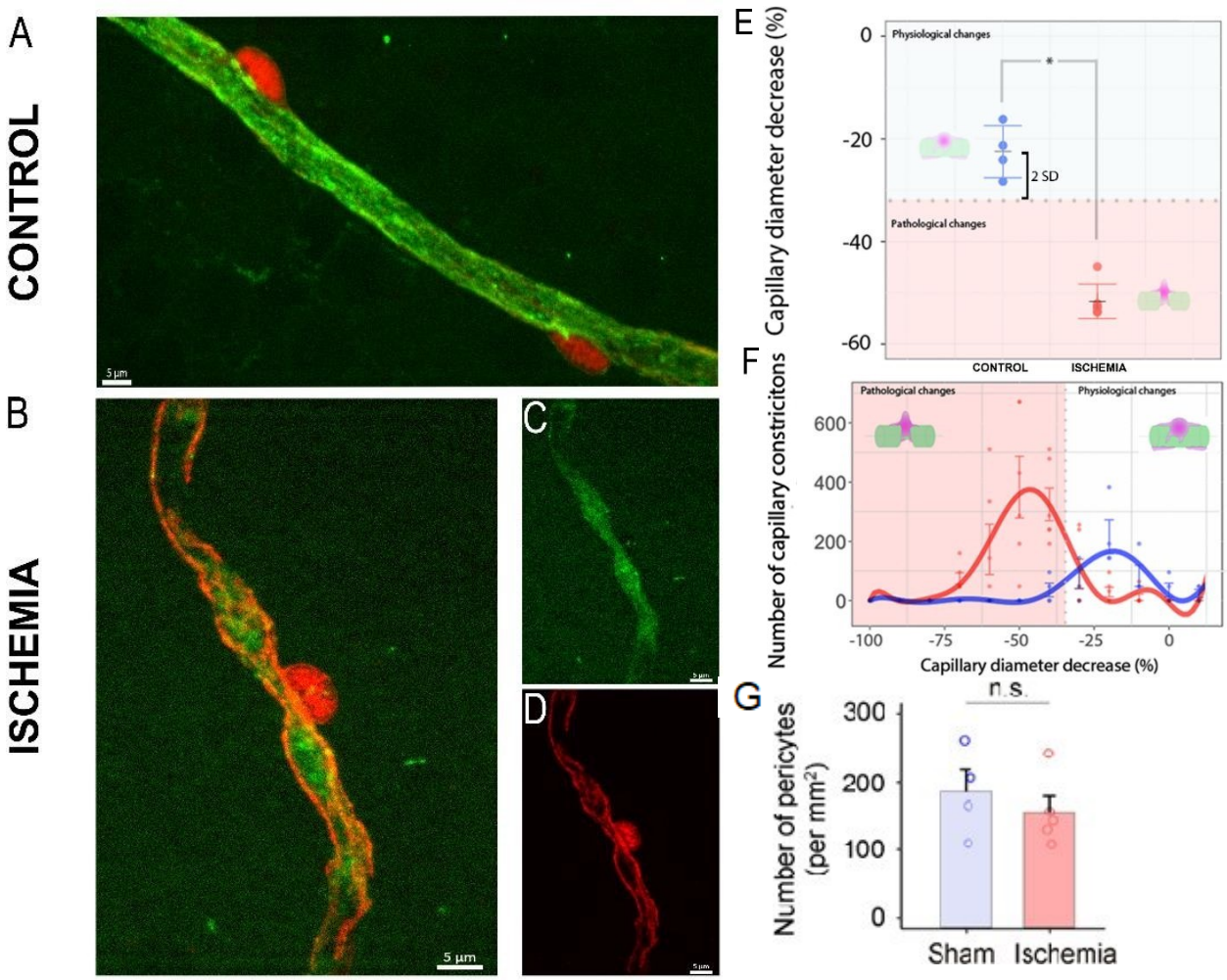

Figure 7. (A) Ex vivo fluorescence imaging of two retinal pericytes (DsRed, red) wrapping a capillary (lectin, green) in a control retina. (B-F) Ischemia (1 hr) led to a reduction in capillary diameter compared to control sham-operated retinas (B-E). (E) We defined capillary constriction as a focal reduction >35% (i.e. 2 SD below the average of the capillary decrease found in control mice). (F) Frequency distribution of the number of constrictions shows a significant increase in constrictions during ischemia (red trace) compared to non-ischemic control retinas (blue trace). (G) Graph showing that the number of pericytes, visualized in Ds-Red retinas (N=4-5 mice/group, Student's t-test, n.s.: not significant, p=0.46) do not change in ischemic retinas relative to sham-operated controls. Scale bars = 5 µm.

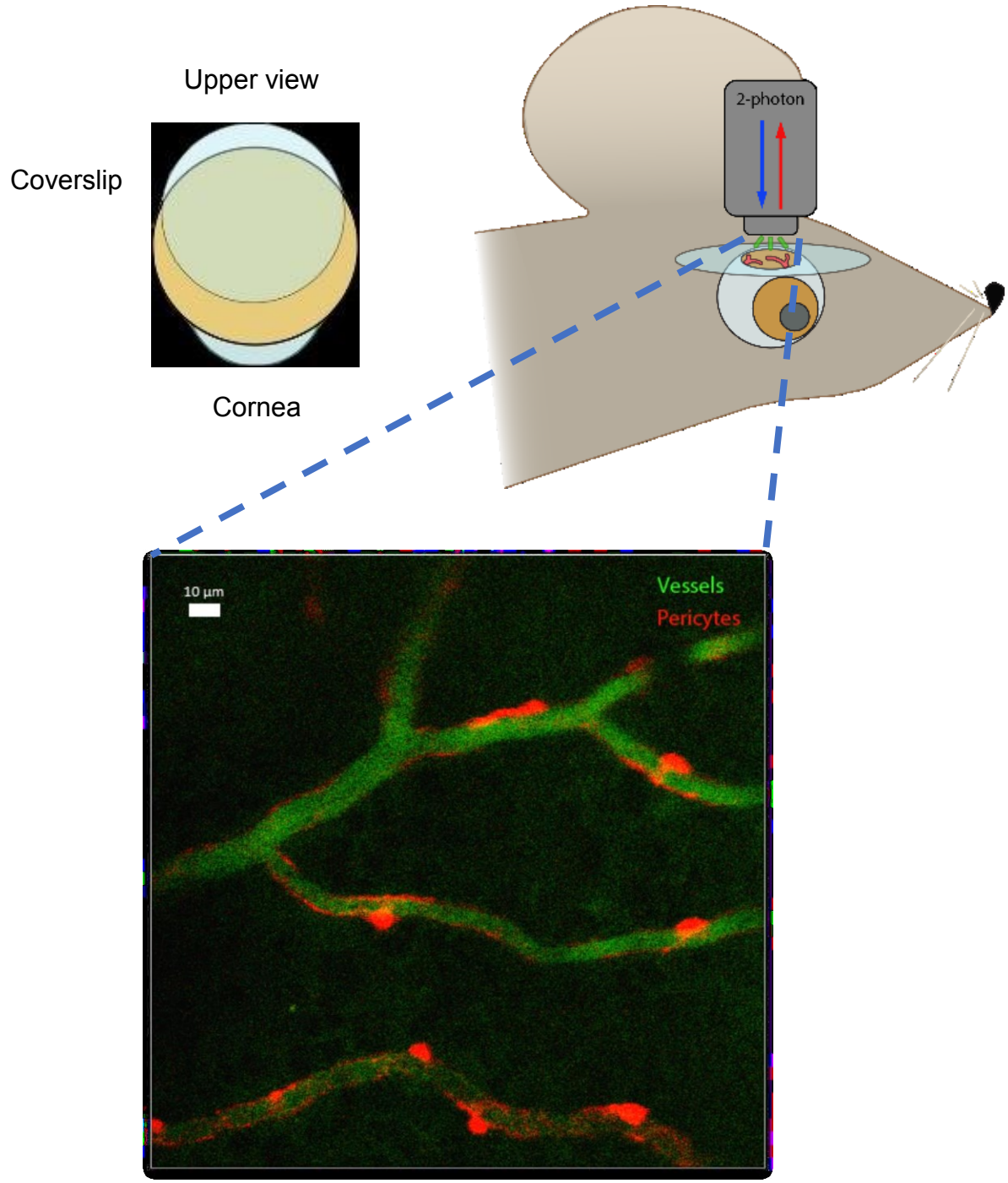

Figure 8. Two-photon laser scanning microscopy (TPLSM) imaging through the sclera of live NG2-DsRed mice was used to visualize pericytes and capillary diameter prior to and during ischemia.

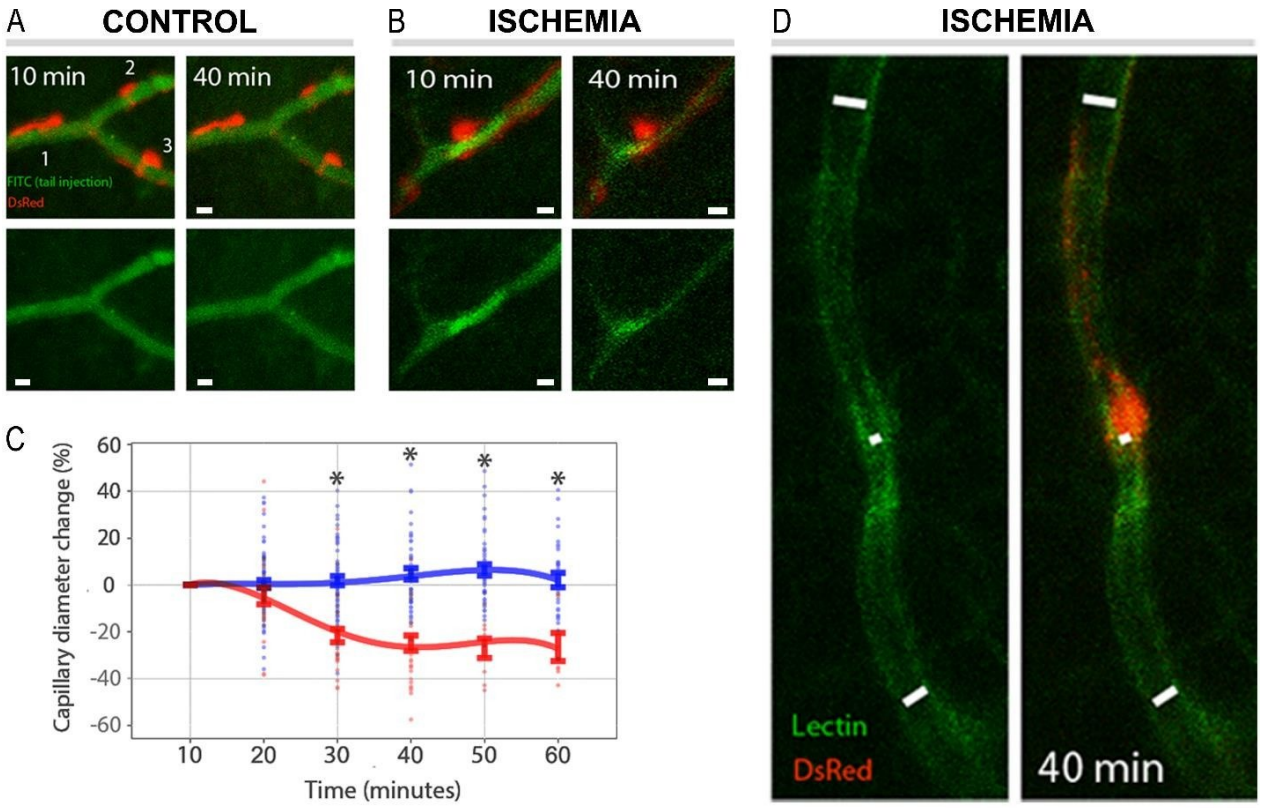

Figure 9. (A-B) In vivo monitoring of DsRed retinal pericytes along the capillaries after intravenous tail injection of FITC-dextran-70S (green) shows a constricting pericyte leading to reduced capillary diameter during ischemia (B). (C) Capillary diameter decreased by 30% during ischemia compared to Sham-operated controls ($p<0.001$; Student's t-test). (D) We confirmed our results with another in vivo vessel marker, lectin, which was injected intravitreally. Scale bars = 10 µm.

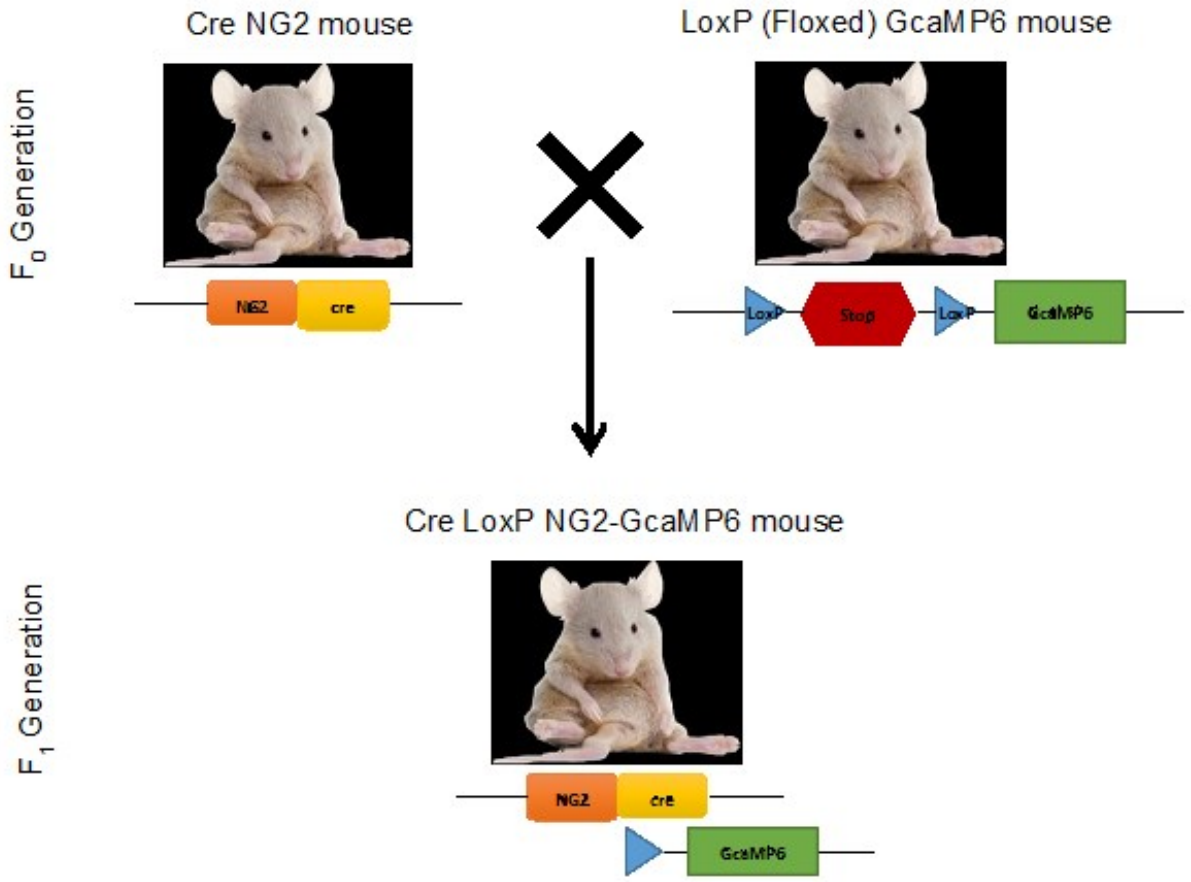

Figure 10. In vivo fluctuations in pericyte intracellular calcium were monitored in transgenic mice expressing the genetically encoded calcium indicator GCaMP6 under the control of the pericyte-specific NG2 promoter (NG2:GCaMP6).

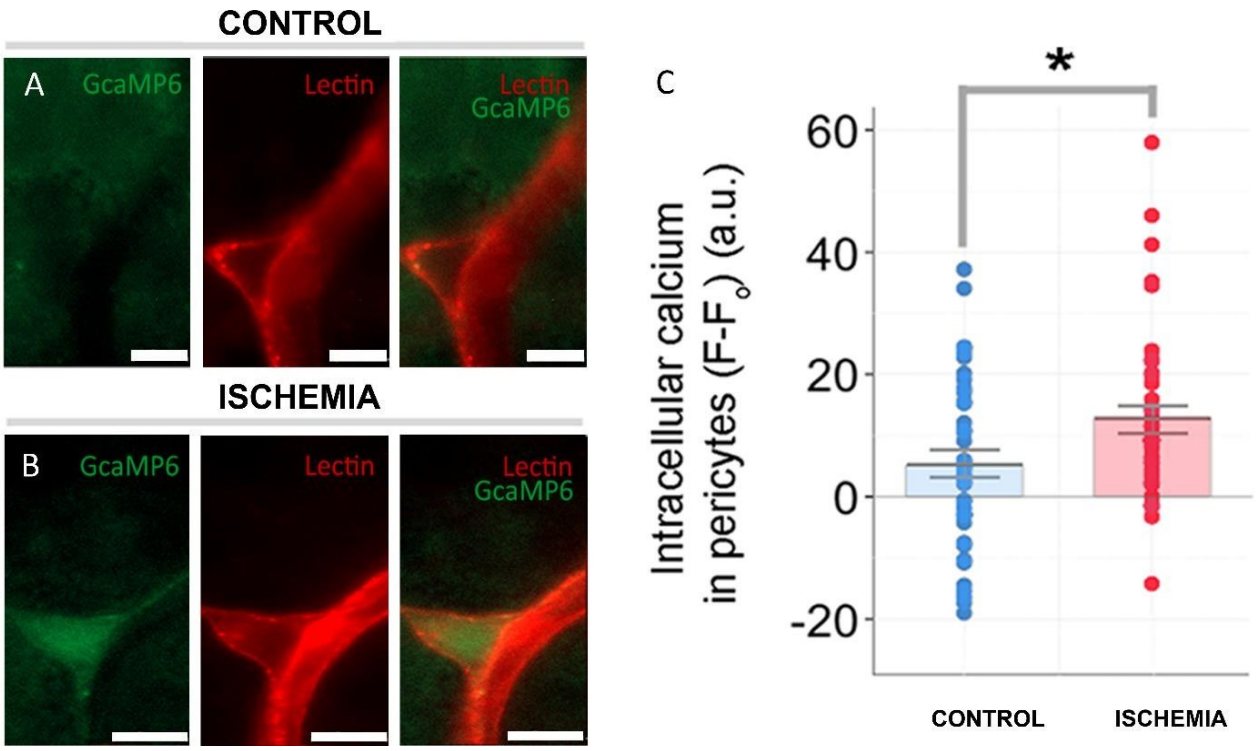

Figure 11. (A-B) Ex vivo fluorescence imaging of intracellular calcium in pericytes (green) from NG2:GcaMP6 retinas. The basement membrane of pericytes and vessels is labeled with lectin (red). Ischemia led to a significant increase of intracellular calcium in pericytes compared to control mice ($p<0.001$), ($n=6$) (B-C). Scale bars = 5 µm.

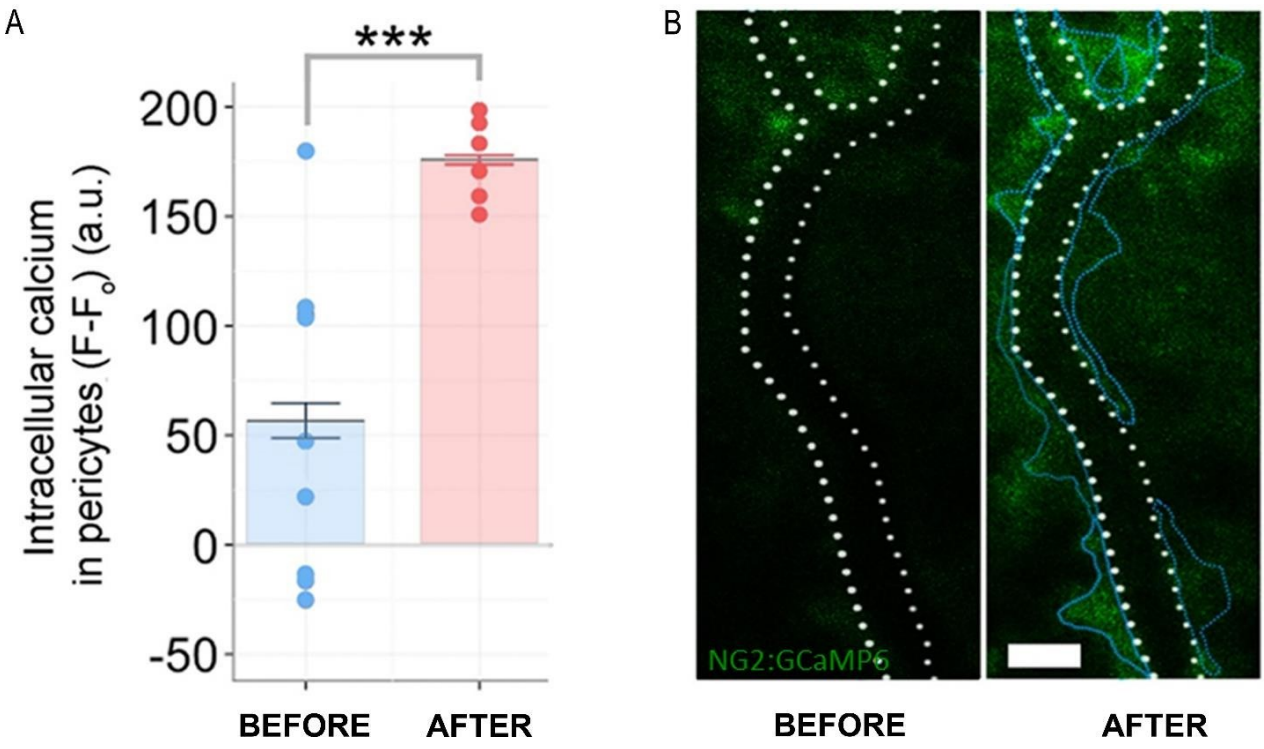

Figure 12. (A-B) Ex vivo fluorescence imaging of intracellular calcium in pericytes (green) from NG2:GcaMP6 retinas. The basement membrane of pericytes and vessels was labeled with lectin (red). Ischemia led to a significant increase of intracellular calcium in pericytes compared to control mice ($p<0.001$), (n=6) (B-C). Scale bars = 5 µm.

CHAPTER 3

3. DISCUSSION

3.1. DISCUSSION

The results from this project indicate that contractile pericytes can control NVC and blood flow by constricting the microvasculature. These data suggest that pericytes are important players in the regulation of microcirculatory blood flow in deeper plexuses of the retinal vasculature. This finding is consistent with studies demonstrating that capillary pericytes contain a contractile apparatus composed of myofibrils and alpha smooth muscle actin (Alarcon-Martinez et al., 2018; reviewed at Sims, 1986) supporting their role as microvasculature modulators.

This study supports several major conclusions. First, there is a significant reduction of capillary diameter, hence increased capillary constrictions, at pericyte locations during retinal ischemia. These findings are consistent with previous studies showing that pericytes control capillary diameter in both the retina and the brain during physiological conditions (Peppiatt et al., 2006). During brain ischemia, pericytes have been suggested to constrict capillaries and remain constricted even after reperfusion, impairing microcirculatory blood flow (Yemisci et al., 2009). Second, ischemia promotes a substantial increase in intracellular Ca^{2+} in pericytes correlating with pericyte-induced capillary constriction. Indeed, intracellular increase in Ca^{2+} was observed in the soma and along the arms of pericytes, visualized in NG2:GcaMP6 retinas, suggesting that Ca^{2+} might be an important regulator of pericyte contractility. The molecular mechanisms underlying capillary constriction are not well established. In this regard, our data suggest that Ca^{2+} increases in pericytes promoting their contraction, a response that leads to capillary constriction and reduced blood flow.

It is well established that glutamate released from presynaptic neurons raises Ca^{2+} concentrations. An increase in extracellular Ca^{2+} during ischemia could drive influx into pericytes promoting capillary constriction. The mechanisms of Ca^{2+} entry into pericytes is still unknown, but could involve Ca^{2+} entry from the extracellular milieu through nifedipine-sensitive L-type voltage-gated Ca^{2+} channels (Borysova et al., 2013; Burdyga and Borysova, 2014) or from other cells. For example, astrocytes accumulate a substantial amount of Ca^{2+} during ischemia (Duffy and MacVicar, 1996) and could potentially be a source of Ca^{2+} for pericytes.

Lastly, we report, a novel non-invasive multi-photon imaging technique that allows to monitor longitudinal changes in pericytes and capillaries in live animals. This technology allows the visualization of subtle microvasculature changes with unprecedented resolution, in the order of a few microns. Our in vivo validation that pericytes constrict capillaries early after retinal ischemia, and that this process is Ca^{2+}-dependent, expand our understanding of the retinal microvasculature response in neurovascular pathologies.

3.2. CONTROVERSY

A tight communication between cells of the NVU allows fine regulation of blood flow to match the energetic demand of active neurons, a process known as functional hyperemia. A better understanding of how the information is processed within the NVU would be useful to identify the signals that initiate blood flow increase in response to

neuronal activity. Yet, the mechanisms regulating functional hyperemia in physiological and pathological conditions are not well understood. Previous work demonstrated a complex set of interactions between neurons, glia and vascular cells including endothelial cells, pericytes, and SMCs (reviewed by Iadecola and Nedergaard, 2007; Itoh and Suzuki, 2012; Roy and Sherrington, 1890). The past few years have witnessed some level of controversy regarding the role of SMCs versus pericytes in the control of the capillary responses. Indeed, a recent study using transgenic mice expressing fluorescent proteins driven by the NG2 or α-SMA promoters proposed a radical redefinition by naming the NG2 and α-SMA-expressing (contractile) cells to be SMCs, rather than pericytes, as they would have conventionally been named under the original Zimmermann definition used since 1923 (Hill et al., 2015). SMCs and pericytes are morphologically and functionally different and so is their location on the vasculature, with SMCs surrounding arterioles while pericytes are exclusively associated with capillaries. Neurons are in much proximity to capillaries than arterioles, which is consistent with the need for energy supply provided at the capillary level (Lovick et al., 1999). Some groups have proposed that blood flow regulation and neurovascular coupling in arterioles precedes the response of capillaries (Fernández-Klett et al., 2010; Hill et al., 2015; Iadecola et al., 1997). In contrast, other groups have reported active capillary diameter changes, notably dilation, much earlier than arterioles, hence allowing increased blood flow in response to neuronal activity (Bell et al., 2010; Hall et al., 2014b; Kisler et al., 2017; Mishra et al., 2016; Rungta et al., 2018). We found that ischemia induces constriction of retinal capillaries at pericyte locations, similar to those

reported for ischemic cortical microvessels (Hall et al., 2014b; Yemisci et al., 2009). Interestingly, another ongoing study in our laboratory did not detect significant changes in arteriolar diameter in the same experimental conditions, supporting the idea that pericytes rather than SMCs are key cells to regulate neurovascular coupling and functional hyperemia (Alarcon-Martinez et al. unpublished).

Another area of interest comprises the mechanisms that mediate functional hyperemia and how these are altered during pathologies. A traditional view has been that blood flow regulation is mediated by astrocytes during neuronal activity (Iadecola et al., 1997). Different glia-derived metabolites including adenosine, lactate, CO_2 or gliotransmitters can modulate the dilation or contraction of vessels. One of the most studied molecules involved in changes in vessel diameter is Ca^{2+}. The increase in free Ca^{2+} within astrocytes in response to neuronal activity promotes the synthesis and release of astroglia-derived factors, including vasodilating arachidonic acid metabolites (Attwell et al., 2010; Iadecola and Nedergaard, 2007; Roy and Sherrington, 1890). Several hypotheses have attempted to address the current data dealing with the involvement of Ca^{2+} as a possible mechanism of blood flow regulation. For example, a recent study reported that Ca^{2+} signaling from Müller cells can generate changes in capillary dynamics (Biesecker et al., 2016). In addition, astrocytic Ca^{2+} excitability has been reported in a number of disease models (Ding, 2014) correlating with our findings that intracellular Ca^{2+} increases in pericytes after ischemia in vivo. Whether Ca^{2+} increase in pericytes derives from astrocytes and is transported to pericytes, or enters from the extracellular space is currently unknown.

Likewise, the contribution of arterioles versus capillaries on blood flow regulation through changes in intracellular Ca^{2+} concentration remains elusive (Attwell et al., 2010). Nevertheless, there is some evidence demonstrating an association between Ca^{2+} fluctuations and changes in the diameter of the vessels in both SMCs and pericytes in the brain and other tissues (Hill et al., 2015; O'Farrell et al., 2017; Rungta et al., 2018; Tykocki et al., 2017). Based on the data accumulated so far, our results support the hypothesis that pericytes are responsible for capillary changes, a response that correlates tightly with intracellular changes in Ca^{2+}. Future studies using pericyte-specific strategies to block Ca^{2+} influx to pericytes from the extracellular milieu and/or adjacent cells including astrocytes should help test this hypothesis (see below).

3.3. LIMITS OF THE STUDY

Our study demonstrates an in vivo role for pericytes in microcirculatory blood flow regulation and suggest a Ca^{2+}-dependent mechanism during pathological ischemic conditions. Two-photon microscopy is a robust technique to monitor the activity of different cell types in the retina as well as in brain tissue in vivo. However, this study was limited by the fact that this technique does not allow visualization of the entire retina. Indeed, our analysis was limited to a small retinal area in the periphery, the site where the microscope objective can be placed directly atop the exposed sclera, while central retinal areas were not accessible. The accessible region of interest typically allows visualization of a handful of pericytes, which allows the examination of a small capillary network, while larger regions are not visible. In spite of this, the information

obtained from analysis of single pericyte reactivity in live animals is invaluable and was complemented by our ex vivo studies using whole-mounted retinas. Other techniques including optical coherence angiography might be used to complement our observations using multiphoton microscopy.

Recently, it has been reported that pericytes at the resting state have spontaneous Ca^{2+} transients in their processes (Rungta et al., 2018). To complete our qualitative study, it would have been appropriate to do some electrophysiology by recording cell spike activity of pericytes in whole-mounted retinas during ischemia. These studies would complement our analysis of Ca^{2+} levels in pericytes with electrophysiological responses in naïve and ischemic retinas. The combination of these imaging and functional techniques would contribute to a better understanding of the response of pericytes during neurovascular coupling and their role in the regulation of physiological and pathological blood flow in retinal and brain diseases.

3.4. PERSPECTIVES

In this M.Sc. project, I demonstrated that during retinal ischemia there is a significant increase in capillary constriction at pericyte locations which correlates with high intra-pericyte Ca^{2+}, suggesting that Ca^{2+} might be an important regulator of pericyte dynamics. Several avenues remain to be explored and this study was intended as a first step in the development of a more in-depth investigation of pericyte biology in the context of retinal damage. In this sense, I have a keen interest in further characterizing

the molecular mechanisms leading to pericyte-mediated capillary constriction in optic nerve pathologies with a focus on retinal ischemia and glaucoma. For example, the role of vasoactive molecules expressed by retinal cells, including Müller cells and pericytes and how their level and function is altered during ischemia would provide useful information about communication within the NVU. Moreover, it is important to identify the molecular mechanisms that mediate Ca^{2+} entry into pericytes leading to capillary constriction and reduction of blood flow. Candidate molecules that can potentially mediate Ca^{2+} influx include membrane receptors and/or Ca^{2+} channels located on pericytes (e.g. L-type channels), as well as other pathways involving intracellular Ca^{2+} homeostasis. These experiments will provide novel knowledge on the response of pericytes to ischemia and might contribute to the development of new therapeutic strategies that limit abnormal Ca^{2+} influx into pericytes restoring capillary dynamics and microcirculatory blood flow in retinal diseases with an ischemic component.

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