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# Influence of chronic intermittent hypoxia (CIH) in retinal neovascularization and vascular remodeling

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Sorbonne Université

Ecole doctorale de Physiologie, Physiopathologie et Thérapeutique

Institut de la Vision

**Influence of chronic intermittent hypoxia (CIH) in  
retinal neovascularization and vascular remodeling**

Doctoral Thesis

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## Abbreviation

ALR: AIM like receptor

AMD: age-related macular dystrophy

AOP: apnea of Prematurity

A $\beta$ : amyloid-beta

BBB: blood-brain barrier

BRB: blood-retina barrier

CAM: cell adhesion molecule

CFB: complement factor B

CFH: complement factor H

CIH: chronic intermittent hypoxia

CNS: central nerve system

CNV: choroidal neovascularization

CR: complement receptor

CRP: C-reactive protein

CSA: central sleep apnea

CSF1: colony-stimulating factor 1

CSF1R: colony-stimulating factor 1 receptor

DR: diabetic retinopathy

DR: diabetic retinopathy

ECM: extracellular matrix

FcR: Fc receptor

GA: geographic atrophy

GCL: ganglion cell layer

GCL: ganglion cell layer

HCVEC: human choroidal vascular endothelial cells

HIF-1 $\alpha$ : hypoxia-induced factor- 1 alpha

ICAM-1: intercellular cell adhesion molecule-1

IFN: interferon  
IGF-1: insulin-like growth factor 1  
IL-1 $\beta$ : interleukin-1 beta  
INL: inner nuclear layer  
INL: inner nuclear layer  
IPL: inner plexiform layer  
IPL: inner plexiform layer  
KKS: kallikrein - kinin system  
LPS: lipopolysaccharide  
MAP: mitogen-activated protein  
MASP1: mannose-associated serine protease 1  
MerTK: Mer tyrosine kinase  
MHCII: major histocompatibility complex class II  
NFL: nerve fiber layer  
NLR: Nod-like receptor  
NPDR: non-proliferative diabetic retinopathy  
NVU: neurovascular unit  
OIR: oxygen-induced retinopathy  
ONL: outer nuclear layer  
OPL: outer plexiform layer  
OSA: obstructive sleep apnea  
PDGFB: platelet-derived growth factor B  
PDGFR $\beta$ : PDGF receptor-beta  
PDR: proliferative diabetic retinopathy  
PIGF: placental growth factor  
PSR: phosphatidylserine receptor  
RA: rheumatoid arthritis  
ROP: retinopathy of prematurity  
ROS: reactive oxygen species  
RPE: retina pigmen epithelium

TGF- $\beta$ : transforming growth factor-beta

TLR: Toll-like receptor

TNF- $\alpha$ : tumor necrosis factor-alpha

TREM2: triggering receptors expressed on myeloid cells-2

VEGF: vascular endothelial growth factor

## **Abstract**

(en français)

Les rétinopathies néovasculaires et l'œdème sont des complications menaçant la vue des rétinopathies ischémiques, telles que la rétinopathie du prématuré (ROP) et la rétinopathie diabétique (RD). L'apnée du sommeil qui conduit à une hypoxie intermittente chronique (HIC) est un facteur de risque indépendant de maladie sévère, mais les mécanismes sous-jacents sont inconnus. Ici, nous montrons que l'HIC expérimentale pendant la phase ischémique de la rétinopathie induite par l'oxygène chez les souris réduit sévèrement la revascularisation bénéfique de la rétine ischémique, et augmente la perte neuronale ainsi que la néovascularisation pathologique. Mécaniquement, nous démontrons que l'HIC réduit à la fois l'expression du facteur stimulant les colonies 1 (CSF-1) et l'augmentation induite par l'ischémie des cellules microgliales rétiniennes qui favorisent la revascularisation de la rétine ischémique en l'absence d'HIC. L'inhibition locale de CSF1R pendant la rétinopathie ischémique a réduit le nombre de cellules microgliales, inhibé la revascularisation et aggravé la néovascularisation pathologique, reproduisant plusieurs effets de l'HIC. Nos découvertes fournissent un nouveau mécanisme par lequel l'apnée du sommeil et l'HIC aggravent les rétinopathies ischémiques, soulignant l'importance de traiter l'apnée dans la ROP et la RD pour aider à prévenir les maladies sévères menaçant la vue.



## **Abstract**

(in English)

Neovascular retinopathies and edema are sight threatening complications of ischemic retinopathies, such as retinopathy of prematurity (ROP) and diabetic retinopathy (DR). Sleep apnea that leads to chronic intermittent hypoxia (CIH) is an independent risk factor for severe disease, but the underlying mechanisms are unknown. Here we show that experimental CIH during the ischemic phase of oxygen induced retinopathy in mice severely reduces beneficial revascularization of the ischemic retina, and increases neuronal loss and pathological neovascularization. Mechanistically we demonstrate that CIH reduces both colony stimulating factor 1 (CSF-1) expression and the ischemia-induced increase of retinal microglial cells that promotes the revascularization of the ischemic retina in the absence of CIH. Local CSF1R inhibition during ischemic retinopathy reduced the number of microglial cells, inhibited revascularization, and exacerbated pathological neovascularization, recapitulating several effects of CIH. Our findings provide a novel mechanism by which sleep apnea and CIH aggravate ischemic retinopathies, underscoring the importance of treating apnea in ROP and DR to help prevent sight threatening severe disease.

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# **Introduction**

# 1. Anatomy of the eye

The eye is one of the most important organs of human which is highly specialized for photo-reception, the process by which light energy from the environment is converted into an electrical impulse. This electrical signal is transmitted to the brain, via the optic nerve, which analyzes it and creates the image that we perceive.

The human eye is divided into two parts (**Figure 1**): the anterior segment (cornea, iris, pupil, anterior chamber, lens, ciliary body) and the posterior segment (retina, choroid, sclera, vitreous humor, optic nerve). In our project we focus on the posterior segment. The anatomical data showed in this chapter is taken from *The Eye* (4th edition) by Forrester J., Dick A., McMenemy P., Roberts F and Pearlman E.

## 1.1 Anatomy of retina

The retina is a complex and highly specialized layer of tissue that lines the inner surface of the back of the eye. It plays a critical role in capturing light and converting it into neural signals that are transmitted to the brain for visual processing. The retina consists of several layers with distinct functions (**Figure 2**), including nerve fiber layer (NFL), ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), photoreceptor layer, retina pigment epithelium (RPE).

Photoreceptors (1<sup>st</sup> neuron), which include two types: rods and cones, locate in the photoreceptor layer in the outer side of retina. Both types of photoreceptors have the shape of a long, narrow cell that includes an inner segment and an outer segment. Light is absorbed by the outer segments of the photoreceptors, and photoreceptors conduct the electrical signal to the outer plexiform layer. Then the information is transmitted to bipolar cells (2<sup>nd</sup> neuron) in the inner nuclear layer via synapses between photoreceptors and bipolar cells. With other two neurons, horizontal and amacrine cells, it is possible to modulate the transmission of information at the

level of the bipolar cells. By synapses in the inner plexiform layer, bipolar cells transmit the nerve impulses to ganglion cells (3<sup>rd</sup> neuron) in the ganglion cell layer. The axons of ganglion cells converge to form the optic nerve. The optic nerve carries the electrical signals to the brain's visual processing centers, where the signals are further processed and interpreted as visual perceptions (Forrester J et al., 2021).

Besides, the retinal glia does not participate in the transmission of nerve impulses but have a role of support and maintenance of the tissue. These cells include Müller cells, astrocytes, and microglia.

1.) The Müller cells are the main supporting cells of the neural retina. The nuclei of Müller cells locate in the inner nuclear layer, but we find their cellular expansions on the thickness of the neural retina up to the outer limiting membrane (**Figure 2**). They have a supporting role and promote conduction of nerve impulses by isolating the cells from each other. They also regulate the level of neurotransmitters at the synapses and participate in the recycling of cone photopigments (Forrester J et al., 2021).

2.) The astrocytes are cells found mainly in the ganglion cell layer and the inner plexiform layer (**Figure 2**). Thanks to their long star-shaped projections, the astrocytes are in contact with the ganglion cells but also with the blood vessels. These cells are part of the inner blood-retinal barrier (BRB): the covering of retinal vessels by the extensions of astrocytes limits the exchanges between the blood circulation and the retina. They spread as a proliferating cell population in a centrifugal fashion across the inner surface of the retina, forming a cellular network that provides a template for the blood vessels in their wake (Fruttiger et al., 1996)

3.) Microglial cells are the main resident immune cells of the central nervous system (Perry and Gordon, 1988). In pathological conditions, they migrate to damaged tissue and initiate inflammation and repair processes (Cuenca et al., 2014). They are involved in the clearance of apoptotic neurons, angiogenesis, as well as in the development of retinal synapses (Wang et al., 2016). The role of microglial cells will be developed in more detail later.

The RPE is the outermost layer of the retina (**Figure 2**). It is involved in the visual cycle. It recycles retinoids that are used in the phototransduction process. This includes the conversion of all-trans-retinol to 11-cis-retinal, which is essential for photoreceptor cells to respond to light. Also, the RPE provides metabolic support for the outer segments of the photoreceptors. The RPE consists of a monolayer of hexagonal pigment epithelial cells, connected to each other by tight junctions, which contain the organelles necessary for the phagocytosis of the photoreceptors' outer segments. The epithelium is extended by apical microvilli. These microvilli make it possible to extend the surface of the RPE by nearly 30 times, which facilitates its interactions with photoreceptors (Boulton and Dayhaw-Barker, 2001). On the side of its basal membrane, the cells of the RPE are in contact with Bruch's membrane, which represents a zone of interactions between the RPE and choriocapillaris.

## **1.2 Vascularization of the posterior segment**

To ensure retinal proper function and to maintain optimal visual health, the retina is supplied by two separate capillary systems, the retinal vascular network and the choriocapillaris.

### **1.2.1 The retinal blood network**

Retinal arteries are the initial vessels that carry oxygen-rich blood from the central retinal artery into the retina. The central retinal artery is a branch of the ophthalmic artery, which originates from the internal carotid artery. The arteries branch off from the central retinal artery and divide into smaller arterioles and capillaries, which feed into the capillary beds of the retina. Capillaries are found in the ganglion cell layer, the inner plexiform layer, and the outer plexiform layer of the retina (Scholfield et al., 2007) (**Figure 2**). These capillaries play a crucial role in facilitating the exchange of oxygen, nutrients, and waste products between the blood and the retinal tissue. After the capillaries, the oxygen-depleted blood flows into venules, which merge to form retinal

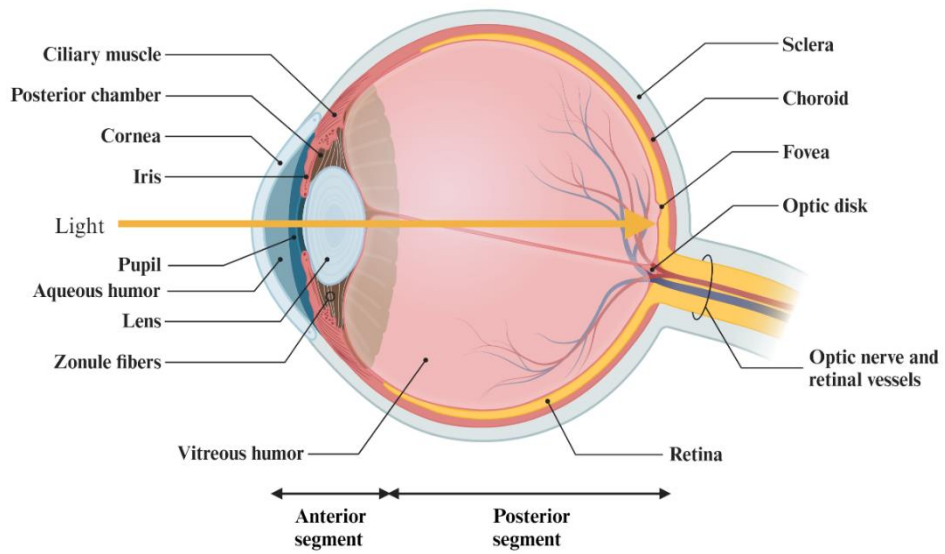


veins. The retinal veins transport deoxygenated blood and waste products away from the retina and back to the heart.

Retinal endothelial cells that line the interior surface of blood vessels within the retina by tight junctions, form a continuous layer known as the retinal endothelium (**Figure 3**). The basement membrane is a thin layer that provides support and anchorage for endothelial cells. Pericytes are present at intervals along the walls of retinal capillaries. The ratio of pericytes–endothelial cells is 1:1 in retinal capillaries (Shepro and Morel, 1993). Astrocytes connect to endothelial cells and pericytes, these three kinds of cells constitute the inner blood-retina barrier.

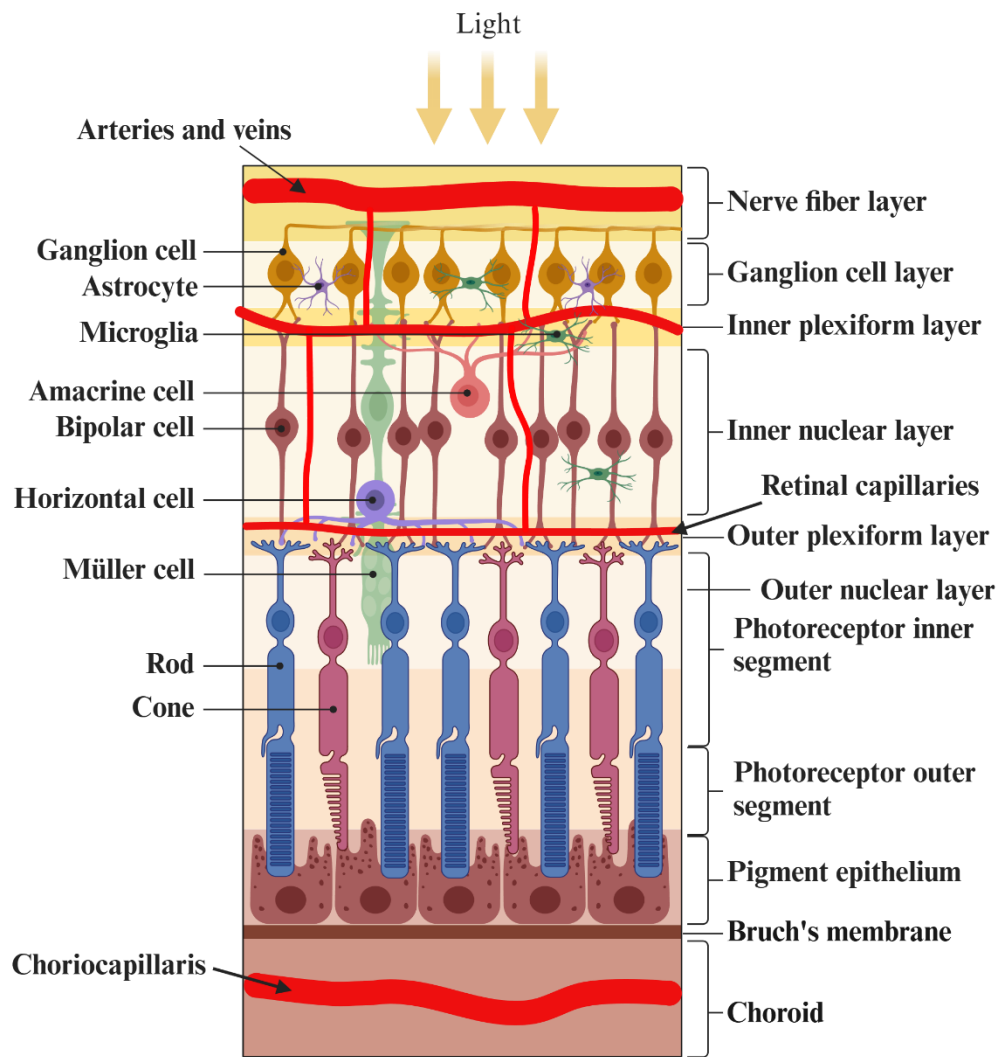
### **1.2.2 The choriocapillaris**

Choriocapillaris are characterized by an extremely high density of capillaries within the choroid. These capillaries are closely spaced and form a dense network that covers the entire choroidal tissue. Choriocapillaris are in close proximity to the outer layers of the retina, particularly the photoreceptor cells and the RPE. Unlike the retinal capillaries, the choriocapillaris are fenestrated due to the fenestrated endothelium, and there are much less pericytes in choriocapillaris. The shortage of pericytes contributes to the high permeability of the choriocapillaris. This high permeability allows for efficient exchange of substances between the blood and these metabolically active retinal layers. Although choriocapillaris are fenestrated, the RPE cells are joined together by tight junctions, which form a selective barrier between the choroid and the neural retina. The RPE and choriocapillaris together constitute the outer blood-retina barrier (Forrester J et al., 2021).



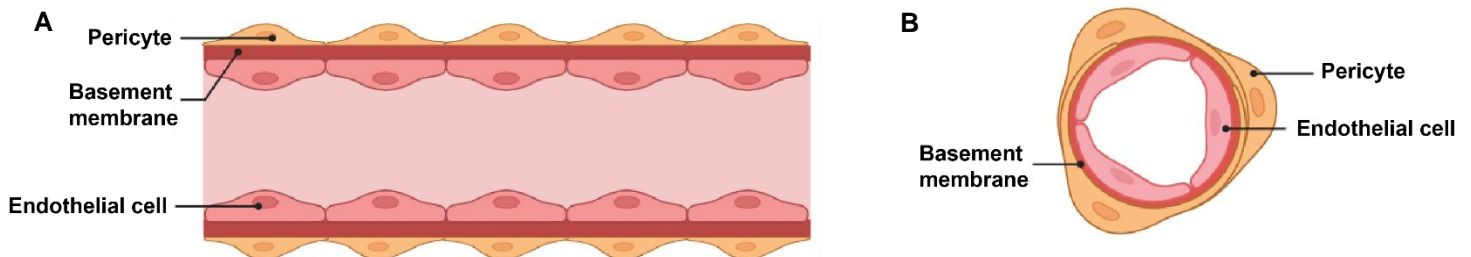
**Figure 1: Human eye anatomy.**

(Created with BioRender.com) The light rays (yellow) pass through the cornea, the aqueous humor, the lens, and the vitreous body to be finally captured by the retina.



**Figure 2: The different layers of retina.**

(Created with BioRender.com) Retinal capillaries (red) are found in the ganglion cell layer, the inner plexiform layer, and the outer plexiform layer of the retina.



**Figure 3: The structure of retinal vessels**

(Created with BioRender.com) (A) A longitudinal section diagram of a retinal capillary covered by pericytes, (B) A cross-sectional diagram of a retinal capillary showing the endothelial cell and pericyte interaction with a common basement membrane.

## **2. Retinal mononuclear phagocytes**

Mononuclear phagocytes include monocytes, monocyte-derived inflammatory macrophages, dendritic cells, and tissue resident macrophages such as microglial cells (Chow et al., 2011, Gautier et al., 2012, Ransohoff and Cardona, 2010, Wynn et al., 2013). Different types of resident and infiltrating mononuclear phagocytes play unique roles in tissue development, inflammation, and homeostasis. In the posterior segment of the eye, numerous resident microphages are located in the choroid and a network of microglia is located in the inner layers of retina, perivascular resident macrophages are along the major retina and choroidal vessels, while dendritic cells are rare in the retina (Forrester et al., 2010, Kumar et al., 2014, Streilein, 2003). In healthy individuals, the adult central layer of photoreceptor cells and the subretinal space typically lack both resident and infiltrating mononuclear phagocytes (Gupta et al., 2003, Combadiere et al., 2007, Eandi et al., 2016, Levy et al., 2015, Sennlaub et al., 2013). However, subretinal mononuclear phagocytes are present at the far periphery and during the fetal growth of the photoreceptor cell layer (McMenamin and Loeffler, 1990).

Microglia are the resident immune cells of the central nerve system (CNS) which constitute 5%-20% of the glial cells' population that also include Müller cells, astrocytes (Langmann. 2007). In the retina, microglia are involved in the clearance of apoptotic neurons, synaptic pruning during development, maintenance of synaptic structure and function, and regulation of inflammatory responses (Huang et al., 2012, Wang et al., 2016, Wang et al., 2014). Moreover, retinal microglia can contribute to retinal angiogenesis which we mainly focus on in our project (Checchin et al., 2006).

### **2.1 Origin of retinal microglia**

The origin of microglia has been a topic of debate and research for many years. Historically, there were various theories about their origin, with some suggesting they arise from the bone marrow, while others believed they have a neural origin. However, recent evidence strongly

supports the idea that microglia originate from progenitors of the hematopoietic system at a very early stage of development, when it is located in the yolk sac (Ginhoux et al., 2015, Hoeffel et al., 2018). These progenitors migrate to the developing CNS including the retina, where they differentiate into microglia under the influence of the CNS microenvironment. This origin sets microglia apart from other immune cells and gives them distinct properties and functions within the CNS.

## **2.2 Distribution of retinal microglia**

Microglia are evenly distributed throughout the early stages of the developing retina (Ashwell et al., 1989), but as the retina matures, their distribution becomes limited to the ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL) and the outer plexiform layer (**Figure 2**) (Santos et al., 2008). In a healthy state, few microglia are located in the outer retinal layers. However, under pathological conditions, microglia can be found not only in the outer nuclear layer (ONL) but also in the subretinal space (Zeng et al., 2000).

## **2.3 Functions of retinal microglia**

As the primary immune cells of the retina, microglia play an essential role in maintaining retinal homeostasis including surveillance, protection, and response activities for injury and inflammation. What's more, retinal microglia participate in the retinal angiogenesis and neovascularization.

### **2.3.1 Immune functions**

The retina's immune protection is, among others, maintained by the blood-retinal barrier (BRB) and an immune-suppressive environment. BRBs act as the primary defense, preventing the retina from pathogens and systemic inflammation. If BRBs are breached, various retinal cells work to defend the retina (Chen et al., 2019). Microglia, considered to be the retina's immune sentinels, engage in immune regulation through processes such as phagocytosis, complement activation, antigen presentation, and involvement in inflammation.

#### **2.3.1.1 Phagocytosis of debris**

Phagocytosis is traditionally viewed as a primary function of retinal microglia. These cells help in synaptic pruning, clearing pathogens, cellular waste, and dead cells, and influence retinal disease progression. Microglia express various receptors, such as Toll-like receptors (TLR), Dectin1, Fc receptors (FcR), complement receptors (CR), and scavenger receptors system (Sierra et al., 2013, Rathnasamy et al., 2019). These receptors play a crucial role in recognizing pathogens in the central nervous system (Sierra et al., 2013, Rathnasamy et al., 2019). After the connection between microglia and phagocytic targets, other receptors expressed on microglia, such as phosphatidylserine receptors (PSR), Mer tyrosine kinase (MerTK), vitronectin ( $\alpha\beta3$  and  $\alpha\beta5$  integrins), and triggering receptors expressed on myeloid cells-2 (TREM2), are involved in the elimination of apoptotic cells by microglia. (Hisatomi et al., 2003, Underhill et al., 2012).

#### **2.3.1.2 Complement activation**

The complement system is a crucial part of the innate immune response that lays a vital role in host defense against pathogens, as well as in mediating inflammatory responses. It consists of three pathways: the classical, lectin, and alternative pathways. All three pathways share the common step of activating the central component C3, but they differ according to the nature of

the target recognition (Bonifati and Kishore, 2007). At inflammatory sites, CFH, an abundant soluble plasma factor participating in the alternative pathway, is secreted by mononuclear phagocytes including microglia and macrophages (Calippe et al., 2017, Gautier et al., 2012, Luo et al., 2011, Schlaf et al., 2001). Also, the expression of C1q, the initiating protein of the classical pathway, is upregulated by activated microglia in retinal ischemia/reperfusion injury (Silverman et al., 2016).

### **2.3.1.3 Antigen presentation**

Antigen presentation is an important immune function that can activate T cells and participates in the pathogenesis of multiple diseases such as diabetic retinopathy, uveitis, and multiple sclerosis (Li et al., 2021, Almolda et al. 2011, Lipski et al., 2017). Retinal microglia express diverse antigenic markers, such as leukocyte and macrophage antigens, CD45 and CD68. A subpopulation of CD45<sup>+</sup> retinal microglia express MHC II (Yang et al., 2000), an important marker restricted to cells that can present antigens to CD4<sup>+</sup> T helper cells. When challenged by IFN- $\gamma$ , microglia isolated from mouse retina displayed enhanced antigen presentation abilities and upregulation of MHC II (Matsubara et al., 1999). CD11c, which is a marker of dendritic cells, can also be expressed by microglia (Immig et al., 2015).

### **2.3.1.4 Inflammation**

Inflammation is the body's defense response to infection and tissue damage. Inflammation is beneficial as it helps fight off invaders and repair damage. However, if it lasts too long, it can be harmful due to the overproduction of bactericidal substances and cytokines and chemokines, which also cause collateral damage.

In the retina, microglia participate in inflammation by synthesizing proinflammatory cytokines, including tumor necrosis factor-alpha (TNF- $\alpha$ ) and interleukin-1 beta (IL-1 $\beta$ ). TNF- $\alpha$  is the

activation of the extrinsic apoptotic pathway, which can lead to the apoptosis of retinal ganglion cells and the optic nerve degeneration (Kitaoka et al., 2006). TNF- $\alpha$  plays a role in the development of choroidal neovascularization (CNV) by increasing the production of vascular endothelial growth factor (VEGF) in RPE cells (Wang et al., 2016, Lichtlen et al., 2010). Patients with monocytes that express the greatest amount of TNF $\alpha$  have a higher prevalence of CNV (Cousins et al., 2004). IL-1 $\beta$  is a potent survival factor for endothelial cells and induces similar genes than VEGF (Schweighofer et al., 2009) and it can exacerbate CNV (Lavalette et al., 2011, Olson et al, 2009). Also, IL-1 $\beta$  can stimulate the growth and activation of microglia (Todd et al. 2019). Microglia-derived IL-1 $\beta$  can increase the production of chemokines in RPE and Müller cells, leading to retinal degeneration (Natoli et al. 2017).

Microglia in the retina also produce chemokines, which are small immune regulatory molecules that can direct the recruitment and migration of leukocytes to the site of injury during immune surveillance and inflammation. Retinal microglial cells express CX3C chemokine receptor 1 (CX3CR1) and homozygosity for the CX3CR1 M280 allele increases the risk of AMD (Christophe et al., 2007). Chemokines secreted by microglia, such as MCP-1 (CCL2), MIP-1 $\alpha$ , CCL5, and MCP-3, are implicated in retinal neuronal apoptosis (Crane et al., 2001, Kohno et al., 2014, Nakazawa et al., 2007, Yang et al., 2007). Contrary to inflammatory monocytes, microglia do not express or induce CCR2, the receptor of the chemokine MCP-1 (CCL2). Therefore, CCR2 becomes a molecular marker that differentiates inflammatory monocytes and inflammatory macrophages from microglia (Sennlaub et al., 2013, Saederup et al., 2010, Mizutani et al., 2011).

Inflammasomes are important components of the body's innate immune system and are related to various inflammatory conditions. They consist of multimeric protein complexes that include an inflammasome sensor molecule. This sensor molecule connects to caspase 1 through an adaptor protein called apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC). In the CNS, the NLRP3 (NOD-like receptors, Pyrin domain containing 3) inflammasome, ASC, and pro-caspase-1, are expressed by activated microglia and macrophages in the brain and retina (Gustin et al., 2015, Halle et al., 2008, Sanz et al., 2009).

Retinal microglia also produce other inflammatory factors, such as nitric oxide (NO) and ROS (Sierra et al., 2014, Wang et al., 2014). Lipopolysaccharide (LPS) stimulation can upregulate



inducible nitric oxide synthase (iNOS) in microglia and then mediate the production of NO (Sierra et al., 2014). In cultured retinal microglia, ROS production was increased in microglia after activation with LPS (Wang et al., 2014).

### **2.3.2 Roles in angiogenesis and neovascularization**

Retinal angiogenesis refers to the formation of new blood vessels from pre-existing ones during retinal development. Microglia move into the retinal tissue before blood vessels do (Arnold et al., 2013). They have a distinct function in both angiogenesis and abnormal vessel growth under disease conditions called neovascularization. The process of angiogenesis involves several steps: (1) Proangiogenic factors that promote vessel growth activate endothelial cells in the existing blood vessels. (2) These activated endothelial cells release enzymes that break down the surrounding material, such as extracellular matrix. (3) The endothelial cells then proliferate and migrate to form new vessels. (4) Maturation of new vessels. (5) Stabilization of new vessels (**Figure 5A**) (Risau, 1997, Kolte et al., 2016).

The role of microglia in retinal angiogenesis can be divided into several parts: vascular branching, vascular anastomoses, and vascular regression.

1.) Microglia can regulate the branching of developing superficial vessels of the retina (Arnold et al., 2013). Microglia are located at the neuron-neurite interface and stimulate the branching of vessels at this interface (Simmons et al., 2016). Decreased retinal microglia numbers can result in a decreased retinal vascular density and reductions in vascular branching (Arnold et al., 2013, Rymo et al., 2011, Kubota et al., 2009).

2.) Microglia also play an important role in the retinal vascular anastomoses. Retinal microglia can be quite close to endothelial tip cells and facilitate the fusion of neighboring tip cells, enabling the formation of anastomoses between adjacent vessels (Rymo et al., 2011).

3.) Microglia can regulate retinal vascular regression. There was decreased retinal vascular regression when microglia number reduced (Fantin et al., 2010). Microglia can also modulate VEGF-mediated angiogenic processes during vascular development and remodeling through the Wnt signaling pathway (Stefater et al., 2011).

## **2.4 Colony-stimulating factor 1 (CSF1)**

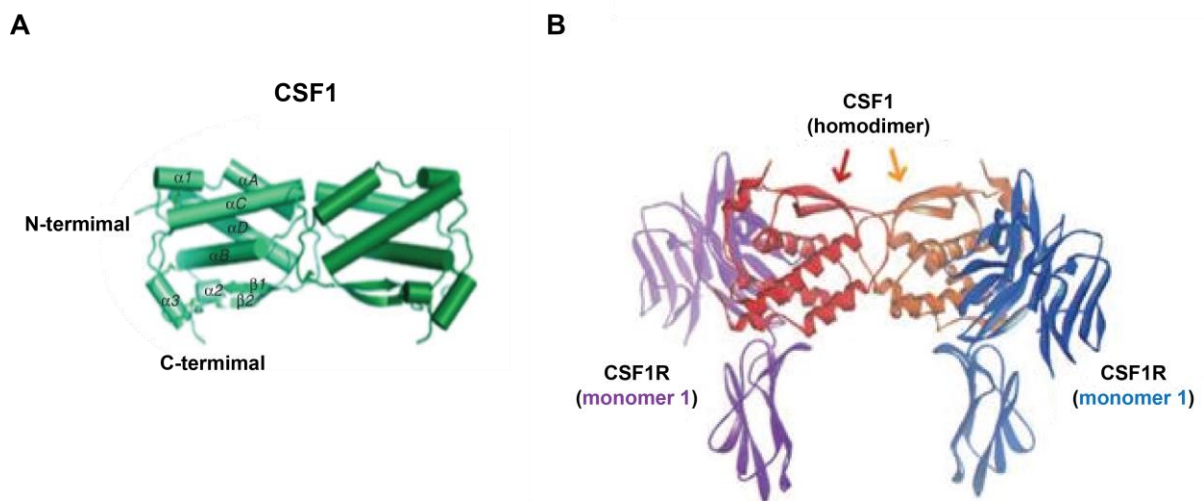
Colony-Stimulating Factor 1 (CSF1), also known as macrophage colony-stimulating factor (M-CSF), is a cytokine that plays a crucial role in the regulation of survival, proliferation, and differentiation of mononuclear phagocytes. CSF1 is a homodimeric glycoprotein which is composed of two identical subunits. Each subunit of CSF1 consists of three distinct domains: an N-terminal domain, a cytokine domain, and a C-terminal domain (**Figure 4A**) (Pandit et al., 1992, Ma et al., 2012).

Colony-Stimulating Factor 1 Receptor (CSF1R), also known as macrophage colony-stimulating factor receptor (M-CSFR) or CD115, a transmembrane receptor with the tyrosine kinase activity (**Figure 4B**). It is the primary receptor for CSF1 and Interleukin-34 (IL-34), both of which are cytokines that regulate the survival, proliferation, and differentiation of mononuclear phagocytes. CSF1R is composed of an extracellular ligand-binding domain, a single transmembrane domain, and an intracellular kinase domain (Pandit et al., 1992, Ma et al., 2012).

In the CNS, CSF1 is widely expressed by microglia (Nandi et al., 2012, Imai and Kohsaka. 2002). The activation of microglia is mediated by the CSF1-CSF1R loop, leading to reactive microgliosis and the release of inflammatory cytokines, such as IL-1 $\beta$ , macrophage inflammatory protein 1 $\alpha$ , IL-6, and CSF1, and nitric oxide (Hao et al., 2002). The lack of CSF1 in mice leads to the absence of microglial cells in the retina during the first week of postnatal development (Rymo et al., 2011). In Alzheimer's disease mouse models, microglia with elevated expression of CSF-1R are found surrounding plaques (Murphy et al., 2000). CSF1 also promotes the clearance of amyloid-beta (A $\beta$ ) by enhancing microglial phagocytic activity (Mitrasinovic et al., 2003). Furthermore, CSF1 plays a role in regulating the population of microglia (Zelante et

al., 2012, V Chitu et al., 2016). For instance, in brain, when CSF1 is blocked in adult mice, it selectively depletes microglia in white and gray matter regions (Easley-Neal et al., 2019). Also, CSF1R-targeted microglial modulation has a significant impact on the development and maintenance of neurons and neural networks (Bollinger and Wohleb, 2019).

Endothelial cells can also express CSF1 under both physiological and pathological conditions (Nakano et al., 2007, Albrecht et al., 2004). For instance, physiologically, endothelial cells produce CSF1 to guide progenitor macrophages towards the alternative differentiation pathway (He et al., 2012). Pathologically, CSF1 that is produced by endothelial cells contributes to osteoclastogenesis and bone destruction in rheumatoid arthritis (RA) (Nakano et al., 2007). CSF1 released from human choroidal vascular endothelial cells (HCVEC) under hypoxic conditions plays a crucial role in boosting the formation of CNV (Zhou et al., 2021). It promotes the proliferation, migration, and tube formation of HCVECs in a CSF1/CSF1R-dependent manner. CSF1/CSF1R blockade has been shown to ameliorate the formation of CNV in a mouse model of laser-induced CNV (Zhou et al., 2021).



**Figure 4: Structure of CSF1 and CSF1-CSF1R complex**

(A) (Modified according to Stanley et al., 2014) CSF1 consists of three distinct domains: an N-terminal domain, a cytokine domain, and a C-terminal domain.

(B) (Modified according to Muñoz-Garcia et al., 2021) One CSF1 homodimer is binding to two CSF1R monomers. The red and orange parts are monomers of CSF1; the purple and blue parts are monomers of CSF1R.

### **3. Angiogenesis and neovascularization**

Angiogenesis, the fundamental biological process involving the formation of new blood vessels from pre-existing ones, plays a crucial role in various tissues and organs, including skin, muscles, reproductive organs, bones, livers, lungs, hearts, eyes, and the CNS, etc. Angiogenesis is important in normal physiology, such as wound healing, tissue growth and development, reproductive system, and physical activity. It also plays a role in pathological conditions, such as cancer, cardiovascular diseases, inflammatory diseases, and ocular diseases (Jeong et al., 2021, Hall, 2005, Takahashi and Shibuya, 2005).

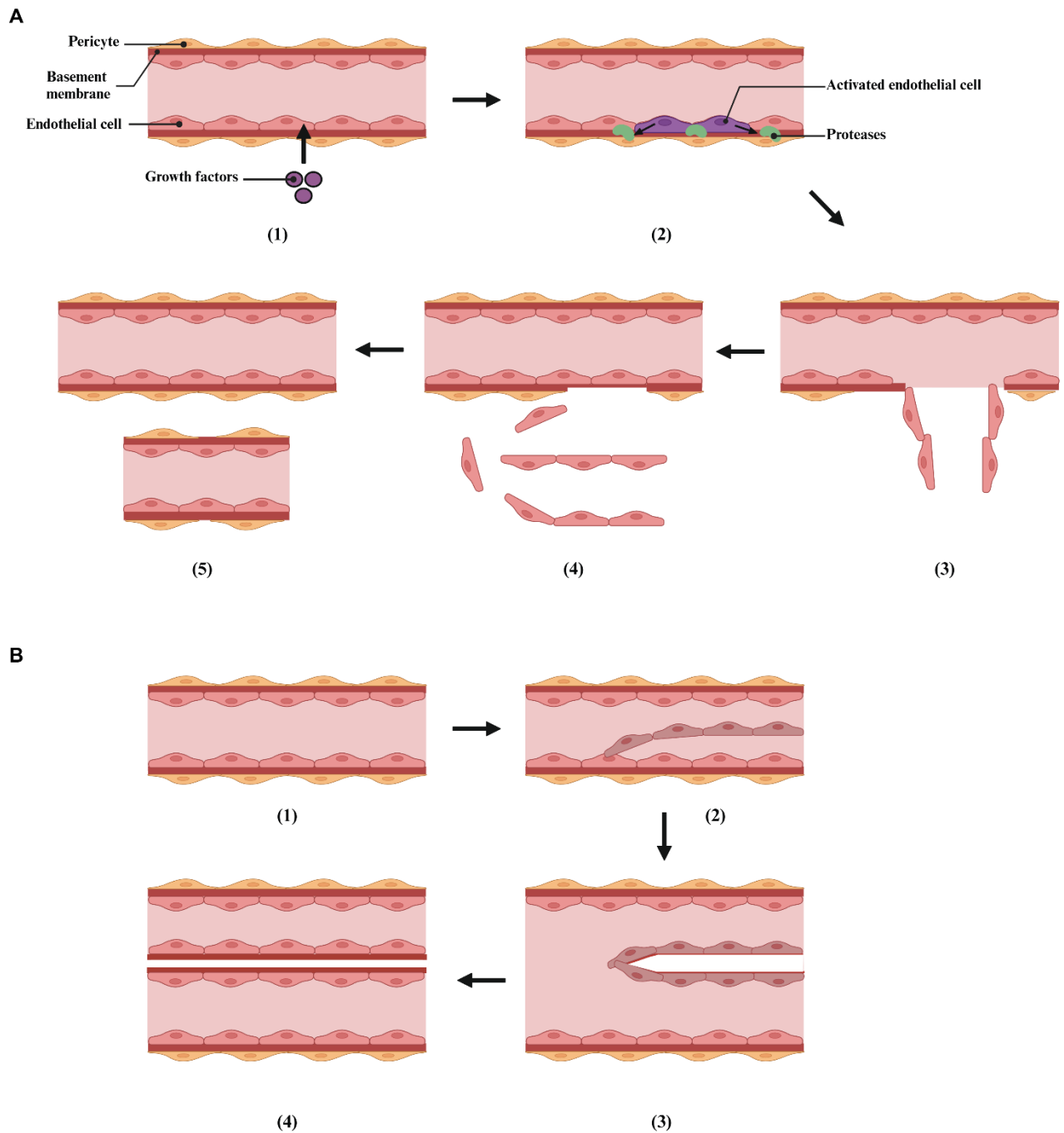
#### **3.1 Types of angiogenesis**

There are two types of angiogenesis: sprouting and intussusceptive (or non-sprouting) angiogenesis (Risau, 1997).

##### **3.1.1 Sprouting angiogenesis**

In sprouting angiogenesis, endothelial cells in pre-existing vessels are activated by growth factors, such as the vascular endothelial growth factor (VEGF). Certain activated endothelial cells then secrete proteases that degrade the pre-existing vessel basement membrane allowing them to pass out of the vessel and migrate and proliferate into the surrounding extracellular matrix (ECM), which includes collagen IV and laminin. The process is initiated by a “tip cell” that leads the sprouting process and the “stalk cells” follow forming a chord. As the vessel matures the stalk cells form a lumen and synthesize a surrounding basement membrane (**Figure 5A**). Some of the nascent vessels are pruned so that an appropriate level of oxygenation is provided to the tissues and others are surrounded by pericytes, stabilized and mature into capillaries (Bishop, 2015). After the primary vascular plexus is formed, more endothelial cells

are generated, producing new capillaries by sprouting or by splitting from their vessel of origin (Risau, 1997).



**Figure 5: Two types of angiogenesis**

(Created with BioRender.com) **(A)** Sprouting angiogenesis. (1) Endothelial cells are activated by growth factors. (2) Activated endothelial cells release proteases that degrade extracellular matrix. (3) Endothelial cells migrate and proliferate. (4) Endothelial cells start to mature into a new vessel. (5) Stabilization of the new vessel. **(B)** Intussusceptive angiogenesis. (1) Stable vessel. (2) Extension of a pre-existing vessel

wall into the lumen. (3) The new vessel wall proliferates and splits the pre-existing vessel. (4) Stabilization of the new vessels.

### **3.1.2 Intussusceptive angiogenesis**

During Intussusceptive angiogenesis, the vessel wall extends into the lumen of a pre-existing blood vessel splitting it into two (**Figure 5B**). Firstly, extension of a pre-existing vessel wall into the lumen occurs. Then the new vessel wall proliferates and splits the pre-existing vessel. Distinct from sprouting angiogenesis, intussusceptive angiogenesis only requires reorganization of existing endothelial cells and does not rely on immediate endothelial proliferation or migration. Therefore, intussusceptive angiogenesis is thought to be faster and more efficient compared with sprouting angiogenesis. However, intussusceptive angiogenesis mainly causes new capillaries to develop where capillaries already exist (Adair et al., 2010).

### **3.1.3. Vasculogenesis**

Unlike angiogenesis, vasculogenesis involves the de novo formation of blood vessels from migrating angioblasts. During embryonic development, vasculogenesis can be described as following steps. 1.) Endothelial progenitor cells (angioblasts) are mobilized from the mesoderm, which is the middle layer of cells in the developing embryo. 2.) These angioblasts migrate to specific areas where they aggregate to form blood islands, the earliest discernible vascular structures in the embryo. 3.) Within these blood islands, some cells differentiate into endothelial cells, which will form the lining of the blood vessel, while others become hematopoietic stem cells, which will eventually give rise to various blood cells. 4.) The endothelial cells then organize themselves into tubular structures, forming the primitive vascular network. 5.) The newly formed vessels undergo further maturation and remodeling to become functional blood vessels. 6.) These new vessels are integrated into the existing vascular network, and blood flow begins (Kolte et al., 2016).

### **3.2 Definition of neovascularization**

Neovascularization refers to the formation of new blood vessels. It can occur through two primary mechanisms: angiogenesis and vasculogenesis.

Neovascularization is essential for many normal physiological processes, including wound healing, embryonic development. However, it can also contribute to various diseases, such as tumor growth, eye diseases (AMD, ROP), and chronic inflammation (rheumatoid arthritis).

### **3.3 Angiogenesis and neovascularization in the CNS**

The central nervous system (CNS), consisting of the brain and spinal cord, possesses a highly specialized vasculature to meet the demands of this metabolically highly active tissue as well as to protect sensitive neurons from toxic metabolites and xenobiotics. The tissue-specific functions of CNS blood vessels are regulated by the neurovascular unit (NVU), which is characterized by cross-talk between neural and vascular cells (**Figure 6**). In the NVU, endothelial cells, pericytes, and vascular astrocytes are closely opposed to each other and interact to form the blood–brain barrier (BBB). These cells play a crucial role in providing the brain with a sufficient supply of oxygen and nutrients.

1.) Endothelial cells in the CNS are distinct from non-CNS endothelia. This distinction is due to the presence of tight junctions between the cells and specific enzymes and transport proteins that are unique to the BBB. CNS endothelial cells express specific transporters on the cell surface to transport small molecules such as glucose, amino acids, vitamins, and nucleosides. They also express transport proteins to actively transport large molecules such as transferrin, insulin, leptin, and LDL from the blood into the brain. On the other hand, they express transporters such as the multidrug resistance protein P-glycoprotein to actively export cell permeable xenobiotics back into the blood (Aller et al., 2009).

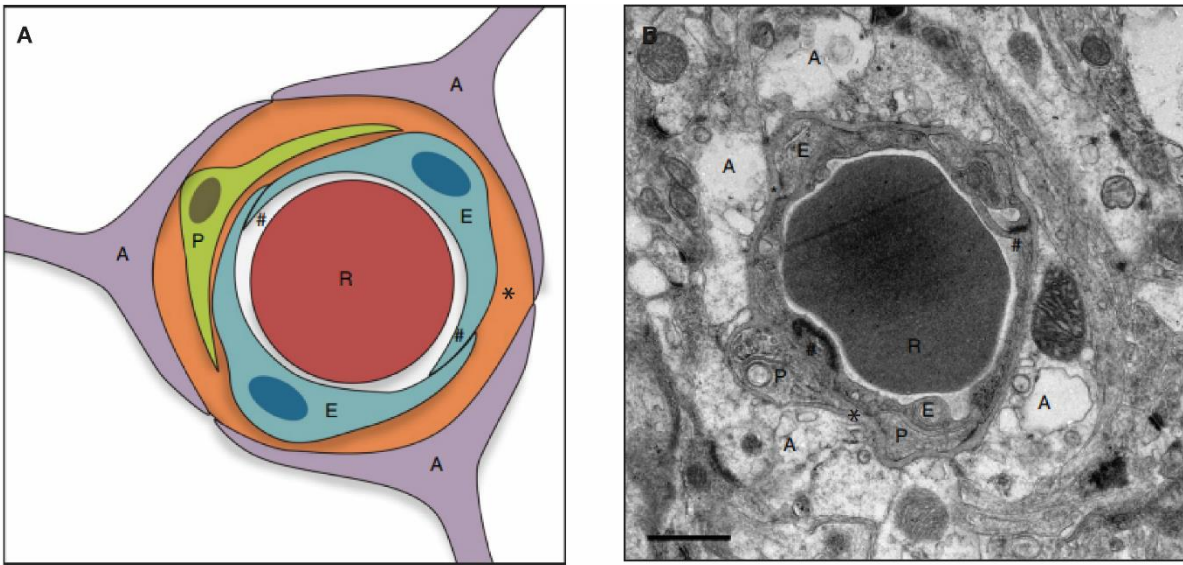
2.) CNS blood vessels have a higher coverage of pericytes than blood vessels of other organs (Shepro and Morel, 1993). CNS pericytes are essential for the formation and maintenance of the BBB. They help stabilize the endothelial cells that form the BBB (Armulik et al., 2010, Daneman et al., 2010). Furthermore, they are located on the walls of capillaries and can regulate blood flow by controlling the diameter of the capillaries. Pericytes also contribute to the production of the extracellular matrix, which provides structural support to the blood vessels.

3.) Astrocytes are star-shaped cells with branching processes that extend out in multiple directions from the cell body. CNS astrocytes extend their processes to blood vessels, where they form end-feet that almost entirely cover the vessel surface. These end-feet play a crucial role in maintaining the integrity of the BBB and regulate BBB function (Abbott et al., 2006). In addition, vascular astrocytes can deliver nutrients from the blood to adjacent neurons (Figley et al., 2011). They can also mediate neuronal regulation of blood flow in blood vessels of the brain (Zonta et al., 2003).

Initial vascularization of embryonic tissues mainly occurs through vasculogenesis, the differentiation of mesoderm-derived angioblasts into blood vessels, whereas at later stages of embryogenesis and in the adult neovascularization mainly occurs through angiogenesis, the sprouting of new blood vessels from pre-existing vessels. The developing CNS is exceptional as its vessels are exclusively formed by angiogenic sprouting of vessels from the perineural vascular plexus (Flamme et al., 1997).

Angiogenesis is regulated by proangiogenic and antiangiogenic factors. These molecules can be released by normal, tumor, endothelial, and stromal cells as well as from leukocytes and the extracellular matrix. Proangiogenic factors include VEGF, fibroblast growth factors, placental growth factor (PlGF), and interleukins. Antiangiogenic factors include angiostatin, endostatin, and thrombospondins 1 and 2 (Fukumura et al., 1998, Fang et al., 2000, Folkman et al., 2006, Carmeliet and Jain, 2000, O'Reilly et al., 1994). The “angiogenic switch” is off while expression of proangiogenic and antiangiogenic factors is balanced. If expression of proangiogenic factors is increased or expression of antiangiogenic factors is decreased, angiogenesis is induced (Vallon et al., 2014).





**Figure 6: The neurovascular unit.**

(Modified by Vallon et al., 2014) **(A)** Schematic representation of the neurovascular unit in a brain capillary of an adult mouse. **(B)** Electron microscopic image of the neurovascular unit in a brain capillary of an adult mouse. *A*: astrocyte end-foot. *E*: endothelial cell. *P*: pericyte. *R*: red blood cell. *Asterisk*: basement membrane. *Hash symbol*: tight junction. *Scale bar*: 1  $\mu\text{m}$ .

CNV angiogenesis is necessary for embryonic development as well as recovery from ischemic stroke and brain injury (Jin et al., 2006, Ye et al., 2010). In contrast, induction of CNS angiogenesis also contributes to pathological conditions such as brain tumor growth, neurodegenerative diseases, and arteriovenous malformations (Papetti and Herman, 2002, Chen et al., 2013, Rothbart et al., 1996).

### 3.4 Important molecules and signaling pathways in CNS angiogenesis

Angiogenesis in the CNS tightly regulated to ensure the proper delivery of oxygen and nutrients to neural tissues and to maintain the BBB. Several molecules and signaling pathways play pivotal roles in CNS angiogenesis, including the VEGF, integrins, TGF $\beta$ , PDGFB and Wnt signaling.

### **3.4.1 VEGF**

The VEGF family has multiple members including VEGF-A, B, C, D, and PlGF which bind to the receptors VEGFR-1, VEGFR-2 or VEGFR-3 and the Neuropilin-1 and Neuropilin-2 (Nrp-1 and Nrp-2) co-receptors (Ferrara et al., 2003). Hypoxia and glucose deprivation are major triggers of VEGF expression during CNS development. Cells respond to hypoxia by stabilizing transcription factors of the hypoxia inducible factor (HIF) family (Breier and Risau, 1996, Wang et al., 2012). As the cortex develops, neurons become the predominant source of VEGF. Once vascular remodeling is complete, glial cells become the predominant producers of VEGF in the CNS (Ogunshola et al., 2000).

VEGF signaling through VEGFR2/Nrp-1 is thought to be the main player in CNS angiogenesis (Raab et al., 2004, Haigh et al., 2003). VEGF can be alternatively spliced into several isoforms, with the larger isoforms having large basic domains binding to heparin and proteoglycans found in the extracellular matrix. The hairpin-binding VEGFs can mediate migration of endothelial cells into avascular areas of the CNS during development (Gerhardt et al., 2003). Activation of VEGFR-2 in endothelial cells can mediate migration, proliferation, and survival of endothelial cells (Olsson et al., 2006)

### **3.4.2 Integrins**

Integrins, which are heterodimeric proteins, are cell adhesion receptors involved in cell-to-cell or cell-to-extracellular matrix interactions, undergo bi-directional signaling, and serve as adaptor molecules. In the CNS, integrins can be expressed by neuroepithelial cells, endothelial cells, and glial cells such as astrocytes and microglia. They play an essential role for proper vascular development and eventual functions, since endothelial cell communication with the neuroepithelium is necessary as the vascular plexus migrates into the CNS (Proctor et al., 2005, McCarty et al., 2005). Mice experiments showed that conditional deletion of integrin  $\beta_8$  or  $\alpha_v$  in neuroepithelial cells caused abnormal brain angiogenesis characterized by dilated vessels, endothelial cell hyperplasia and hemorrhage (McCarty et al., 2005, Proctor et al., 2005).

### **3.4.3 TGF $\beta$**

The three isoforms of transforming growth factor  $\beta$  (TGF $\beta$ 1, TGF $\beta$ 2, and TGF $\beta$ 3) are multifunctional cytokines involved in development, cell differentiation, immune function, and cell cycle control. TGF $\beta$  is expressed in glial cells including astrocytes and microglia and endothelial cells of the developing CNS (Kriegstein et al., 2002, Pelton et al., 1990, Pelton et al., 1991). TGF $\beta$  signaling plays a critical role in vascular development and function by regulating endothelial cell proliferation and migration, fibronectin synthesis, and mural cell differentiation (Bohnsack et al., 2004, Perrella et al., 1998). Interestingly, integrins  $\alpha_v\beta_6$  and  $\alpha_v\beta_8$  affect TGF $\beta$  activity directly by activating latent forms of TGF $\beta$ 1 and TGF $\beta$ 3 (Annes et al., 2002, Mu et al., 2002). Integrin  $\alpha_v\beta_8$  expressed on astrocytes surrounding blood vessels has been proposed to act as an “angiogenic switch” in the CNS by activating TGF $\beta$  (Cambier et al., 2005).

### **3.4.4 PDGFB**

The ligand/receptor pair platelet-derived growth factor B (PDGFB)/PDGF receptor-beta (PDGFR $\beta$ ) a critical role in the recruitment of pericytes to newly formed vessels (Hoch et al., 2003). Initially, pericytes are recruited independent of PDGFR $\beta$  signaling (Hellstrom et al., 1999), while as angiogenesis starts, sprouting endothelial cells secrete PDGFB, which signals through PDGFR $\beta$  expressed by pericytes, resulting in their proliferation and migration during vessel maturation. In the postnatal mouse retina, PDGFB is expressed by the tip cells of the migrating vascular plexus, which generates a PDGFB gradient driving pericytes recruitment to stabilize the newly formed blood vessels as they penetrate into the CNS (Gerhardt et al., 2003).

### **3.4.5 Wnt signaling**

The canonical Wnt signaling pathway involves Wnt ligand-driven activation of Frizzled and LRP cell surface co-receptors, with Dishevelled-dependent stabilization of cytoplasmic  $\beta$ -catenin.

Subsequent  $\beta$ -catenin translocation into the nucleus and binding to Lef-1/TCF transcription factors then induces transcription of Wnt-responsive genes (van Amerongen et al., 2009). This pathway is an essential regulator of developmental CNS angiogenesis and BBB formation. Mice experiments showed that loss-of-function of either Wnt7a/Wnt7b (ligands of Wnt expressed in neural progenitor cells during CNS angiogenesis) or of  $\beta$ -catenin/Ctnnb1 in endothelial cells leads to angiogenic defects and subsequent vascular hemorrhage (Stenman et al., 2008, Daneman et al., 2009). Wnt signaling also express the BBB components Glut-1 and Claudin-3, which play an important role in BBB formation (Liebner et al., 2009).

### **3.5 Microglia in CNS vascular development**

In mice, yolk-sac derived macrophage precursors migrate into and colonize the whole embryo between E9.5 and E10.5 (Ginhoux et al., 2010, Schulz et al., 2012) and the brain is the first organ to be colonized. Microglia invade the brain through the pial surface, then migrate and proliferate, populating the brain. During this time, microglia associate with radial glia and blood vessels, and are found in close proximity to dying cells. Eventually, microglia are notably excluded from the neuroepithelium and cortical plate, and then are widely distributed in the adult brain, except in areas of densely packed neuron cell bodies. As for retina, microglia populate the embryonic retina well before post-natal retinal angiogenesis occurs (Fantin et al., 2010, Santos et al., 2008, Rymo et al., 2011). Shortly after entering the neuroepithelium, microglia associate with the developing blood vessels in the brain and retina (Kurz et al., 1998, Cuadros et al., 1993, Herbomel et al., 2001). Microglia and vessels may therefore directly influence each other's development (Rymo et al., 2011).

As microglia migrate into the CNS and retinal neuroepithelium before vessels do, microglia are uniquely positioned to influence the early sprouting, migration, anastomosis, and refinement of the growing CNS and retinal vascular systems. A study of rats showed that reducing microglia retinal microglia numbers was associated with a decrease in retinal vascular density, and intravitreal co-administration of microglia with clodronate restored vascularity of the developing retina (Checchin et al., 2006). Similarly, mice lacking retinal microglia had a significant decrease

in branching of the primary vascular plexus, and the angle of filopodia extending from tip cells was reduced (Rymo et al., 2011). Branching in these mice recovered as development progressed, suggesting that microglia principally effect developmental vascular remodeling (Kubota et al., 2009). Also, mice with pharmacologic microglial depletion had comparatively normal numbers of endothelial tip cells and filopodia, which suggests that microglia facilitate branching anastomosis, but not tip cell extension (Kubota et al., 2009). In the hindbrain, loss of microglia was associated with a significant decrease in the numbers of vascular branch points in the brain periventricular vascular plexus, without significant effects on the numbers of tip cells, filopodia, or radial vessels (Fantin et al., 2010). Furthermore, during retinal angiogenesis, Notch signaling may play a role in the microglia-endothelial cell interaction (Outtz et al., 2011, Hofmann et al., 2007).

## **4. Ocular angiogenesis and pathological neovascularization**

As we describe in Chapter 1, the retina is supplied by two separate vascular systems: the retinal vascular network which supplies the inner retina and the choriocapillaris which supplies the retinal pigment epithelium and outer retina. Retinal vasculature is perhaps the most studied vascular bed in the entire body, thanks to the increasingly advanced imaging technology in living patients (Bajwa et al., 2015). To study the biology of vascular growth both in physiological and pathological is valuable as there are numerous human conditions affected by pathological angiogenesis (Coorey et al., 2012, Ye, 2016, Stahl et al., 2010).

### **4.1 Ocular vasculature during development**

During the development of the ocular vasculatures in embryo, the hyaloid vessels develop first, followed by the choroidal vessels, and finally the retinal vascular network. Before the retinal vasculature finishes, the hyaloid vessels provide the inner retina and maturing lens with oxygen and nutrients, and choroidal vessels supply nutrients and oxygen to outer retina (Lutty and McLeod. 2018). Originating at the 4<sup>th</sup> week of gestation, the hyaloid vessels peak by the 9<sup>th</sup> week in human fetuses. As the retinal vasculature forms around mid-gestation, the hyaloid vessels simultaneously diminish. The central hyaloid, which is connecting the optic disk to the lens's rear, is the last to regress (Balazs et al., 1980, Zhu et al., 1999). The hyaloid artery enters the embryonic fissure, extends through the vitreous humor, and surrounds the lens (Balazs et al., 1980). Within this hyaloid network, efferent blood enters via a central hyaloid artery within the optic nerve, ramifies through hyaloid vessels in the vitreous, finally exiting via a venous choroidal net on the external eye.

Human choriocapillaris start to develop via vasculogenesis between 6th and 8th week of gestation (Hasegawa et al. 2007). At 11th or 12th week of gestation the development of intermediate choroidal vessels occurs by angiogenesis and forms the layer of medium-sized choroidal vessels named as Sattler's layer. It allows the choriocapillaris to connect with the

larger, outer choroidal vessels of Haller's layer, which is defined as the outer choroidal layer containing the large choroidal vessels. At 21st week of gestation, there are three distinct layers of choroidal vessels (Haller's layer, Sattler's layer, and the small-vessel layer located at inner choroid which contains small capillaries), which coincides with the beginning of photoreceptor cell differentiation (Baba et al., 2009, Hasegawa et al., 2007). At 22nd week of gestation, the choriocapillaris appears fully mature, with flat, thin-walled fenestrated vessels (Baba et al., 2009, Hasegawa et al., 2007, Luty et al. 2010). The general principles of choriocapillaris development are conserved between mice and humans, but there are different developmental timelines. In mice, the choriocapillaris exhibits a more mature and denser network of capillaries by 1 week after birth. The full maturation and stabilization of the choriocapillaris may continue for several weeks postnatally.

In humans, the process of retinal blood vessel formation begins around the 16<sup>th</sup> week of gestation and finishes roughly at the 40<sup>th</sup> week, just before birth (Provis et al., 2001, Roth, 1977). The most superficial layer of retinal vessels forms initially, originating from the optic nerve head and extending to the peripheral edge of the retina. By the 36<sup>th</sup> week, it reaches the retina's nasal boundary (Hughes et al., 2000). As the superficial layer nears completion, vessels dive deeper into the retina, creating the deep vascular layer at the outer plexiform layer's base. Subsequently, an intermediate layer emerges between the superficial and the deep layers, positioned above the inner plexiform layer, culminating in a three-layered vascular network (Dorrell et al., 2009). This three-layered vascular pattern is also observed in other mammals, although with different developmental timelines (Gariano and Gardner, 2005). In humans, retinal vascular development concludes before birth, contrasting with mice and rats where it happens after birth structurally similar to that in primates (Stone et al., 1995). In mice, the superficial vascular network starts to form during the first week after birth. In the following week, vessels from the superficial layer penetrate deeper into the retina, establishing the deep and then the intermediate layers. The entire retinal blood vessel system in mice reaches maturity three weeks after birth (Stahl et al., 2010).

## **4.2 Retinal pathological vasculature in diseases**

Angiogenesis is important in both physiological vascular development and pathological neovascularization in eyes. Endothelial cells proliferate and migrate to form new vessels from pre-existing vessels following guidance cues and angiogenic stimulators and inhibitors (details in Chapter 3). Dysregulated angiogenesis disrupts delivery of oxygen and nutrients, resulting in unbalanced metabolic demand and supply and disturbed neural retinal function. Pathological retinal neovascularization is characterized by leaky and tuft-like vessels, which are associated with retinal exudates and hemorrhage, leading to retinal damage, retinal detachment, or both (Al-Latayfeh et al., 2012). Abnormal ocular angiogenesis can occur in various eye disorders, such as retinopathy of prematurity (ROP), diabetic retinopathy (DR), and age-related macular degeneration (AMD) (Foos, 1987, Cunha-Vaz, 1978, Young, 1987).

### **4.2.1 Retinopathy of prematurity (ROP)**

#### **4.2.1.1 Basic description of ROP**

Retinopathy of prematurity (ROP) is a leading cause of visual impairments and even blindness affecting premature infants. In the United States, ROP affects approximately 16,000 infants per year (Good, 2004) and contributes to 6–18% of cases of blindness in this country (Lad et al., 2009). Supplemental oxygen administration for premature infants is the main risk factor for ROP (Patz et al., 1952, Patz et al., 1953, Silverman, 1980). Also, infants with low birth weight (<1500g) or young gestational age (<32 weeks) have higher risk of developing ROP (Bardin et al., 1997, Darlow et al., 2005).

As we mentioned above, retinal blood vessel formation begins around the 16th week of gestation and finishes just before birth, so premature infants have incompletely vascularized retinas with a peripheral avascular zone at birth (Foos, 1987). There are two phases of ROP (**Figure 7**). In the first phase, the relative hyperoxic environment after birth compared with that in utero suppresses the normal secretion of growth factors, such as VEGF and insulin-like growth factor 1 (IGF-1)



(Pierce et al., 1995, Hellström et al., 2001, Bang et al., 1994). This leads to the cessation of retinal vessel growth and loss of existing vessels (Pierce et al., 1996, Hellström et al., 2001). During the second phase of severe ROP, the poorly vascularized retina becomes ischemic due to increased metabolic activity, leading to tissue hypoxia. This triggers the revascularization in response to elevated levels of hypoxia-driven angiogenic growth factors such as VEGF, resulting in pathological neovascularization (Aiello et al., 1995). These new vessels are leaky and fail to adequately supply the retina, leading to the hemorrhage, fibrosis, and retinal detachment.

Oxygen-induced retinopathy (OIR) model is a well-established model for ROP nowadays. As we mentioned above, distinct from that in humans, in rodents the retinal vascular network develops after birth. To induce OIR, young mice or rats are kept in hyperoxia with their nursing mother from postnatal day 7 (P7) to postnatal day 12 (P12), then they are transferred into normal air. In the mouse model, within the retinal vasculature only capillaries in the center of the retina are obliterated by hyperoxia whereas capillaries in peripheral regions are spared (Smith et al., 1994). However, in rat model it is converse that vessels in retinal periphery regions are obliterated but not in the center region (Alon et al., 1995). Similar to human ROP patients, OIR rodents model involves two phases of pathological processes, vaso-obliteration and vaso-proliferation.

#### **4.2.1.2 Pathological vasculature in ROP**

Hyperoxia induces obliteration of retinal capillaries by apoptosis of endothelial cells and downregulates production of VEGF (Alon et al., 1995, Pierce et al., 1996., Dammann et al., 2023). Exogenous VEGF administration compensate for the deficit in endogenous VEGF and rescue the vasculature in OIR rat (Alon et al., 1995). Retinal VEGF is mainly produced by astrocytes, the Müller cells, and RPE, and hyperoxia downregulates VEGF expression in astrocytes and the Müller cells (Alon et al., 1995). Hence, the VEGF withdrawal caused by hyperoxia could induce the vaso-obliteration. Interestingly, there could be a gradient of VEGF in the retina due to the regulation of VEGF mRNA expression by oxygen tension (Provis et al., 1997). VEGF mRNA expressed by astrocytes is reduced in the vascularized, normoxic central retina, while it is not decreased in the non-vascularized (hypoxic) peripheral retina (Provis et al.,

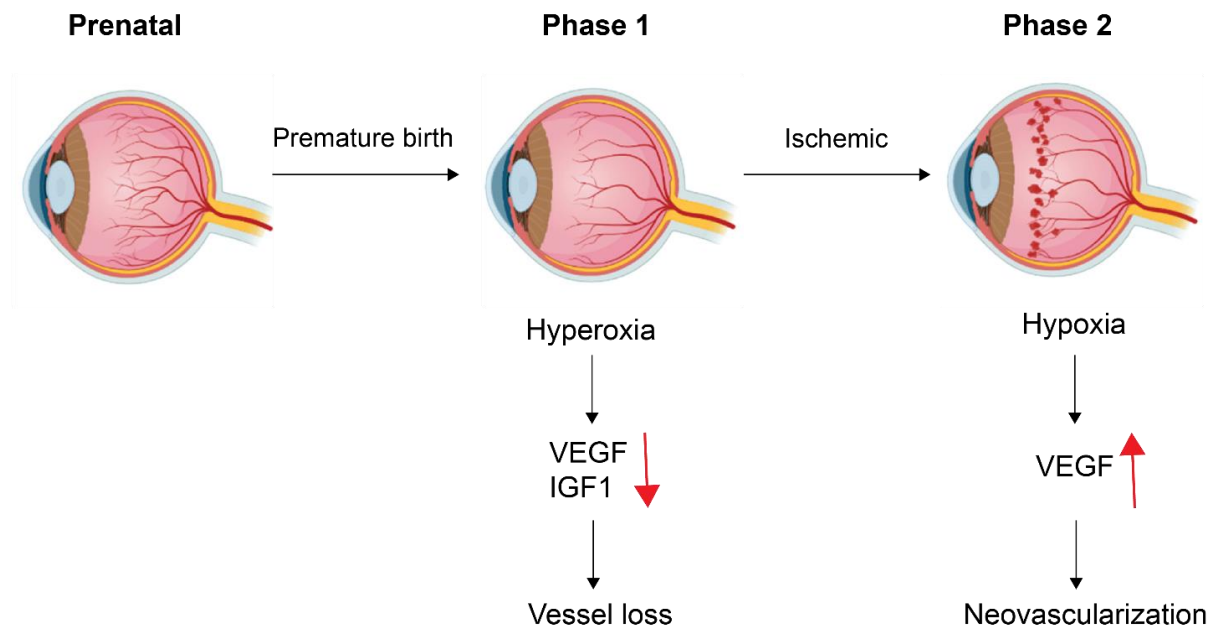
1997, Fruttiger, 2007). Intraocular injection of VEGF can "flood" the gradient and slow down vascular growth (Gerhardt et al., 2003). Additionally, mutant mouse strains with altered VEGF isoforms that affect its affinity for the extracellular matrix have shown flattened VEGF protein gradients and slowed vascular growth in the retina (Ruhrberg et al., 2002, Stalmans et al., 2002).

Vaso-proliferation initiates when the mice or rats are removed from hyperoxia environment into normal air. Retinal blood vessel proliferation can manifest as neovascularization or revascularization. When the mice return to normal air, the sudden lack of oxygen that triggers overexpression of growth factors such as VEGF (Gu et al., 2002). The upregulation of VEGF contributes to the formation of abnormal tufts of blood vessels, which leads to the pathological neovascularization (Ozaki et al., 2000, Sone et al., 1999). A gene-expression profile shows that, in OIR mice, the retinal expression of VEGF-A rises from P12 upon transfer from hyperoxia to room air, peaks at P16, and returns to P12 levels by P21 (Sato et al., 2009). This trend in VEGF-A expression is consistent with the observed trend in neovascularization, since neovascularization reaches its maximum at P17 and after P17 it spontaneously regresses between P17 and P25. (Sato et al., 2009, Connor et al., 2009). Therefore, there may be three conditions:

- 1.) under normoxia VEGF is at a balanced level required to maintain existing retinal vessels.
- 2.) Under hyperoxia VEGF is downregulated to a level below the 'maintenance dose', resulting in vessel regression.
- 3.) Under hypoxia, VEGF is upregulated to a level of 'angiogenic dose', resulting abnormal vessel growth.

Compare to neovascularization as pathological angiogenesis, revascularization is beneficial angiogenesis that can help nourish the retina. Astrocytes may play a role in revascularization, the persistence of retinal astrocytes within the vaso-obiterated areas may help facilitate normal revascularization and contribute to reduce the pathological neovascularization in OIR mice (Downie et al., 2008, Dorrell et al., 2010). Immune cells such as microglia and macrophages also participate in revascularization process. OIR mice show a decrease in microglia along with a reduction in vasculature (Checchin et al., 2006). Transplanted BM-derived progenitors into

microglia-lacked OIR mice accelerate retinal revascularization and reduce neovascular tuft formation (Ritter et al., 2006). Depletion of resident retinal microglia suppress normal angiogenesis, which can be restored by intravitreal microglial injection, suggesting that microglia have a role in guiding vessel growth in revascularization process (Checchin et al., 2006). M2 macrophages, the anti-inflammation subsets, are associated with neovascular tufts in OIR mice retina and depletion of M2 macrophages suppresses pathological neovascularization and promote physiological revascularization (Zhou et al., 2015).



**Figure 7: Pathogenesis of retinopathy of prematurity**

(Modified by Ryu, 2022) In the first phase, due to the premature birth, the relative hyperoxic environment compared with that in utero suppresses the normal secretion of VEGF and IGF-1, which leads to the cessation of retinal vessel growth and loss of existing vessels. In the second phase of severe ROP, the poorly vascularized retina becomes ischemic due to increased metabolic activity, leading to tissue hypoxia. This triggers the revascularization in response to elevated levels of VEGF, resulting in pathological neovascularization.

## **4.2.2 Diabetic retinopathy (DR)**

### **4.2.2.1 Basic description of DR**

Diabetic retinopathy (DR), a major ocular complication of diabetes mellitus, affects approximately 30 to 40% of diabetic individuals, which means more than 100 million people globally suffering from this condition (Yau et al., 2012, Ruta et al., 2013), and DR is a leading cause of vision loss especially in working-age population (Ting et al., 2016). There are two subtypes of DR, non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR). NPDR is the early stage of DR, hemorrhages and exudates are found in retina but there is no significant growth of new blood vessels. NPDR can progress to a more severe pathological state over time. PDR is the late stage of DR, and it is characterized by the formation of new abnormal blood vessels (neovascularization). These new vessels are fragile so they can rupture and bleed (Solomon et al., 2017). In this stage, patients might suffer severe vision loss due to bleeding of the new abnormal vessels into the vitreous (vitreous hemorrhage) or from tractional retinal detachment. The same as ROP, OIR model is also a widely used animal model for study in DR.

### **4.2.2.2 Pathological vasculature in DR**

DR is recognized as a microvascular disease. Hyperglycemia is believed to be a key factor in the pathogenesis of retinal microvascular damage. The initial reactions of retinal blood vessels to hyperglycemia include vessel dilation and alterations in blood flow, which are thought to be a metabolic autoregulation to enhance retinal metabolism in diabetic individuals (Bek, 2017, Ansari et al., 2022). Hyperglycemia also causes apoptosis of pericytes in the early stage of DR (Naruse et al., 2000, Romeo et al., 2002). Since pericytes are crucial for structural supporting for blood vessels, the loss of pericytes can lead to localized outpouching of capillary walls and breakage of blood vessels (Ejaz et al., 2008). In addition to loss of pericytes, there is also apoptosis of endothelial cells in DR, which contributes to the impairment of BRB (Beltramo and Porta, 2013). Loss of pericytes and endothelial cells together leads to capillary blockage and

ischemia, and the retinal ischemia/hypoxia causes increased VEGF production by activating HIF-1 (Huang et al., 2015). It has been shown that increased phospholipase A2 (PLA2) levels in diabetes also triggers the upregulation of VEGF (Lupo et al., 2013). Increased VEGF expression is observed in the retina of diabetic mice and in the vitreous of patients with PDR (Li et al., 2010, Aiello et al., 1994, Adamis et al., 1994). VEGF plays an important role in the progression of DR. VEGF is thought to enhance vascular permeability through the phosphorylation of tight junction proteins such as occludin and zonula occludens-1, leading to vascular leakage (Antonetti et al., 1999). Furthermore, VEGF increases neovascularization by enhancing proliferation of endothelial cells via activation of mitogen-activated protein (MAP) (Rousseau et al., 1997).

### **4.2.3 Age-related macular degeneration (AMD)**

#### **4.2.3.1 Basic description of AMD**

Age-related macular degeneration (AMD) is a common eye disease and a leading cause of vision loss worldwide among old people. Approximately 10 million people in the United States suffer from AMD (Friedman et al., 2004). The occurrence of AMD consistently rises with age, impacting 2% of individuals at 40 years old and reaching one out of every four individuals by the age of 80 (Friedman et al., 2004). AMD has risk factors such as age, smoking, and obesity (Smith et al., 1996, Vingerling et al., 1996, Hirvela et al., 1996, Zhang et al., 2016). There are two types of AMD: "dry" and "wet" (**Figure 8**). Dry AMD is a long-term condition that typically leads to some degree of vision loss gradually and can occasionally advance to severe blindness. On the other hand, wet AMD, which impacts only 10%–15% of those with AMD, appears suddenly and can quickly lead to blindness if left untreated (Guyer et al., 1986, Wong et al., 2008). In AMD's early stage, which shows no symptoms, the retina accumulates insoluble extracellular aggregates known as drusen (Bird, 2010). The late stage of dry AMD, often referred to as geographic atrophy (GA), is characterized by scattered or merging regions of degeneration of the RPE cells or the adjacent light-sensing retinal photoreceptors, which depend on the RPE for nourishment. The other late stage of AMD, the wet type, is distinguished by choroidal neovascularization (CNV). This involves the growth of newly immature blood vessels from the

underlying choroid, moving towards the outer retina. These immature blood vessels release fluid either below the retina or within it.

The rodent laser trauma model is a commonly used model to mimic the CNV in wet AMD. In this model laser photocoagulation is applied to rupture of Bruch's membrane, which leads to the growth of new blood vessels from the choroid into the sub-retinal space. The rodent laser trauma model has advantages such as its lower cost, capability to produce high numbers of CNV lesions for statistical evaluation, and its short time course. However, the main limitation of this model is that it doesn't fully mimic the complex sequence of events that leads to CNV in AMD. It represents a model of immediate damage and inflammation, rather than a result of stemming from prolonged age-related degeneration and chronic inflammation.

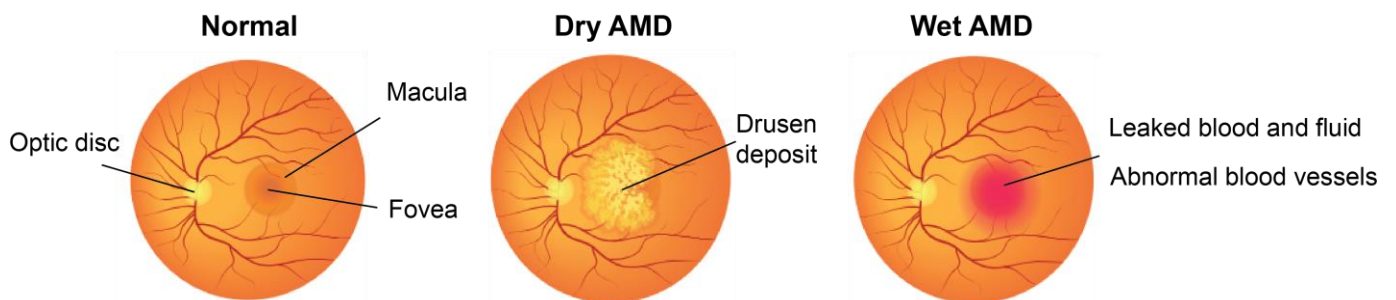
#### **4.2.3.2 Pathological vasculature in AMD**

As we mentioned above, the CNV is a pathological process involving the abnormal growth of new blood vessels from the choroid into subretinal space. Eyes exhibiting CNV show a reduction in choriocapillaries in the surrounding areas (McLeod et al., 2009). The CNV can lead to various complications and visual impairments including:

- 1.) Retinal edema: the accumulation of fluid in the retina, causing retinal edema. This can result in distorted or blurred vision.
- 2.) Subretinal exudation: the leakage of fluid, blood, and lipids into the subretinal space. This can lead to the formation of deposits and exudates, further affecting vision.
- 3.) Formation of fibrous membrane: If left untreated, CNV can result in the formation of a neovascular fibrous membrane. This membrane can cause scarring and further damage to the retina, leading to vision loss.

4.) Disciform subretinal scar: CNV can progress to the formation of a disciform subretinal scar. This scar can cause permanent damage to the retina and result in severe vision impairment (Sarks, 1976).

As previously discussed, VEGF plays a significant role in ocular angiogenesis and contributes to CNV. VEGF-A was identified in surgically acquired CNV samples from humans with AMD (Frank et al., 1996, Lopez et al., 1996). Increased VEGF secreted by RPE toward its basal side may contribute to the development of choroidal neovascularization (Blaauwgeers et al., 1999). Significant CNV was observed in the rats that were given subretinal injection of vectors expressing VEGF (Baffi et al., 2000, Spilsbury et al., 2000, Wang et al., 2003). Therefore, Anti-VEGFa therapy for CNV significantly enhances or stabilizes vision in most wet AMD patients nowadays (Gragoudas et al., 2004, Brown et al., 2006, Rosenfeld et al., 2006).



**Figure 8: Dry and wet type of AMD**

(Created with BioRender.com) In dry AMD, tiny yellow protein deposits called drusen form and accumulate under macula. The built-up deposits dry and thin your macula. In wet AMD, abnormal blood vessels develop under your retina and macula. These abnormal blood vessels leak blood and fluid.

## **5. Apnea and chronic intermittent hypoxia**

Sleep apnea is a common sleep disorder in which an individual's breathing repeatedly stops and starts during sleep. This interruption in breathing can occur hundreds of times in a night and often lasts for a duration of 10 seconds or more, leading to disrupted sleep and potential health complications (Wright et al., 1997, Ross et al., 1998). There are three main types of sleep apnea: obstructive sleep apnea (OSA), central sleep apnea (CSA), and complex sleep apnea. OSA is a prevalent sleep-related breathing disorder which is characterized by repetitive pharyngeal airway narrowing or collapse. In contrast, CSA, often seen in congestive heart failure (Lanfranchi et al., 2003, Javaheri et al., 1998), involves a lack of breathing drive during sleep, leading to repetitive periods of insufficient ventilation. Complex sleep apnea is a combination of both OSA and CSA.

Chronic intermittent hypoxia (CIH) refers to be repeated episodes of low oxygen levels (hypoxia) followed by periods of normal oxygen levels (normoxia). While sleep apnea is the condition characterized by the recurrent significantly reduced (hypopnea) or absent (apnea) ventilation, CIH is one of the primary physiological consequences, and it plays a significant role in the health risks associated with untreated sleep apnea (Neubauer, 2001, Malhotra and White, 2002, Labarca et al., 2020).

A report of 67 786 OSA patients has been shown that OSA patients are considerably more likely to be subsequently diagnosed with AMD independently of obesity (Keenan et al., 2017), suggesting that OSA may be a strong environmental risk factor for AMD. A recent study of 115 preterm infants in India has been shown that premature infants with apnea of prematurity (AOP) has a higher risk for developing ROP (Kumar et al., 2023). Hence, apnea/CIH may become a risk factor for eye disease such as AMD in adults and AOP in infants.



## 5.1 Apnea in adults

Obstructive sleep apnea (OSA) is a common sleep disorder in human adults worldwide. It is estimated that around 936 million adults aged 30–69 years experience mild to severe OSA, while approximate 425 million in the same age range have moderate to severe forms of OSA globally (Benjafeld et al., 2019). In patients with OSA, airway obstruction leads to hypopnea or apnea, even with ongoing respiratory efforts. This causes hypoxemia and hypercapnia, intensifying the respiratory drive. However, without the airway reopening, this drive doesn't improve ventilation. The apnea/hypopnea persists until the patient wakes up and terminates the obstruction. Once the airway is open, there is a rush of breathing to correct the blood gas imbalances. The patient then falls back asleep, and the cycle repeats (Eckert et al., 2009). This repetitive pattern causes sleep disruption and, combined with intermittent hypoxia, contributes to daytime sleepiness, fatigue (Punjabi et al., 1999), neurocognitive dysfunction (Kim et al., 1997), cardiovascular issues (Shamsuzzaman et al., 2003, Arzt and Bradley, 2006), and a higher risk of car accidents (Young et al., 1997). People with excess weight have higher risk of developing OSA (Peppard et al., 2000, Young et al., 2002), and there is a higher prevalence of OSA in older persons ( $\geq 65$  years old) compared to middle-aged (30-64 years) individuals (Bixler et al., 1998, Young et al., 2002), indicating that obesity and age could be risk factors for OSA. Also, males are more likely to develop OSA compared to females (Bixler et al., 2001, Young et al., 1998), and smoking may contribute to the risk of OSA (Young et al., 2002, Wetter et al., 1994).

Central sleep apnea (CSA) is defined by cessation of airflow without respiratory effort. Unlike OSA, where there is continuous effort to breathe against a blocked airway, CSA is characterized by an absence of the drive to breathe during sleep. Between CSA and OSA there is considerable overlap in the pathophysiology, making them sometimes hard to distinguish. CSA is typically diagnosed when over 50% of apneas are central (meaning a pause in breathing for over 10 seconds without respiratory effort) during an overnight sleep study (Eckert et al., 2009). Similar to OSA, CSA disrupts sleep due to frequent awakenings, leading to daytime sleepiness and a higher risk of cardiovascular issues (Lanfranchi et al., 2003, Javaheri et al., 1998, Khayat et al., 2012).

## 5.2 Apnea in infants

Apnea of prematurity (AOP) is commonly defined as a temporary cessation of breathing that lasts for more than 20 seconds, or for more than 10 seconds with accompanying bradycardia and/or oxygen desaturations, in infants born before 37 weeks' gestation (Blackmon et al., 2003; Finer et al., 2006; Eichenwald et al., 2016). AOP is a frequently diagnosed condition in the Neonatal Intensive Care Unit (NICU) (Eichenwald et al., 2016), affecting almost half of infants born before 32 weeks' gestation and nearly all infants whose birthweight is below 1000g (Henderson - Smart. 1981). The majority of AOP is central apnea, as AOP is believed to be a result of immature respiratory centers in the brain, leading to insufficient ventilation of the lungs (Poets. 2010, Milner et al., 1980). Mixed apneic episodes (combination of central and obstructive events) can also occur in premature infants, since obstruction in airways may also be present during AOP (Milner et al., 1980, Ruggins et al., 1991, Dransfield et al., 1983). However, purely obstructive apneas are uncommon in premature infants with apnea (Al-Saedi et al., 1997).

The central respiratory center, which is located in the medulla oblongata, play a role of in regulating the rhythm and expansion of the chest during breathing. At rest, the dorsal respiratory group, which is located in the distal portion of the medulla, controls the normal breathing rhythm. Exhalation occurs due to decreased activity from the dorsal group and natural recoil of the chest wall. The ventral respiratory group of the medulla mainly handles active exhalation and rapid breathing, typically during exercise or when stimulated by external chemoreceptors, and the pre-Bötzing complex within the ventral respiratory group is essential for generating the breathing rhythm (Smith et al., 1991; Rekling et al., 1998, Smith et al., 2000).

Breathing movements stretch the lungs for growth during fetal development, and it does not functionally support gas exchange until after birth (Kitterman, 1996, Sanchez-Esteban, 2013). The immature respiratory center in the medulla of premature infants has difficulties in maintaining stable breathing patterns or respond well to respiratory stimuli from chemoreceptors, lung stretch receptors, or other regions of the brain (MacLean et al., 2015). This physiological immaturity is believed to be the primary cause of AOP (Fiore et al., 2012). For instance, the central response to CO<sub>2</sub> in the medulla primarily regulates respiratory drive (Darnall, 2010), but

premature infants can still have unstable breathing rhythms when exposed to high CO<sub>2</sub> levels (MacFarlane et al., 2012). Full-term infants adjust to high CO<sub>2</sub> by increasing both tidal volume and breathing rate. In contrast, premature infants only increase tidal volume, leading to slower breathing and oxygen desaturation (MacFarlane et al., 2012). This raises their CO<sub>2</sub> apneic threshold close to regular CO<sub>2</sub> levels, making them more prone to apneic events (Mathew, 2011, Khan et al., 2005).

Besides the brain's central respiratory centers, other mechanisms can also contribute to AOP. Premature birth causes dramatic changes in the physiology of the peripheral chemoreceptors, which are sensors in the carotid and aortic bodies. These sensors detect changes in O<sub>2</sub> and CO<sub>2</sub> levels and send signals through specific nerves (hypoglossal (IX) and vagus (X) nerves) to regulate breathing (Darnall, 2010). At birth, peripheral receptors are inactive in response to changes in O<sub>2</sub> saturations with the infant's first breath, and then their functions are restored gradually in full-term infants in order to respond appropriately to changes in blood gases (Gauda et al., 2004, Cohen et al., 2005, Blanco et al., 1984). However, CIH makes these receptors overly active, especially in babies with AOP (Gauda et al., 2004). This can lead to irregular breathing and poor response to high carbon dioxide levels in infants suffered from AOP (Rigatto et al., 1975, Nock et al., 2004).

Apneic events can be triggered by spontaneous neck flexion, and it is recommended to position prone infants on their bellies to prevent this (Mathew. 1985), since the airway requires consistent stimulation from neck muscles to stay open. Also, sleep plays a significant role in respiratory control. Premature infants experience more rapid eye movement sleep (REM, also known as active sleep) (Parmelee et al., 1967; Anders et al., 1985), which weakens the contraction of pharyngeal muscles and can lead to obstruction and apnea in infants with hypercompliant, narrow airways (Poets. 2010, Anders et al., 1985, Schulte et al., 1977).

### **5.3 Apnea/CIH and retinopathy**

Several studies have shown the relationship between intermittent hypoxemia and ROP. Intermittent hypoxemia refers to intermittent hypoxemic events where there is a decrease in the level of oxygen in the blood. Incidence of hypoxemic events was higher in infants with ROP, and severe ROP was associated with intermittent hypoxemia, making intermittent hypoxia a risk factor for ROP (Fiore et al., 2010, Fiore et al., 2012, Martin et al., 2011). Also, fluctuations in oxygen saturation in blood are an important factor in the development of ROP (York et al., 2004, Saito et al., 1993, Anderson et al., 2004). Therefore, optimized oxygen saturation technology, which is used for measuring blood oxygen levels to obtain essential information about a patient's respiratory status, may help to prevent ROP in premature infants (Castillo et al., 2010). Furthermore, a recent study in India showed that infants with AOP are more likely to be diagnosed as ROP, also indicating that apnea is a risk factor for ROP (Kumar et al., 2023).

In addition to ROP, severe hypoxemia during sleep is an important factor for developing DR (Banerjee et al., 2013). Patients with OSA are at a higher risk to be diagnosed as DR (Prudon et al., 2017, Chang et al., 2018, Kaba et al., 2022). Also, patients OSA have a significantly higher likelihood of being diagnosed with AMD, regardless of obesity (Keenan et al., 2017).

### **5.4 Apnea/CIH and inflammation**

Over the last 30 years numerous studies have explored the relationship between sleep apnea and inflammation in both adults and children. For example, CIH can cause inflammatory process in the carotid body which may contribute to upper airway dysfunction in both OSA in adults and AOP in infants (O'Halloran et al., 2017, O'Halloran, 2016, McDonald et al., 2015, Gauda et al., 2013). In animal, simulating sleep apnea by exposure to CIH induces inflammation in the lung and liver of mice (da Rosa et al., 2012). Intermittent hypoxia may also cause adipose tissue inflammation in rat model (Murphy et al., 2017, Gileles-Hillel et al., 2017).

TNF- $\alpha$ , a classic pro-inflammatory cytokine, plays a role in regulation of sleep, promoting non-rapid eye movement sleep, and its concentration in human body displays circadian patterns (Churchill et al., 2008, Jewett et al., 2015, De Araújo Freitas et al., 2015, Rockstrom et al., 2018). A study of 50 newly diagnosed OSA male patients showed that the plasma levels of TNF- $\alpha$  in the severe OSA group were significantly higher than those in the moderate group and healthy control group (Bozic et al., 2018). In mice, sleep fragmentation resembling OSA-induced disruptions leads to increased TNF- $\alpha$  expression in the CNS. This results in heightened sleep propensity, cognitive issues, and mood disturbances, similar to OSA, without reduced sleep duration (Ramesh et al., 2007, Kaushal et al., 2012). What's more, when treated with a TNF- $\alpha$  neutralizing antibody, or in double TNF- $\alpha$  receptor null mice with fragmented sleep, there is a significant reduction in increased sleep propensity and related cognitive and behavioral issues caused by sleep disruption (Ramesh et al., 2012, He and Kapur, 2017). As the main characterization of OSA, CIH induces the activation of TLR-4-NF- $\kappa$ B pathway, a critical signaling mechanism in the immune system to produce pro-inflammatory cytokines, along with increased cellular and extracellular levels of TNF- $\alpha$  in mice (Nácher, et al., 2009, Taylor et al., 2014, Poulain et al., 2015, Dyugovskaya et al., 2011).

Besides TNF- $\alpha$ , other inflammatory biomarkers such as Interleukin 6 (IL-6), C-reactive protein (CRP), and Tau are found to play a role in the OSA, which suggests the presence of chronic systemic inflammation in OSA patients. IL-6, belonging to the so-called IL-6 family of cytokines, has the ability to cause vascular inflammation and increase the risk of cardiovascular disease, diabetes, and cognitive decline (Kosmas et al., 2019, Akbari et al., 2016, Tegeler et al., 2018). CRP is a prototypic marker of inflammation, which is mainly produced in the liver in response to IL-6 (McNicholas, 2009). The level of plasma CRP is increased in OSA (Nadeem et al., 2013, Yardim-Akaydin et al., 2014). Tau, a microtubule-related protein, is vital for neuronal function and, alongside amyloid beta (A $\beta$ ), is associated with neurodegeneration and neuronal death (Wang et al., 2016). It has been found that CIH would total tau level by upregulating Tau phosphorylation in OSA (Daulatzai, 2015, Bubu et al., 2019). A study of 24 healthy controls, 22 mild, and 28 moderate-severe OSA subjects showed that Tau and IL-6 levels were significantly higher in the severe group compared to the control and mild OSA groups (Motamedi et al., 2018).

Adherence of leukocytes to the vascular endothelium is an important step in the inflammatory process. In a rat model, recurrent obstructive apnea caused a significant increase in leukocyte-endothelial cell interactions such as leukocyte rolling and firm adhesion of leukocytes (Nacher et al., 2007). Cell adhesion molecules (CAMs) are proteins located on the cell surface involved in binding with other cells or with the extracellular matrix. Several studies have demonstrated increased plasma level of CAMs in OSA, which may support an association of CAMs with inflammation in OSA (Ohga et al., 1999, El-Solh et al., 2002, Ursavas et al., 2007, Zamarron-Sanz et al., 2006).

Hypoxia-induced factor - 1 $\alpha$  (HIF-1 $\alpha$ ), a key transcription factor under hypoxia, promotes gene transcription for glycolysis and angiogenesis (such as VEGF) to deliver oxygen to hypoxic tissues (Abe et al., 2017). HIF-1 $\alpha$  can enhance inflammation by influencing the inflammatory functions of monocytes/macrophages and polymorphonuclear cells, including their infiltration and activation in vivo (Cramer et al., 2003). Activation of HIF-1 $\alpha$  was primarily observed in rodents exposed CIH and tissue culture of intermittent hypoxia models (Semenza and Prabhakar, 2007, Yuan et al., 2005, Toffoli et al., 2007).

### **5.5 Apnea/CIH and angiogenesis**

Several studies have been shown that endothelial dysfunction is present in OSA patients (Atkeson et al., 2009, Budhiraja et al., 2007, Foster et al., 2007, Atkeson et al., 2008). Endothelial dysfunction occurs when vasoconstrictor effects dominate vasodilator ones, damaging the arterial wall. It is characterized by decreased vasodilation, increased vasoconstriction, and persistent prothrombotic and inflammatory activities (Hirata et al., 2010). Repetitive hypoxia-reoxygenation and frequent arousals in OSA impair endothelial function (Atkeson and Jelic, 2008, Atkeson et al., 2009). Endothelial dysfunction disrupts blood vessel homeostasis, marked by decreased NO availability. NO plays a crucial role in vasodilation, preventing platelet and leukocyte adhesion, and inhibiting smooth muscle cell growth (Lurie, 2011a, 2011b). In OSA, this NO reduction is associated with vasoconstriction processes involving angiotensin II or endothelin (Fletcher, 2003, Narkiewicz and Somers, 2003, Durgan et

al., 2015). Furthermore, in a study of mice, CIH decreased the myocardial angiogenesis in the left ventricle by disturbing the interaction of the VEGF with the kallikrein - kinin system (KKS), an interaction essential for angiogenesis under hypoxic conditions (Visniauskas et al., 2021).

On the other hand, intermittent hypoxia can also influence the angiogenesis in tumor as a pro-angiogenic factor (Toffoli and Michiels, 2008). Tumor blood vessel formation is essential for tumor development, and in the absence of angiogenesis and new blood vessel formation, tumor growth is restricted (Brem et al., 1976, Gimbrone and Leapman 1972). In vitro and in vivo experiments in mice model showed that intermittent hypoxia promoted endothelial cells survival and angiogenesis in liver tumor (Martinive et al., 2006, Dong et al., 2019). What's more, liver tumor cells expression of VEGF was increased by intermittent hypoxia compared to normoxia (Dong et al., 2019). HIF -1 $\alpha$  is thought to be responsible for these pro-angiogenic effects of intermittent hypoxia (Moeller et al., 2004, Dong et al., 2019).

CIH also has pro-angiogenic functions in CNS vascular network. Intermittent hypoxia plays a protective role in cardio and cerebral microvasculature by promoting angiogenesis (Manukhina et al., 2016, Mallet et al., 2018). In a mouse model of AOP, a significant increase of vessel density in the cerebral cortex was observed after 5 days CIH exposure (Bousslama et al., 2015). In another study on CD1 mice, an increased capillary density in the cerebral cortex and hippocampus was observed over 2-4 weeks of CIH since postnatal day 2 (Kanaan et al., 2006). Also, in a rat model of ROP, higher cortical vessel density was measured after 14 days of intermittent hyperoxia-hypoxia (per day 4 hours) (Morken et al., 2013). A study of 12 weeks' CIH exposure for rats exhibited that expression of intercellular cell adhesion molecule-1 (ICAM-1) and VEGF was increased in the prefrontal cortex area and the corpus striatum (Yang et al., 2018).

## **5.6 Apnea/CIH and microglia**

As the major inflammatory cells in the CNS, microglia are found to induce pathogenic neuroinflammation in diseases such as Alzheimer's disease and Parkinson's disease (Khan et al.,

2011, Glezer et al., 2007, Van Eldik et al., 2007, Rogers et al., 2007). Intermittent hypoxia may also cause structural neuron damage in the CNS (Rae et al., 2009). Microglia primarily induce oxidative stress and inflammation in the CNS via mitochondria, NADPH oxidase, and the secretion of harmful excitatory neurotransmitters (Yang et al., 2013). In the CNS, mitochondria and NADPH oxidase are the primary reactive oxygen species (ROS) producers and are vital in the neuronal damage caused by CIH (Wang et al., 2010). CIH can lead to a surge in intracellular ROS. When ROS production exceeds its elimination, it overwhelms the capacity of antioxidative enzymes and natural antioxidants, culminating in oxidative harm to lipids, proteins, and nucleic acids (Feng et al., 2012, Gozal et al., 2001). In primary cortical neurons, the shift from hypoxia to normoxia during CIH seems to produce more ROS compared to the change from normoxia to hypoxia or just hypoxia. All these conditions produce more ROS than the standard normoxic state (Shan et al., 2007).

When stimulated by environmental factors (such as lipopolysaccharide, diethyl phthalate, rotenone, paraquat), endogenous protein toxins (such as  $\beta$ -amyloid peptide,  $\alpha$ -synuclein), or neuronal damage, microglia in the CNS can consistently produce ROS (Block et al., 2004, Yang et al., 2013). Interestingly, intracellular ROS can also activate microglia and increase the production of pro-inflammatory factors such as NO and TNF- $\alpha$  (Block and Hong, 2005, Pineau and Lacroix, 2009), and those pro-inflammatory factors from microglia can further activate other microglia and produce ROS (Block et al., 2004). Therefore, consider about the increased level of ROS by CIH we described above, in sleep apnea or CIH condition, microglia may cause impairments and dysfunction of the CNS (especially in the hippocampus) by microglia-mediated oxidative stress and inflammation (Yang et al., 2013).

Furthermore, microglia can have both beneficial and pathological effects under CIH. For instance, following the initial damage from CIH, which leads to neuronal cell death and cognitive decline, there is a subsequent phase of neural repair and functional recovery (Gozal et al. 2001, Zhu et al. 2005, Tsai et al. 2011). It is not clear yet about the mechanisms that drive this compensation and partial recovery, but it may be associated with the microglia functions in neuroplasticity, neuroprotection and neurogenesis (Parkhurst and Gan, 2010, Franco and Fernandez-Suarez, 2015, Sierra et al., 2014).



Moreover, an in vitro study showed that CIH reduced microglia proliferation by inhibiting cyclin D1 and cyclin E2 expression, which are involved in cell cycle transition and cellular proliferation, and increased inflammatory factors were observed in these microglia including up-regulated IL-6, TNF- $\alpha$  and iNOS (Liu et al., 2016). However, in a CIH mouse model, an increased microglia density was observed in the dorsal hippocampus after 6 and 24 weeks of CIH exposure, indicating that CIH may induce microglia priming in the dorsal hippocampus (Sapin et al. 2015).

## **5.7 Animal model of Apnea/CIH**

As the primary physiological consequences of sleep apnea, CIH is thought to be the major contributor to lung, heart, vascular, and brain dysfunction in OSA by most experimental research in both animals and humans (Dewan et al., 2015). Numerous animal and human models have been established to study the harmful impact of CIH on OSA patients. There are three most frequent model types, spontaneous models, surgical/mechanical models, and CIH exposure models (Dematteis et al., 2009).

### **5.7.1 Spontaneous models**

The first animal spontaneous model of OSA is the English bulldog model (Hendricks et al., 1987). They have both central and obstructive breathing disturbances, which intensify during rapid eye movement sleep and elevate the risk of obesity. In the case of Yucatan miniature pigs, two obese pigs were found to have OSA, and one of them also suffered from central sleep apnea (Lonergan III et al., 1998). The obese Zucker rats had reduced cross-sectional areas of their small airways during inhalation due to fat infiltration in the tongue, which is similar with what is seen in OSA patients (Brennick et al., 2006). Generally, these models have been primarily used to investigate the anatomy and physiology of the upper airway, as well as to evaluate the effects of various medications (Veasey et al., 2001).

### 5.7.2 Surgical/mechanical models

Several experimental models have been developed to artificially induce OSA. These models, applied in animals such as dogs, lambs, baboons, and rats, involve a tracheotomy procedure where the endotracheal tube is blocked at intervals (Brooks et al., 1997, Kimoff et al., 1997, White et al., 1995, Chen and Scharf, 2000, Fletcher et al., 1992, Náchér et al., 2007). A computer-controlled system induces periods of upper airway blockage by closing a valve connected to the tracheotomy. When the subject awakens, the valve opens, reversing the pharyngeal obstruction. These models offer a closer simulation of human OSA, as they permit the study of various OSA-related factors, including the awakening process, negative intrathoracic pressure, the duration of apnea, and the reduction in blood oxygen levels.

### 5.7.3 CIH exposure models

Both of spontaneous models and surgical models are limited by the number and size of the animal studies. Additionally, the outcomes could be influenced by the animal stress levels and the necessity to adapt them to the experimental environment. In order to reduce the impact of these factors and increase the animal number size, the CIH exposure models were developed. Commonly, the IH-reoxygenation is induced by delivery of a hypoxic mixture (such as high concentration nitrogen) to a sealed chamber to create a hypoxic environment, and then filling the chamber with normal room air for the normoxic status. Rodents are exposed to fixed periods of IH during their daytime sleep and to sustained normoxia during the night which corresponds to their awake period. This noninvasive rodent model can enhance the comprehension of the impacts of intermittent hypoxia on the cellular, tissue, and organ levels, as well as allowing for the investigation of the effects of different degrees of hypoxia severity. For example, researchers have used different hypoxia concentration, hypoxia exposure time, and hypoxia-normoxia cycles to study the influence of CIH in cardiovascular issues, dysfunctions in the CNS and eyes, disorders in lung etc. (summarized in **Tabel 1**). However, CIH exposure models are not able to mimic all the physiological changes in patients with OSA, including respiratory efforts and the hypercapnia that arises during upper airway blockage, two factors that are not consistently

present in the CIH rodent model. Moreover, CIH models may lead to more severe hypoxemia than that occurring in OSA patients. Despite these shortcomings, this hypoxia-reoxygenation model used in mice or rats better characterizes the typical features of OSA, such as the exposure to CIH resulting in cardiovascular effects that are similar to those present in OSA patients (Sforza et al., 2016).

**Table 1: CIH murine models**

<b>Reference</b>	<b>Model</b>	<b>Program</b>	<b>Results</b>
Kang et al. 2017	C57BL/6J mice High fat diet 7-8 weeks old Male	Hypoxia: 7% last 0s A cycle 1 min 12h per day 4 weeks	Liver fibrosis ↑ Body weight ↓ Expression of TLR4 and MyD88 ↑
Ali et al. 2019	C57BL/6J mice C57BL/KaLwRij mice Multiple myeloma-induced 8 weeks old Females	Hypoxia: 10% last 90s A cycle 5 min 10h per day 4 weeks	Myeloma cells in bone marrow ↑ Lytic bone lesions and osteopenia ↑
Macheda et al. 2019	C57BL/6J mice Alzheimer's disease-relevant 10-11 months old Males and females	Hypoxia: 10% last 0s A cycle 4 min 10h per day 4 weeks	Amyloid load → Astrogliosis ↑ Expression of genes associated with A1-type and A2-type astrocytes ↑
Gille et al. 2018	C57BL/6J mice Bleomycin-induced pulmonary fibrosis 2-3 months old Males	Hypoxia: 6% last 0s A cycle 90s 8h per day 3 weeks	Survival ↓ Lung pulmonary edema ↑ Lung antioxidant enzymes ↓ Lung cell apoptosis, lung fibrosis and inflammation ↑
Fu et al. 2017	Balb/C mice Radioactive particulate matter-inserted 8 weeks old Males	Hypoxia: 7% last 10s A cycle 1 min 9h per day 6 weeks	Lung radioactive deposit ↑ Airway resistance ↑ Lung inflammation ↑ Activation of PI3K/AKT pathway ↑
Polšek et al. 2017	C57BL/6J mice 65-105 days old Males	Hypoxia: 5.7% last 90s A cycle 3 min 8h per day 21 days	Hippocampus neuronal apoptosis ↑
Sapin et al. 2015	C57BL/6J mice 9 weeks old Males	Hypoxia: 5% last 30s A cycle 90s 8h per day 6 and 24 weeks	Hippocampus microglia density ↑ Hippocampus low-grade neuroinflammation ↑

Wang et al., 2013	C57BL/6J mice 20-30g Males	Hypoxia: 5% last 15s A cycle 7 min 8h per day 10 days	Insulin secretion in pancreatic islets ↑ Mitochondrial ROS in pancreatic β-cells ↑
Drager et al., 2012	ApoE <sup>-/-</sup> mice Angiopoietin-like 4 (Angptl4) treated	Hypoxia: 6.5% last 0s A cycle 1 min 12h per day 4 weeks	Fasting plasma triglycerides and very low density lipoprotein cholesterol ↑ Adipose Angptl4 ↑ HIF-1α induced Angptl4 ↑
Shiota et al., 2013	3xTg-AD mice 6 months old Males	Hypoxia: 5% last 0s A cycle 10 min 8h per day 8 weeks	Brain Aβ <sub>42</sub> levels ↑ Brain HIF-1α levels and expression → Brain cortex intracellular Aβ levels ↑
Beharry et al., 2013	Sprague-Dawley rats P21 pups OIR models Males and females	50% to 12% last 60s, repeat 3 times in 2 hours as an episode 12 episodes per day P14-P21	Choroid H <sub>2</sub> O <sub>2</sub> levels ↑ Retina and choroid SOD levels ↑ OIR severity ↑
Beharry et al., 2018	Sprague-Dawley rats P21 pups OIR models Males and females	50% to 12% last 60s, repeat 3 times in 2 hours as an episode 8 episodes per day P7-P21	Retina VEGF levels ↑ Astrocyte disorganization ↑ Retina thickness ↓ OIR severity ↑
Li et al., 2005	C57BL/6J mice Lep <sup>ob</sup> obesity model Males	Hypoxia: 5% last 0s A cycle 1 min 12h per day 12 weeks	Liver triglyceride and phospholipid ↑ Genes controlling lipogenesis ↑
Nisbet et al., 2009	C57BL/6J mice gp91phox knockout 8–10 weeks Males	Hypoxia: 10% last 0s A cycle 90s 8h per day 5 days per week 12 weeks	Pulmonary vascular remodeling ↑ Lung vascular NO bioavailability ↓
Joyeux-Faure et al., 2005	Wistar rats 220-240g Males	Hypoxia: 5% last 40s A cycle 1 min 8h per day 35 days	Body weight ↓ Heart infarct sizes ↑ Arteries vascular reactivity

Savransky et al., 2007a	C57BL/6J mice High cholesterol diet 8 weeks old Males	Hypoxia: 5% last 0s A cycle 60s 12h per day 12 weeks	Serum lipoproteins ↑ Atherosclerosis ↑
Savransky et al., 2007b	C57BL/6J mice 6-8 weeks old Males	Hypoxia: 5% last 0s A cycle 60s 12h per day 12 weeks	Liver weight → Fasting blood glucose ↑ Fasting serum insulin ↓ Acetaminophen hepatotoxicity ↑
Chen et al., 2005	Sprague-Dawley rats 225-275g Males	Hypoxia: 4-6% last 0s A cycle 60s 8h per day 5 days per week 5 weeks	Systolic blood pressure ↑ Left-ventricular (LV) function ↓
Greenberg et al., 2006	Mice	Hypoxia: 6.5-7% % last 5-7s A cycle 1 min 8h per day 14 days	Aorta NF-κB binding activity ↑ Cardiac and vascular iNOS expression ↑
Greenberg et al., 1999	Sprague-Dawley rats 2 months old Males	Hypoxia: 7% last 0s A cycle 60s 8h per day 30 days	Body weight → Systemic hypertension ↑
Li et al., 2007	C57BL/6J mice 10-11 weeks old Males	Hypoxia: 5% last 0s A cycle 60s 12h per day 28 days	Fasting serum cholesterol ↑ Lipoprotein secretion ↑ Hepatic stearyl coenzyme A desaturase 1 ↑ Liver
Peng et al., 2006	C57BL/6J mice Partially deficient in HIF-1α 10-15 weeks old Males	Hypoxia: 5% last 15s A cycle 7 min 8h per day 10 days	Brian ROS levels ↑ Brian HIF-1α levels ↑
Zoccal et al., 2007	Wistar rats 291-295g Males	Hypoxia: 6% last 40s A cycle 9 min 8h per day 45 days	Arterial pressure ↑ Plasma noradrenaline and adrenaline ↑

# Objectives

Retinopathy of prematurity (ROP) is an ocular disorder where there is a loss of existing retinal blood vessels and abnormal vessel growth. Apnea in premature infants is a temporary cessation of breathing that lasts for more than 20 seconds, characterized by chronic intermittent hypoxia (CIH). Both ROP and apnea are common diseases in premature infants (Good, 2004, Blackmon et al., 2003). Some studies have already shown that CIH is a risk factor for ROP (Fiore et al., 2010, Fiore et al., 2012, Martin et al., 2011, Kumar et al., 2023). However, the mechanism remains unknown. Therefore, we raised our first question: to what extent CIH impacts the ischemic retina? To answer this question, we used the well-established oxygen-induced retinopathy (OIR) mouse model for ROP and designed a real-time nitrogen input system to create a chronic intermittent hypoxia (CIH) environment, simulating obstructive sleep apnea (OSA). Microglia, the resident immune cells of the central nervous system (CNS), have been shown to play a role in enhancing retinal vessel growth (Checchin et al., 2006, Rymo et al., 2011). We wanted to determine if microglia are influenced by CIH, which leads us to our second question: what are the underlying molecular mechanisms? To address the second question, we considered the use of RNA sequencing and quantitative PCR. Colony-Stimulating Factor 1 (CSF1), a cytokine that plays a crucial role in the regulation of survival, proliferation, and differentiation of mononuclear phagocytes including microglia. CSF1 was identified as a key molecule and we further inhibited CSF1/CSF1R signaling to reveal the role of CSF1.

Taken together, the aims of this thesis were 1) To determine to what extent CIH impacts CIH impacts on retinal angiogenesis and neovascularization, and 2) To determine the mechanisms by which CIH affects retinal angiogenesis and neovascularization.



# Results

# Article

At the time of submitting this manuscript (06/2024), our article, *Chronic intermittent hypoxia disrupts protective microgliosis in ischemic proliferative retinopathy*, is under review by the editors of *Molecular Neurodegeneration*.

## Chronic intermittent hypoxia disrupts protective microgliosis in ischemic proliferative retinopathy

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## Abstract

### Background

Sleep apnea that leads to chronic intermittent hypoxia (CIH) is an independent risk factor for advanced, debilitating ischemic proliferative retinopathies, such as diabetic retinopathy (DR) and retinopathy of prematurity (ROP). The underlying mechanisms are unknown.

### Methods

We investigated the consequences of CIH on the ischemic retina of the oxygen-induced retinopathy (OIR) model in mice using immunohistochemistry (retinal vasculature and microglia), flow cytometry (microglia), optical coherence tomography (retinal structure) and RNA sequencing. Retinal function was evaluated by electroretinography. Finally, CSF1-CSF1R signaling was blocked by intravitreal injections of CSF1R blocking antibodies.

### Results

Here we show that experimental CIH inhibited colony stimulating factor 1 (CSF-1) expression, blunted the reactive microgliosis during the ischemic phase of OIR. CIH severely delayed beneficial revascularization of the ischemic retina and increased pathological neovascularization. CIH also induced photoreceptor thinning and protracted inner and outer retinal functional deficits. Mechanistically we demonstrated that local CSF1R inhibition during ischemic retinopathy reduced the number of microglial cells, inhibited revascularization, and exacerbated pathological neovascularization, recapitulating the effects of CIH.

### Conclusions

Our findings provide a novel mechanism by which sleep apnea and CIH aggravate ischemic retinopathies, underscoring the importance of treating apnea in ROP and DR to help prevent sight threatening severe disease.

Keywords: ischemic retinopathy, sleep apnea, hypoxia, microglia

## Background:

Ischemic proliferative retinopathies, such as retinopathy of prematurity (ROP) and diabetic retinopathy (DR), remain a major threat to vision despite major advances in the treatment of late neovascular complications by anti-VEGF therapies (1-3). They are characterized by an ischemic first stage, in which there is retinal microvascular degeneration and associated neuronal degeneration. This occurs in ROP due to the premature interruption of the maternal metabolic-endocrine support of the immature fetus with exposure to supra-physiological extra-uterine oxygen levels, and in diabetes due to metabolic dysregulation. In both diseases this first stage can evolve into advanced stages characterized by neovascularization that can lead to inner retinal edema and fibrosis (1-3). Additionally to neovascularization, pathological neuronal degeneration occurs in the inner retina {Barber, 1998 #2426;Bek, 1994 #2495;Murakami, 2013 #2492} and the photoreceptors in DR (4-7) and photoreceptor dysfunction remains detectable years after the preterm days of ROP patients (8).

Interestingly, the risk of severe ROP is increased in infants suffering from apnea of prematurity (9, 10), and decreased when the apnea is treated with caffeine (11). Similarly, obstructive sleep apnea, characterized by recurrent upper airway collapse and interruptions in breathing during sleep, is independently associated with an increased prevalence of advanced DR, proliferative DR, and diabetic macular edema (DME) after adjustment to confounders including age, sex, body mass index, hypertension etc. {Chang, 2018 #2378;Kaba, 2022 #2380;West, 2010 #2432} . Of note, sleep apnea is also associated with other neurodegenerative diseases such as Alzheimer's- and Parkinson's-disease (12, 13). Sleep apnea produces repeated episodes of hypoxemia followed by re-oxygenation, called chronic intermittent hypoxia (CIH), which is believed to be the major contributor to sleep apnea-related morbidity and mortality (14).

Ischemic proliferative retinopathy can be experimentally induced in mice by exposing pups to 75% O<sub>2</sub> for five days during their second week of life. This oxygen-induced retinopathy (OIR) model (15) provokes vaso-oblivation and subsequent capillary loss of the central retinal vasculature leading to an ischemic retina when the mice are returned to room-air conditions. The oxygen-induced central avascular area subsequently, and invariably, triggers VEGF-dependent growth of pathological intravitreal neovascular tufts (15) that reach a maximum a further five days into the ischemic phase of the model (PN 17-18). In parallel, the avascular area disappears as the ischemic retina revascularizes (16) and the neovascular tufts resorb by PN25 (16). This

phenomenon is also observed in most ROP patients. It is not clear to what degree intra-retinal revascularization occurs in DR in which potential revascularization is outweighed by continued microvascular degeneration. In rodents, this revascularization has been shown to be at least in part dependent on microglial cells, the resident macrophages of the central nervous system (17, 18).

Contrary to the mild retinal changes of animal models of diabetes (19), the mouse OIR model results in sizeable ischemic avascular zones and neovascularization as in severe human ROP and DR. The clear separation of the hyperoxic, vaso-obliterative phase from the ischemic phase in the mouse OIR model also helps to specifically address mechanisms in the ischemic retina compared to rat models in which oscillating hyper- and hypoxic exposures are necessary to trigger pathological changes (20).

To investigate how CIH influences ischemic proliferative retinopathy we exposed mice with oxygen-induced ischemic retinopathy to experimental CIH. The CIH was simulated by exposing experimental animals to 10h daily of 266 cycles of quick oscillations of the O<sub>2</sub> concentration in the cage during their sleep, mimicking CIH in severe obstructive sleep apnea (21). Here we show that experimental CIH greatly delayed beneficial revascularization of the ischemic retina, and increased pathological neovascularization. CIH also affected neuronal signaling with lasting functional consequences for retinal function. Additionally, CIH blunts the increase of microglial cell population otherwise observed in the ischemic retina. We demonstrate that the CIH-associated reduced microglial cell count is coincident with diminished colony stimulating factor 1 (CSF-1) expression, known to control microglial cell populations. Local inhibition of CSF1R, the CSF-1 receptor, during ischemic retinopathy reduced the number of microglial cells and inhibited revascularization similar to CIH. Together, these findings provide a mechanistic explanation of how apnea and CIH aggravate ischemic proliferative retinopathies and emphasize the importance of monitoring and treating apnea in ROP and DR to help prevent severe, sight threatening disease.

## Methods:

### Animals

C57BL/6J pups and nursing mothers were obtained from Charles River. All mice were housed in pathogen-free conditions with water and chow available ad libitum. All experimental protocols and procedures were approved by the French Ministry of Higher Education, Research and Innovation (APAFIS authorization #43233 2023042416144897).

### Oxygen induced retinopathy and chronic intermittent hypoxia

In an adaptation of the widely used OIR model (15) C57BL/6J mice at postnatal (PN) day 8 (PN8) were exposed, with their mothers, for 5 days to hyperoxic conditions (75% O<sub>2</sub>), inducing vaso-obliteration and subsequent capillary loss of the central retinal vasculature (15). At PN13, mice were returned to room-air conditions which induces extensive neovascularization in all experimental mice with maximum effects on PN18.

CIH was induced in C57BL6/J mice using a Hycon0520 simulator (22) that can oscillate the cage O<sub>2</sub> concentration extremely quickly. For 10 hours during the daytime (corresponding to the mouse sleep period) CIH experimental animals were exposed daily to 266 cycles of a 135s program of oscillating O<sub>2</sub> concentration as follows: decrease of 20.95% O<sub>2</sub> to 5% O<sub>2</sub> in 45s, 5% O<sub>2</sub> for 30s, increase to 20.95% O<sub>2</sub> in 15s, 45s of 20.95% O<sub>2</sub> (adapted from Xu et al. (21)). Control animals were exposed to the same flow (and noise) of gasses, without affecting O<sub>2</sub> concentrations.

### Immunohistochemistry

For immunohistochemical analyses, eyes were immediately placed in 4% PFA (ThermoFisher) following CO<sub>2</sub> narcosis and cervical dislocation, and eyes were allowed to fix at room temperature for 45 minutes. After fixation, eyes were transferred to PBS (Gibco) the anterior segments and connective tissues were removed, four curvature-relieving cuts were made into the eye cups, and the retinas were gently separated from the RPE/choroid/sclera. Retinas and RPE/choroid/sclera complexes were first incubated overnight at room temperature with AlexaFluor488-conjugated isolectin (diluted 1:200, Invitrogen) and goat or rabbit anti-IBA1 (diluted 1:400, final concentration 1.25 ug/ml, Abcam goat antibody, Wako rabbit antibody) in PBS with 0.1% Triton. Tissues were then rinsed with PBS and then incubated for 2 hours at room temperature with a donkey anti-goat or anti-rabbit IgG antibody (diluted 1:400, final

concentration 5 ug/ml, Invitrogen). After final rinses in PBS, retinas and RPE/choroid/sclera complexes were mounted on glass slides with Fluoromount aqueous mounting media (Sigma). Images were captured with a Leica DM550B fluorescence microscope (Leica Biosystems). Image analysis was performed using FIJI (23).

### Flow cytometry

For flow cytometry, retinas were collected in PBS (Gibco) and placed on ice immediately following CO<sub>2</sub> narcosis and cervical dislocation. Retinas were first enzymatically dissociated by incubation with Liberase TL (final concentration 0.15 mg/ml, Roche) at 37 C for 30 minutes, and then dissociation was completed mechanically by gently pipetting with a P1000 pipette. The resulting cell suspensions were filtered by passing through a 70 µm filter (Miltenyi) and rinsed with PBS. Cells were labelled with a viability dye (Viability 405/520 fixable dye, diluted 1:100, Miltenyi) following manufacturer recommendations for 15 minutes at room temperature. After a PBS wash, cells were then labelled for 30 minutes on ice with the relevant flow cytometry antibodies: VioBlue-conjugated anti-mouse CD45, APC-conjugated anti-mouse CD11b, FITC-conjugated anti-mouse MHCII, PE-conjugated anti-mouse CD31, and PE-Vio770-conjugated anti-CD115 (all antibodies diluted 1:50 in PBS for a final concentration of 3 µL/ml, all purchased from Miltenyi). Finally, cells were fixed in 1% PFA (ThermoFisher). All samples were run on a FACSCelesta Cell Analyzer (BD Biosciences, USA). The software FlowJo (BD, Oregon, USA) was used for all flow cytometry analysis.

### Optical coherence tomography

Optical coherence tomography (OCT) was performed similar to (24, 25). In brief, pupils were dilated using tropicamide (Mydriaticum, Théa, France) and phenylephrine (Neosynephrine, Europhta, France), and animals were anesthetized using isoflurane (Piramal, 5% to induce and under 2% to maintain anesthesia). SD-OCT scans were acquired using a Bioptigen SDIOS-HHP system fitted with an objective to allow imaging the mouse posterior segment, using the Bioptigen InVivoVue Clinic software (Bioptigen, North Carolina, USA). OCT volumes were exported for processing and analysis using FIJI (23). B scans were registered to remove breathing artifacts using the MultiStackReg plugin (a modification of the TurboReg plugin (26), written by Brad Busse), then 3 consecutive B scans were averaged, and the retinal layers were measured manually using the measurement tools in FIJI.

### Electroretinography

After overnight dark adaptation, pupils were dilated using tropicamide (0.5%, Mydraticum, Théa) and phenylephrine (5%, Neosynephrine, Europhta), corneas were anesthetized with oxybuprocaine eye drops (Théa, France), and animals were anesthetized with an IP injection of a mixture of ketamine (80 mg/kg, Axience) and xylazine (8 mg/kg, Bayer HealthCare). Electroretinograms (ERGs) were recorded using a Lab Cradle system fitted with a Color Dome Ganzfeld white (6500k) LED stimulator (Diagnosys). Gold wire loop corneal electrodes were placed on both eyes, a reference electrode was placed subcutaneously between the eyes, and a ground electrode was placed subcutaneously at the base of the tail. Transparent ophthalmic gel (Lubrithal, Dechra) was placed on both eyes to maintain corneal moisture and good contact with the corneal electrode throughout recordings. Animals were kept on a heating pad to maintain normal body temperature throughout experimentation. Scotopic retinal function was probed using 4 ms flashes of 3 cd\*s/m<sup>2</sup> with 60 seconds between flashes. Resulting responses were amplified and passed through a 0-300 Hz bandpass filter with a one-channel DC-/AC-amplifier. The collected waveforms were further processed using the Espion software V6 (Diagnosys) including the identification of the a- and b-wave amplitudes. A-wave amplitudes were measured from baseline to the peak of the first negative deflection, and b-waves were measured from the a-wave to the peak of the following positive deflection. A- and b-wave amplitudes were then normalized to the average normoxia a- and b-waves for presentation.

### RNA sequencing

Fresh eyes (3 eyes from the OIR-Norm group, 3 eyes from the OIR-CIH group, all at PN18) were dissected on ice to isolate retinas immediately after enucleation. Retinal RNA was extracted using the NucleoSpin RNA kit (Macherey-Nagel) according to the Midi protocol in the manufacturer's instructions. RNA quality was confirmed using a Bioanalyzer RNA Analysis kit (Agilent). RNA sequencing libraries were prepared from 250 ng total RNA using the TruSeq Stranded mRNA Library Prep kit (Illumina) and sequenced at the Institut du Cerveau iGenSeq sequencing core facility. The RNAseq dataset generated during the current study is available in the GEO repository, accession number GSE268420 (reviewer token: cdabucoytzwxdsx).

After sequencing, fastq files were aligned to the *mus musculus* reference genome from Ensembl (Ensembl 105, December 2021) using STAR (version 2.7.9a) with the option “- - quantMode GeneCounts” to extract the raw counts for each gene, and all count files were



concatenated into a single file. The count file and a descriptive sample file were loaded into our in-house R Shiny application “EyeDVseq” for analysis: the genes with a total count below 10 across samples were filtered out and the differential gene expression analysis was performed using the version 1.40.2 DESeq integrated into EyeDVseq, comparing OIR-Norm as control and OIR-CIH as the condition. The resulting gene list was then filtered, with  $|\text{Log}_2(\text{Fold Change})| > 0.5$  and  $p\text{Adj-values} < 0.05$  being considered significant.

### qPCR

Retinal RNA from 32 fresh eyes (16 eyes from the OIR-Norm group, 16 eyes from the OIR-CIH group) was collected at PN18, as above for RNA sequencing. Extracted RNA was reverse-transcribed into cDNA with the QuantiTech reverse transcription kit (Qiagen) following the quick-start protocol in the manufacturer's instructions. The qPCR was conducted using Power SYBR Green PCR Master Mix (Applied Biosystems) on a QuantStudio 5 Real-Time PCR System (Applied Biosystems). The cycling conditions were as follows: initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The qPCR data was analysed using the relative quantification method ( $2^{-\Delta\Delta C_t}$ ) on Design & Analysis Software v2.6.0 (Applied Biosystems). The forward primer of *Csfl* was 5'-TGACCCAGGATGAGGACAGA-3', the reverse primer of *Csfl* was 5'-GGTAGTGGTGGATGTTCCCA-3'. Both primers were custom designed by Primer-BLAST webtool, and synthesized by IDT (Integrated DNA Technology, Europe).

### Intraocular injections

Mice were anesthetized using isoflurane (Piramal, 5% to induce and under 2% to maintain anaesthesia). We injected rat anti-CSF1R (Bio-technie) or rat IgG isotype (InvivoGen) intravitreally using glass capillaries (Eppendorf) and a microinjector. Each mouse's left eye was injected with 3  $\mu\text{L}$  rat igG isotype (10  $\mu\text{g}/\text{ml}$ ) as a control, and the right eye was injected with 3  $\mu\text{L}$  anti-CSF1R antibody (10  $\mu\text{g}/\text{ml}$ ).

### Statistics

All statistical comparisons were performed in Prism v9.02 (GraphPad, Massachusetts, USA). Outliers were identified using the ROUT (Robust regression and Outlier removal) method with the false discovery rate (Q) set at 1% to balance between sensitivity and specificity in outlier detection. Two group comparisons were done using a Mann-Whitney test, unpaired when

comparing between animals and paired when comparing data from contralateral eyes. Multiple comparisons were performed using one- or two-way ANOVAs, as appropriate and indicated in figure legends, followed by Šídák's multiple comparisons post hoc test. P values less than 0.05 were considered significant.

## Results:

### Chronic intermittent hypoxia alone does not affect retinal vascularization or microglia cell counts

First, we examined if chronic intermittent hypoxia (CIH) affects retinal vasculature or microglia in the absence of retinal ischemia. From PN8 to PN18 pups were placed in specialized cages and exposed to CIH or normal oxygen conditions (normoxia). CIH was simulated by rapidly oscillating the cage O<sub>2</sub> concentration for 10 hours during the normal sleep hours of the animals mimicking CIH of severe obstructive sleep apnea (266 cycles over 10 hours, each 135s cycle: decrease of 20.95% to 5% O<sub>2</sub> in 45s, 5% O<sub>2</sub> for 30s, increase to 20.95% O<sub>2</sub> in 15s, and 45s of 20.95% O<sub>2</sub>; adapted from Xu et al. (21))(schematized in Fig. 1A). Normoxic animals were exposed to the same flow and noise as CIH mice but without oscillating O<sub>2</sub> concentrations. Retinal vessels were examined histologically in flatmounts labeled with isolectin at PN18. Qualitatively the vascular bed was unchanged by the CIH, and indeed the density of superficial inner retina vessels was unchanged (Fig. 1B). Retinal microglia were assessed using flow cytometry, with microglia identified as single, alive, CD45<sup>med</sup>CD11b<sup>+</sup> cells. There was no difference in microglia numbers between normoxia and CIH retinas (Fig. 1C). Additionally, there was no difference in MHCII expression, an indicator of microglial activation, between groups (Fig. 1D). Finally, there was only a mild, non-significant reduction in body weight between groups, suggesting CIH alone does not cause growth retardation (Fig. 1E).

These results demonstrate that our CIH regimen had no effect on the normal vascular maturation and development that takes place in mouse pups at this stage of development.

### CIH inhibits revascularization and promotes pathological neovascularization in the ischemic retina, and induces a transient growth retardation

Clinical studies showed that obstructive sleep apnea is associated with proliferative DR, and DME after adjustment to confounders including age, sex, body mass index, and hypertension {Chang, 2018 #2378; Kaba, 2022 #2380 ;West, 2010 #2432}, but the underlying mechanism is unknown. To investigate if CIH exacerbates advanced ischemic proliferative retinopathy we used

the mouse model of oxygen induced retinopathy (OIR), which induces microvascular degeneration followed by neovascularization contrary to diabetic animal models that only develop mild microvascular degeneration. From PN8-PN13 OIR was induced by exposing the mice to hyperoxic conditions (75% O<sub>2</sub>) for 5 days in an adaptation of the previously described PN7-PN12 exposure due to ethical regulations (15). This hyperoxia provokes vaso-obliteration and subsequent capillary loss of the central retinal vasculature leading to an ischemic retina when the mice are returned to room-air conditions. Following the hyperoxia, pups were exposed to normoxia or CIH conditions until sacrifice, as described above. Again, to control for noise and air-flow induced disturbances and sleep fragmentation, normoxic animals were exposed to the same gas flows than CIH animals but with a constant 20.9% of O<sub>2</sub> (Fig. 2A).

In this model neovascular tuft development reaches a maximum five days after return to room air (PN 17-18). In parallel, the avascular area disappears as the ischemic retina revascularizes (16) and the neovascular tufts resorb by PN25 (16). In our hands, isolectin-stained retinal flatmounts revealed a similar sized avascular area at PN14, after 24h of CIH or normoxia exposure. However, while the avascular area of control mice quickly disappeared over the observation period and neovascular tufts were regressing, CIH significantly inhibited the revascularization of the ischemic retina, associated with delayed and exaggerated neovascularization (Fig. 2B). Quantification of the size of the central avascular area revealed significant differences at PN18, PN21, and PN24, with an over three-fold bigger avascular area in the CIH group at PN24 (Fig. 2C). While neovascular tuft formation reached a maximum at PN18 in control mice, CIH exposed animals reached a maximal pathological neovascular response only at PN24 at which point the surface covered by neovascular tufts was twice as large as in control mice (Fig. 2D). At PN30 retinas from the CIH group were still very recognizable by the irregularity of the intra-retinal revascularization and remaining neovascular, while control retinas had, other than some degree of increased vessel tortuosity, renormalized their retinal vasculature, as previously described (16)(Fig. 2B).

Interestingly, the transient growth retardation that is observed in OIR-norm mice compared to mice kept under normoxic conditions, was also prolonged significantly in OIR-CIH mice reaching significance at PN21 and 24 but their growth curve, despite continued CIH had caught up with the other conditions by PN30 (Fig. 2E).

Taken together, although CIH did not affect normal vascular development (Fig.1) it profoundly inhibited the revascularization of the ischemic retina. The resulting prolongation of the ischemia was associated with more persistent and exaggerated pathological neovascularization. Interestingly, the CIH also accentuated the transient growth retardation induced by OIR, suggesting a possibly more generalized effect on angiogenesis in the growing animals.

### CIH photoreceptor segment degeneration and irreversible retinal dysfunction

Additionally to neovascularization pathological neuronal degeneration occurs in the inner retina during OIR (27, 28), ROP (29) and DR (4, 30). Furthermore, optical coherence studies revealed that the photoreceptor outer segments undergo degenerative changes in DR. The line that represents the junction of the mitochondria-containing inner segments and photo-sensitive outer segments (IS/OS) gets disrupted (5), and photoreceptor outer segment shorten (6), which strongly correlates with visual function in these patients (7).

To investigate whether CIH affects the neuronal structures in the ischemic retina we visualized retinal layers using optical coherence tomography (OCT) at PN24 and PN30 from normally raised mice and mice that had undergone OIR followed by CIH or oscillating normoxic air as described above (Fig. 3A). The thickness of the inner and outer retinal layers was clearly visible on OCT (Fig. 3B). Quantification of the inner retina confirmed earlier reports (27) of neuronal degeneration in the ischemic retina compared to the normal retina, and this inner retinal thinning was not affected by CIH (Fig. 3C, left). However, although the outer nuclear layer that contains the photoreceptor nuclei was not affected by the model (data not shown), CIH consistently and significantly worsened the OIR-induced thinning of the photoreceptor segment layer at PN24 and PN30, which include the mitochondria containing inner segments and photo-sensitive outer segments (IS/OS) (Fig. 3C, right).

Functionally, electroretinography (ERG) examination at PN30 revealed that CIH significantly reduced the amplitude of OIR-induced scotopic negative a-waves, a measure of rod photoreceptor function, and the subsequent positive b-waves, a measure of transmission and function of the inner retina (Fig. 3D and E). Importantly, these CIH-induced reduction of the a-waves and b-waves were still detectable 30 days after cessation of CIH in animals that had been transferred from PN30 to PN60 to the normal animal facility (Fig. 3 F and G)

In summary, our data demonstrates that CIH induces the degeneration of photoreceptor segments additionally to the previously described OIR-induced thinning of the inner retina. Further, these cause lasting deficits in retinal signaling, which are exacerbated by CIH even after return to normal oxygen conditions.

#### CIH dampens the reactive microglial expansion in ischemic retinopathy

In rodents, revascularization of the ischemic retina in OIR has been shown to be dependent on microglial cells (17, 18). Given the vascular and neuronal changes observed in OIR-Norm and OIR-CIH retinas, we next examined the distribution of mononuclear phagocytes, including resident microglial cells and infiltrating immune cells in the model. Using flow cytometry, we did not detect a significant infiltration of monocytes or other leukocytes (data not shown). Quantification of retinal microglia numbers revealed no difference between groups immediately following OIR induction. There after the number of microglia in OIR-Norm retinas quickly increased, peaking at PN21, and then returning to baseline by PN27. In contrast, the size of the microglia population in OIR-CIH retinas was largely stable other than a small swell from PN18-PN24 (Fig. 4A). Additionally, microglia visualized in IBA1-labelled retinal flatmounts at PN18 of the central retina revealed that microglia of the ischemic retina of OIR-CIH retinas retained a more homeostatic morphology (long, fine primary processes and small cell bodies) compared to OIR-Norm microglia with shortened and thickened primary processes and larger cell bodies. By PN30, these differences disappeared and retinal microglia were morphologically similar between OIR-Norm and OIR-CIH retinas (Fig. 4B).

Given the degenerative changes and functional deficits of the photoreceptors (Fig. 3), we further examined macrophages in the subretinal space (Fig. 4C). The subretinal space is normally devoid of immune cells, but accumulation of protective microglial cells and pathogenic monocyte-derived macrophages in the subretinal space is a hallmark of photoreceptor disease (31, 32). Immunohistochemical labeling of RPE/choroid/sclera complexes at PN30 revealed significantly more IBA1+ cells in the subretinal space of OIR-Norm retinas compared to OIR-CIH (Fig. 4C). Given that we did not detect monocyte-derived macrophages but only microglial cells in flow cytometry these IBA1+ cells are likely microglial cells.

In summary, these data demonstrate that CIH inhibits the expansion and activation of retinal microglia in the ischemic retina that has been shown to be associated with the reparative

revascularization of the ischemic retina (17) and could have a protective role on photoreceptors (31).

### CIH reduces retinal *Csf1* transcription

To identify potential underlying transcriptional differences, we next performed bulk RNA sequencing (RNAseq) on OIR-Norm and OIR-CIH retinas at PN18 (Fig. 5A). We identified many differentially expressed genes, most of which were downregulated in OIR-CIH retinas compared to OIR-Norm (Fig. 5B, Supplemental Table 1). Several of these genes, including *Hmox1*, *C4b*, *Cd84*, and *Csf1*, have been previously associated with immune functions. Considering the dampened microglial response in OIR-CIH retinas, we decided to further examine the expression of CSF1 and its receptor CSF1R (CD115), which are important in the regulation of microglia survival (33). RT-QPCRs confirmed a very significant down-regulation in *Csf1* mRNA in OIR-CIH compared to OIR-norm mice at PN18 (Fig. 5C). Cytometric analysis of CD115 expression on both retinal microglia and endothelial cells from OIR-Norm PN18 retinas showed that endothelial cell CD115 expression was negligible and that microglia were the main CD115 expressing cells (Fig. 5D), suggesting that any effects of decreased *Csf1* expression are mediated via signaling on retinal microglia.

### CSF1R inhibition during the ischemic phase of OIR recapitulates the effect of CIH on microglia and vasculature

To determine if decreased CSF1-CSF1R signaling could drive several of the effects of CIH on the ischemic retina, we used an anti-CSF1R blocking antibody to inhibit CSF1R. We first assessed the effect of the CSF1R blocking antibody on microglial populations. Immediately following the induction of OIR (PN13), pups received an injection of the anti-CSF1R antibody in one eye and an IgG isotype control in the contralateral eye (Fig. 6A). The CSF1R blocking in the OIR-norm group decreased the microglia population to the equivalent size as in OIR-CIH, but the injection of the anti-CSF1R antibody had no effect on the number of retinal microglia in the OIR-CIH group (Fig. 6B group comparison, Fig. 6C right-eye versus left-eye paired comparison).

We then sought to determine if modulation of microglia via CSF1-CSF1R signaling in ischemic retinas under normoxic conditions could recapitulate the exacerbated vascular pathology observed during CIH. Immediately following OIR induction, pups received injections of the anti-CSF1R antibody in one eye and isotype control in the contralateral eye, and were then

kept in normoxia conditions (Fig. 6D). As observed in OIR-CIH, there was a delay in the revascularization of the retina and larger avascular areas were observed in retinas treated with the CSF1R blocking antibody than controls (Fig. 6E-F). Additionally, there was more persistent neovascularization in the retinas treated with the antibody than controls (Fig. 6F).

Taken together our data shows that the inhibition of CSF1-CSF1R signaling in OIR-Norm animals recapitulates the vascular phenotype that is observed in OIR-CIH mice in which we showed the *Csfl* expression was decreased.

## Discussion:

Using a combination of oxygen-induced microvascular degeneration that induces an ischemic retina and experimental chronic CIH, we here investigated the mechanisms that might link apnea of prematurity with the risk of severe proliferative ROP (9, 10), and obstructive sleep apnea with an increased prevalence of severe proliferative DR {Chang, 2018 #2378; Kaba, 2022 #2380 ;West, 2010 #2432}.

The OIR mouse model uses continuous oxygen exposure in the vaso-obliterative phase, contrary to the rat model which necessitates alternating hyperoxia–hypoxia cycles, where the oxygen levels cycle between 50 and 10% every 24 h for the first 14 d after birth (for review see Liu et al (20)). The mouse model thereby more clearly separates the hyperoxic, vaso-obliterative phase from the ischemic phase, contrary to the rat model in which an aspect of intermittent hypoxia is already introduced from the beginning. The OIR mouse model also very reproducibly develops neovascularizations of the ischemic retina that are typical for severe human ROP and DR, contrary to rodent animal models of diabetes that only model early stages of non-proliferative retinopathy (19).

Apnea of prematurity and obstructive sleep apnea (OSA) produce repeated episodes of hypoxemia followed by re-oxygenation, called chronic intermittent hypoxia (CIH). CIH is believed to be the major contributor to apnea-related morbidity and mortality (14). To investigate how CIH influences ischemic proliferative retinopathy we therefore exposed mice with an oxygen-induced central avascular area to experimental CIH. The CIH was simulated by exposing experimental animals to 10h daily of 266 cycles of quick oscillations of the O<sub>2</sub> concentration in the cage during their sleep (21), which corresponds to severe obstructive sleep apnea rather than the central apnea of prematurity. Remarkably, this CIH regimen on its own did not induce any

phenotype in normal mice (Fig. 1), similar to human patients in whom even severe OSA only causes mild vascular changes in the absence of other underlying retinal diseases (28).

In contrast, CIH profoundly inhibited the previously described revascularization of the ischemic retina (16), resulting in an important, over three-fold bigger avascular area at PN24 (Fig.2). This vascular growth retardation was mirrored by a general growth retardation in the CIH exposed OIR pups, a phenomenon we did not observe in pups without prior hyperoxia exposure (Fig. 1). The CIH-associated general growth retardation might be the result of altered food intake, but could also suggest that CIH can more generally inhibit angiogenesis after the vasculature has been rendered more vulnerable by the hyperoxic phase. In the retina, the resulting prolongation of the ischemia by CIH was likely responsible for the more persistent and exaggerated pathological neovascularization observed at PN24 and PN27 (Fig. 2), a previously described mechanism (34). While the vascular changes in the OIR-norm group were largely reversible, as previously described (16) and much like vascular changes in non-severe ROP, the vasculature of the CIH group remained still very recognizable due to the irregularity of the intra-retinal revascularization and remaining neovascular tufts at PN30.

In addition to the vascular changes, CIH also aggravated the OIR-induced thinning of the layer of the photoreceptor segments (Fig. 3) in addition to the thinning of the inner retina previously known to be associated with the avascular area in the OIR model (27, 28). Interestingly, photoreceptor segment changes have also been described in severe DR and DME (5-7), which are associated with sleep apnea {Chang, 2018 #2378; Kaba, 2022 #2380 ;West, 2010 #2432}. Although the human macular, and in particular the fovea with its foveal cones, are anatomically quite different from the mouse retina, it is tempting to speculate that photoreceptor segment changes in severe DR might also be linked to CIH or its associated hypoxemia. In our model, these observed photoreceptor segment morphological changes were likely responsible for the further reduction of the scotopic a-wave amplitude and likely contributed to the b-wave amplitude reduction in the OIR-CIH group compared to the OIR-norm group. The ERG alterations we observed in the OIR-norm groups had been previously reported (8). Interestingly, the OIR-CIH-induced scotopic a-wave amplitude reduction remained detectable after 30 days of recovery in room air contrary to the OIR-norm group that had fully recovered. The persistent reduction of the b-wave amplitude in both groups can likely be attributed to the inner retinal degeneration, accentuated in the OIR-CIH group by the additional photoreceptor dysfunction.



In the periphery, CIH is often associated with inflammation and macrophage activation (35). To our surprise, our bulk RNA sequencing of CIH-exposed mice and their controls revealed a downregulation of a number of inflammation-associated genes in the CIH-exposed retina. Interestingly, our analysis revealed a strong downregulation of the macrophage colony-stimulating factor *Csf1*, a cytokine that regulates microglial cell populations (33), which we confirmed by RT-PCR (7-fold decrease; Fig. 5). Our analysis revealed that CSF1R was mainly expressed by microglia while its expression on vascular endothelial cells was negligible, making a direct effect of the lack of CSF-1 on endothelial cells unlikely (Fig. 5). Indeed, in terms of microglial cell populations, the decreased CSF-1 expression in the CIH group was associated with a reduction of subretinal microglial cell infiltration and an attenuation of the microglial cell increase that is observed in the ischemic retina under normoxic conditions (Fig.4). These lower microglial cell counts were observed across several time points in three independent experiments and are in accordance with a previous study that showed that CIH reduced microglia proliferation *in vitro* (36). In this model we do not observe an infiltration of monocytes and monocyte-derived macrophages. This is a marked difference to human DR where the number of cells positive for the mononuclear phagocyte (MP) markers HLA-DR, CD45 and CD68 markedly increases (37). In human DR, the MP population likely represents a mixture of resident microglial cells and perivascular macrophages as well as Pin2<sup>+</sup> monocyte derived macrophages that have been shown to drive the disease (38). CIH in DR, might therefore reduce the number of reparative microglial cells and push the balance to a more monocyte-derived macrophage infiltration.

Others and we have previously shown that the revascularization of ischemic retina in OIR is dependent on microglial cells (17, 18). To test whether the CIH-induced reduction of CSF-1 could be responsible for the inhibition of retinal revascularization via a decreased microglial cell population, we treated OIR mice during the ischemic phase with intravitreal CSF1R-blocking antibody in one eye and an isotypic control antibody to the other eye. In OIR-Norm animals blocking CSF1R induced a decrease in the microglial populations to similar levels observed in OIR-CIH mice. Blocking CSF1R in OIR-CIH mice had no effect, in accordance with the observed down-regulation in this group. Interestingly, the CSF1R-blocking antibody also inhibited the revascularization of the ischemic retina and aggravated pathological neovascularization compared to the control injected contralateral eyes in the OIR-Norm group.

The observation that local blocking of CSF1-CSF1R signaling alone is sufficient to recapitulate many of the effects of CIH on the ischemic retina highlights the importance of this local deregulation. Indeed, these experiments suggest that the inhibition of retinal revascularization is not the consequence of the observed systemic growth retardation in the model and also independent of hypertension or eventual pro-inflammatory changes in the periphery.

Interestingly, a previous study that used osteopetrotic mice (op/op mice carrying a mutation of the *Csf1* gene) and a c-fms tyrosine kinase inhibitor reported that CSF-1 inhibition strongly inhibited vitreal neovascularization, but not retinal revascularization (39). These differences might be explained by the fact that their study analyzed retinæ at an earlier time point, PN16, only. Furthermore, it is not clear to what degree op/op mice that lack of CSF-1 expression congenitally compensate by the expression of the alternative CSF1R ligand IL34.

## Conclusions:

In our study, we uncovered a novel mechanism by which chronic intermittent hypoxia (CIH), which is believed to be the major contributor to sleep apnea-related morbidity, exerts its detrimental effect on neurodegeneration and pathological vascular alterations in ischemic proliferative retinopathy. Our findings, unveil that CIH disrupts a protective reactive microgliosis via the downregulation of colony stimulating factor 1 (CSF-1) in ischemic proliferative retinopathy. Thereby CIH reduces beneficial revascularization and increases pathological neovascularization, and exacerbates photoreceptor degeneration and retinal dysfunction. Our findings help explain the previously unexplained association of sleep apnea with the increased prevalence of severe DR (40, 41 , 42) and retinopathy of prematurity (9, 10). These results may also have implications for other neurodegenerative diseases linked to sleep apnea, such as Alzheimer's and Parkinson's diseases (12, 13), which involve a protective microgliosis (43-45). Together, our study underscores the importance of detecting and treating apnea in ROP and DR to help prevent sight threatening severe disease.

## List of abbreviations

Ab - antibody

CIH – chronic intermittent hypoxia

DR – diabetic retinopathy

ERG – electroretinogram  
INL = inner nuclear layer  
IPL = inner plexiform layer  
IR = inner retina  
IS/OS = inner- and outer-segment  
MP = mononuclear phagocyte  
Norm - normoxia  
OCT – optical coherence tomography  
OIR – oxygen-induced retinopathy  
OPL = outer plexiform layer  
OSA – obstructive sleep apnea  
PN – postnatal  
PR = photoreceptors  
ROP – retinopathy of prematurity  
RPE = retinal pigment epithelium

## Declarations:

## Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request. The RNAseq dataset generated during the current study is available in the GEO repository, accession number GSE268420 (reviewer token cdabucoytzwxdsx).

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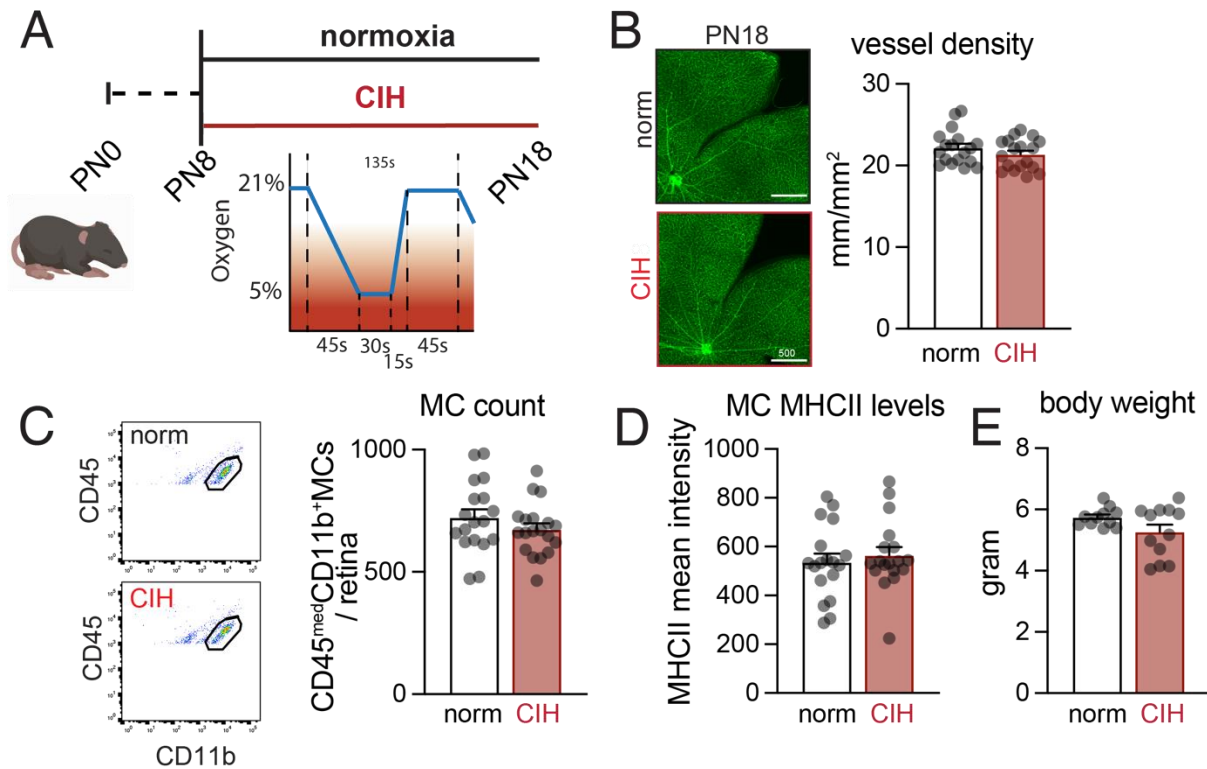
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## Author contributions

TY, ST, and FS conceived of the project.  
CD, XG, and FS secured funding.  
TY, KER, CD, XG, and FS designed experiments.  
TY, KER, SA, CN, and FA carried out experiments.  
TY, KER, FB, CN, CD, XG, and FS analyzed data.  
TY, KER, and FS wrote the manuscript.

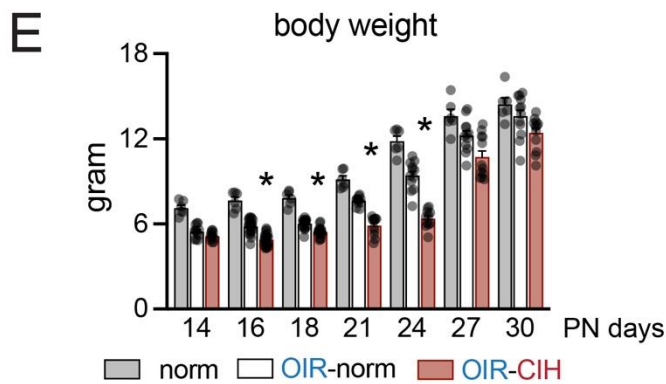
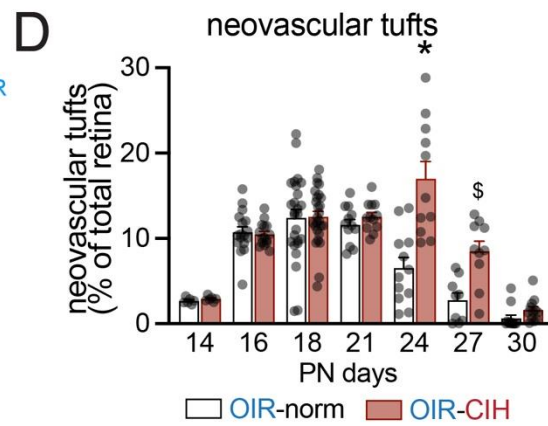
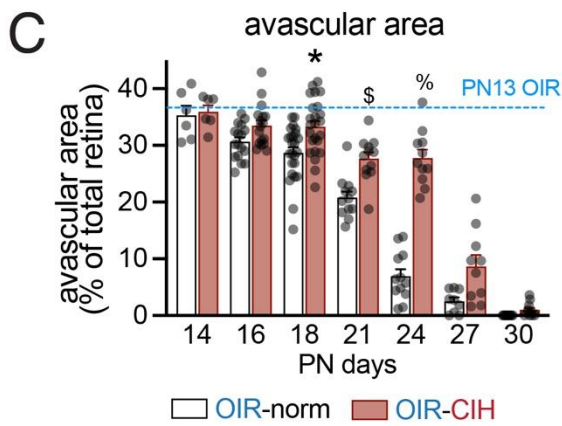
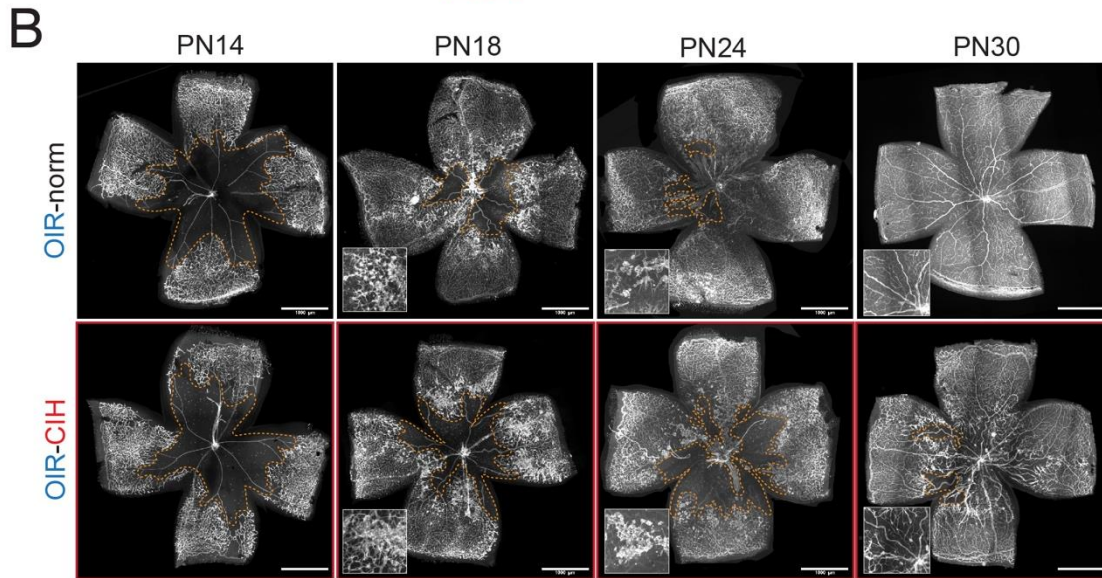
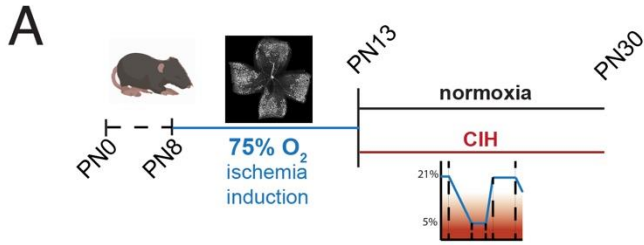
## Competing interests

None of the authors have competing interests  
Ethics approval and consent to participate – Not applicable  
Consent for publication – Not applicable



**Figure 1.** Chronic intermittent hypoxia alone does not affect retinal vascularization or microglial cell counts:

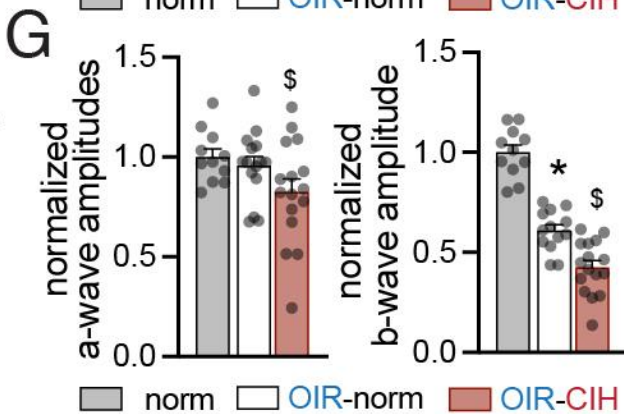
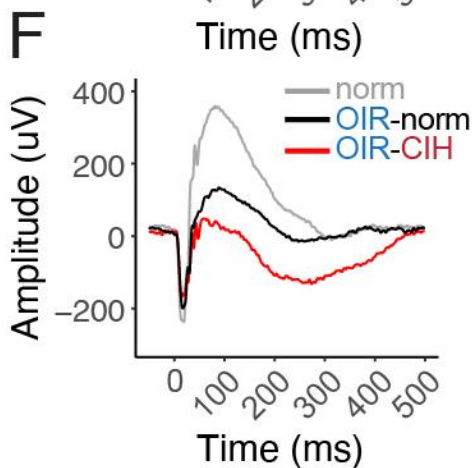
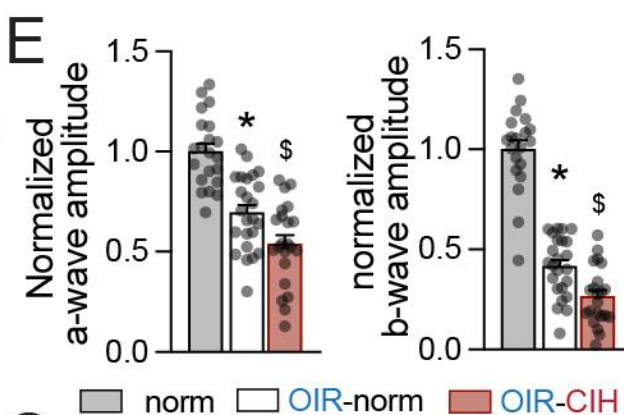
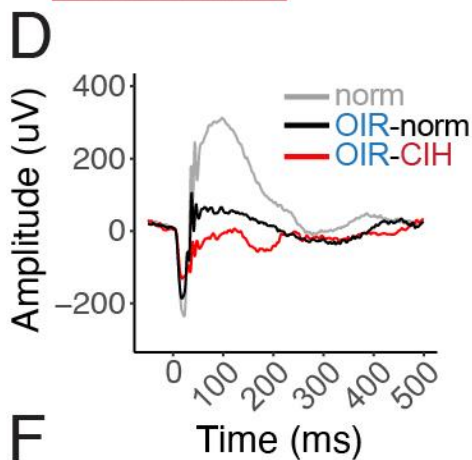
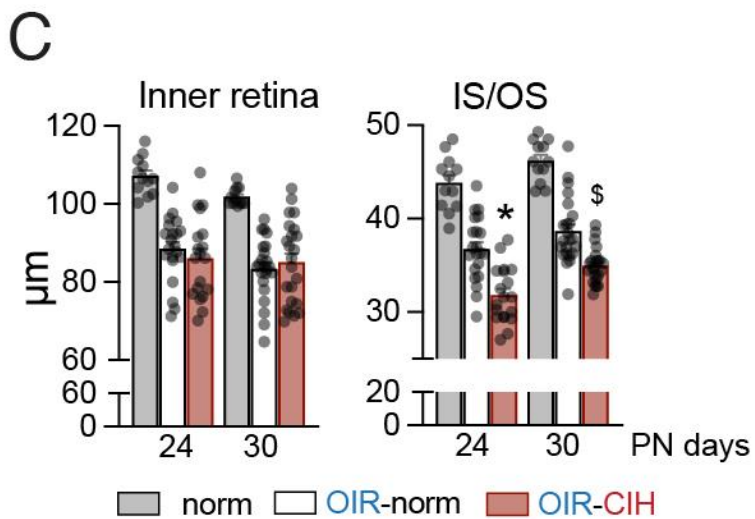
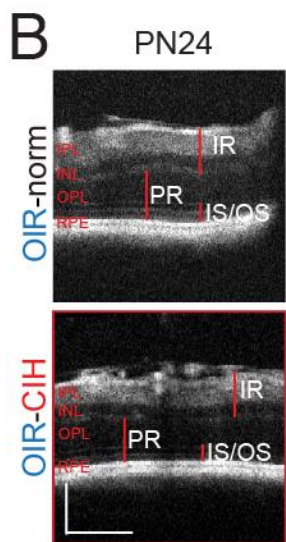
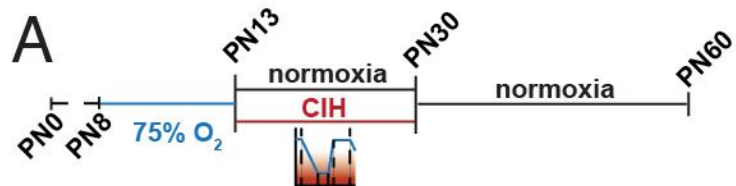
(A) Schematic of the time course of normoxia and CIH conditions. (B) Left: Representative retinal flatmounts labeled with isolectin (green) to visualize vessels. Right: Quantification of vessel density (mm vessel per mm<sup>2</sup> retinal area,  $n=18$  retinas per group). (C) Left: Representative gating of CD45<sup>med</sup>CD11b<sup>+</sup> microglial cells (MC). Right: Quantification of the number of microglia (single, alive, CD45<sup>med</sup>CD11b<sup>+</sup> cells) per retina ( $n=18-19$  retinas per group). (D) Mean intensity of MHCII labeling on MC detected via flow cytometry ( $n=18-19$  retinas per group). (E) Body weight of pups at PN18 ( $n=11-12$  animals per group). Scale bars = 500  $\mu$ m; all comparisons Mann-Whitney test, all  $p$ -values > 0.05; all values displayed as mean  $\pm$  SEM, individual points indicate values per retina.



**Figure 2.:** CIH inhibits the revascularization of ischemic retina, promotes pathological intravitreal neovascularization, and induces a transient growth retardation

(A) Schematic of the time course of OIR, normoxia, and CIH conditions. (B) Representative retinal flatmounts labeled with isolectin (green) to visualize vessels. Avascular areas are indicated with a dashed orange line. PN18 insets show examples of neovascularization. PN30 insets highlight abnormal vasculature in some revascularized areas in OIR-CIH retinas. Quantification of (C) avascular and (D) neovascular regions in retinal flatmounts ( $n = 14-30$  retinas per timepoint per group; two-way ANOVA followed by Šídák's multiple comparisons test **C** \*  $p = 0.0125$  PN18 OIR-Norm versus OIR-CIH, \$  $p = 0.0014$  PN21 OIR-Norm versus OIR-CIH, %  $p < 0.0001$ , **D** \*  $p = 0.0028$  PN24 OIR-Norm versus OIR-CIH, \$  $p = 0.0089$  PN27 OIR-Norm versus OIR-CIH). In C the average percentage of avascular area at the end of OIR (PN13) is indicated with a blue dashed line. (E) Body weight in grams of pups from PN14-PN30 ( $n = 6-12$  animals per group; two-way ANOVA followed by Šídák's multiple comparisons test \*  $p < 0.0001$  PN16 OIR-Norm versus OIR-CIH, \*  $p = 0.0001$  PN18 OIR-Norm versus OIR-CIH, \*  $p < 0.0001$  PN21 OIR-Norm vs OIR-CIH, \*  $p < 0.0001$  PN24 OIR-Norm versus OIR-CIH). Scales bars = 1000  $\mu\text{m}$ ; all values displayed as mean  $\pm$  SEM, individual points indicate values per retina (C-D) or per animal (E).

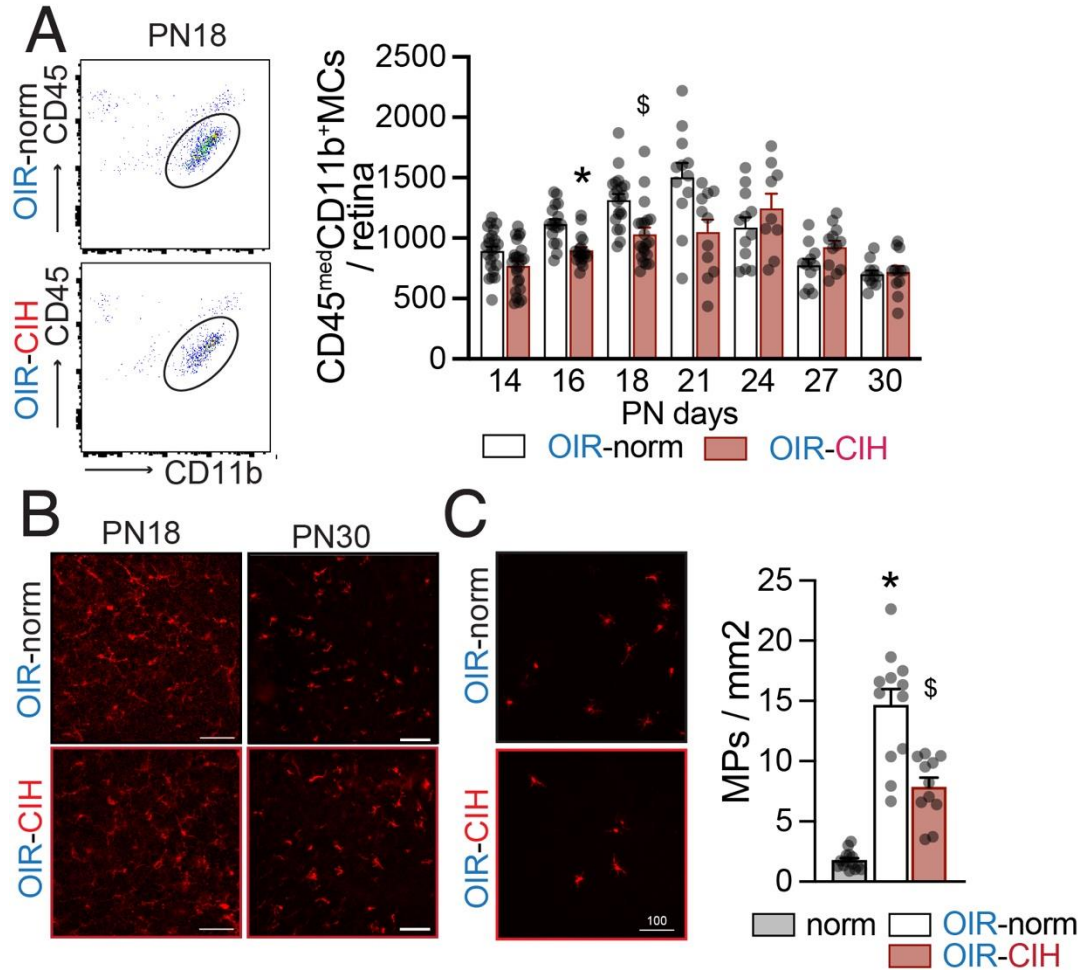




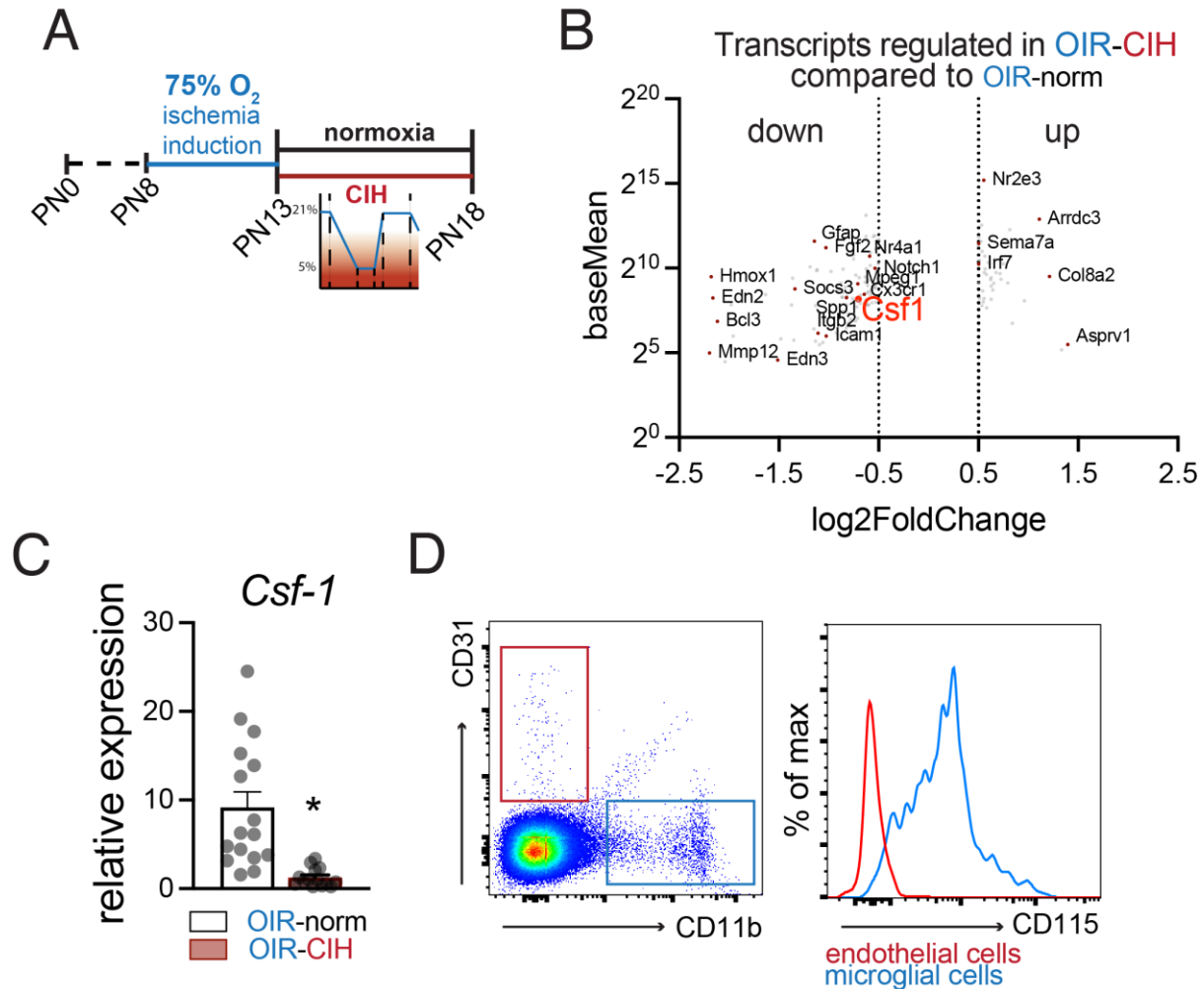


**Figure 3. OIR induces neurodegeneration and CIH exacerbates functional deficits in retinal signaling.**

(A) Schematic of the time course of OIR, normoxia, and CIH conditions. (B) Representative B scans of OIR-Norm and OIR-CIH retinas at PN24. B scans are from the central retina, near the optic nerve head. Horizontal scale bar = 200  $\mu\text{m}$ , vertical scale bar = 150  $\mu\text{m}$ . (C) Quantification of the thickness of the inner retina, ONL, and IS/OS layers, measured from the OCT B scans ( $n=12-24$  eyes per group; one-way ANOVA followed by Šídák's multiple comparisons test, inner retina \*  $p < 0.0001$  PN24 and PN30 Normoxia vs OIR-Norm and Normoxia vs OIR-CIH; ONL \*  $p = 0.0034$  PN24 Normoxia vs OIR-CIH; IS/OS \*  $p < 0.0001$  PN24 Normoxia vs OIR-Norm, Normoxia vs OIR-CIH, and OIR-Norm vs OIR-CIH, \*  $p < 0.0001$  P30 Normoxia vs OIR-Norm and Normoxia vs OIR-CIH, \$  $p = 0.0007$  P30 OIR-Norm vs OIR-CIH). (D) Representative ERG waveforms of normoxia (grey), OIR-norm (black), and OIR-CIH (red) at PN30 in response to 4 ms flashes of 3  $\text{cd}^*\text{s}/\text{m}^2$ . (E) Quantification of a- (left) and b-wave (right) amplitudes at PN30 in response to 4 ms flashes of 3  $\text{cd}^*\text{s}/\text{m}^2$  ( $n = 20-24$  eyes per group; one-way ANOVA followed by Šídák's multiple comparisons test, normalized a-wave amplitude \*  $p < 0.0001$  Normoxia vs OIR-Norm, \$  $p < 0.0001$  Normoxia vs OIR-CIH and  $p = 0.0205$  OIR-Norm vs OIR-CIH; normalized b-wave amplitude \*  $p < 0.0001$  Normoxia vs OIR-Norm, \$  $p < 0.0001$  Normoxia vs OIR-CIH and  $p = 0.0096$  OIR-Norm vs OIR-CIH). (F) Representative ERG waveforms of normoxia (grey), OIR-norm (black), and OIR-CIH (red) at PN60 in response to 4 ms flashes of 3  $\text{cd}^*\text{s}/\text{m}^2$ . (G) Quantification of a- (left) and b-wave (right) amplitudes at PN60 in response to 4 ms flashes of 3  $\text{cd}^*\text{s}/\text{m}^2$  ( $n = 11-16$  eyes per group; one-way ANOVA followed by Šídák's multiple comparisons test, normalized a-wave amplitude all  $p$  values  $> 0.05$ , normalized b-wave amplitude \*  $p < 0.0001$  Normoxia vs OIR-Norm, \$  $p < 0.0001$  Normoxia vs OIR-CIH and  $p = 0.0008$  OIR-Norm vs OIR-CIH). All values displayed as mean  $\pm$  SEM, individual points indicate values per eye. IPL = inner plexiform layer, INL = inner nuclear layer, OPL = outer plexiform layer, ONL = outer nuclear layer, RPE = retinal pigment epithelium, IR = inner retina, PR = photoreceptors, IS/OS = inner and outer segments.

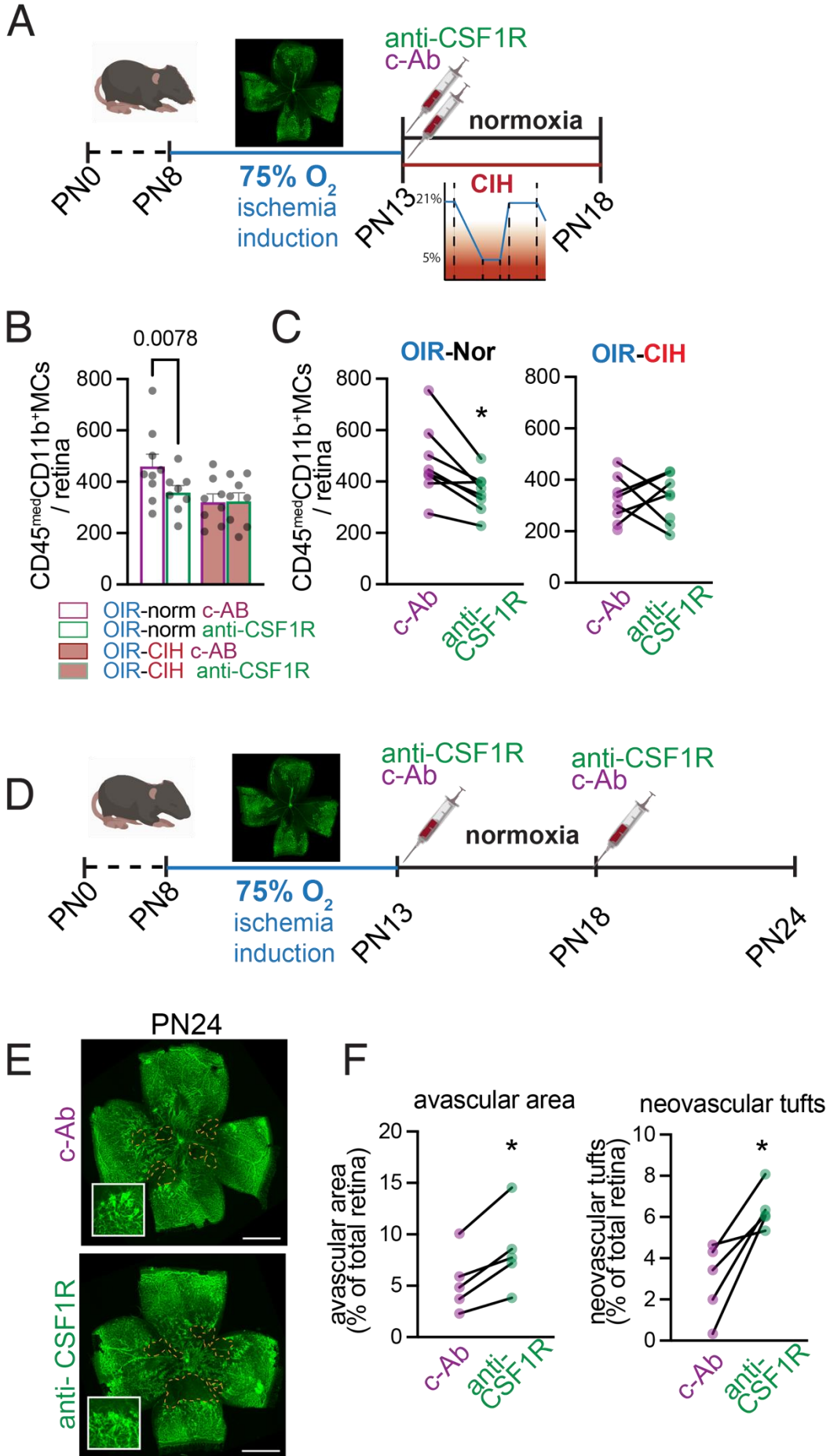


**Figure 4.** CIH dampens the reactive microglial expansion in ischemic retinopathy (A) Left: Representative gating of CD45<sup>med</sup>CD11b<sup>+</sup> microglial cells (MC). Right: Quantification of single alive CD45<sup>med</sup>CD11b<sup>+</sup> retinal microglia ( $n = 11-24$  retinas per group; two-way ANOVA and Šídák's multiple comparisons test \*  $p = 0.0006$  PN16 OIR-Norm versus OIR-CIH, \$  $p = 0.0039$  PN18 OIR-Norm versus OIR-CIH). (B) Representative images of central inner retinal microglia, labeled with IBA1 (red) in retinal flatmounts at PN18 and PN30. (C) Left: Representative images of subretinal mononuclear phagocytes (MPs), labeled with IBA1 (red) on RPE/choroidal flatmounts at PN30. Right: quantification of subretinal IBA1<sup>+</sup> MPs at PN30 ( $n = 11-14$  eyes per group; one-way ANOVA followed by Šídák's multiple comparisons test, \*  $p < 0.0001$  Normoxia vs OIR-Norm, \$  $p < 0.0001$  Normoxia vs OIR-CIH and OIR-Norm vs OIR-CIH). Scales bars = 100  $\mu\text{m}$ ; all values displayed as mean  $\pm$  SEM, individual points indicate values per retina.



**Figure 5. CIH reduces retinal *Csf1* transcription:**

(A) Schematic of the time course of OIR, normoxia, and CIH conditions. (B) Volcano plot of differentially expressed genes detected in bulk RNA sequencing of OIR-Norm and OIR-CIH retinas. (C) Comparison of *Csf1* expression in OIR-Norm and OIR-CIH retinas at PN18 by qPCR ( $n = 16$  retinas per group; Mann-Whitney test \*  $p < 0.0001$  OIR-Norm versus OIR-CIH; all values displayed as mean  $\pm$  SEM, individual points indicate values per retina). (D) Left: Flow cytometry gating scheme for CD31<sup>+</sup> endothelial cells and CD11b<sup>+</sup> microglia. Right: CD115 (CSF1R) expression on single alive CD31<sup>+</sup> endothelial cells (red) and single alive CD45<sup>med</sup>CD11b<sup>+</sup> microglia (blue).



**Figure 6: CSF1R inhibition during the ischemic phase of OIR recapitulates the effect of CIH on microglia and vasculature:**

(A) Schematic of the time course of injections, and OIR, normoxic, and CIH conditions for the data shown in B-C. (B) Quantification of CD45<sup>med</sup>CD11b<sup>+</sup> microglia by flow cytometry at PN18, 5 days after intravitreal antibody injection, comparing across all experimental groups ( $n = 8$  retinas per condition; values displayed as mean  $\pm$  SEM, individual points indicate values per retina). (C) Quantification of CD45<sup>med</sup>CD11b<sup>+</sup> microglia by flow cytometry, as in B, but showing the paired comparisons between contralateral eyes within the OIR-Norm (left) and OIR-CIH (right) groups ( $n = 8$  pups per group; paired Mann-Whitney test \*  $p = 0.0078$  OIR-Norm c-Ab versus anti-CSF1R). (D) Schematic for the time course of injections and OIR and normoxic conditions for the data shown in E-F. (E) Representative flatmounts of PN24 retinas after anti-CSF1R antibody or control injections. Vessels are labeled with isolectin (green) and avascular areas are indicated with dashed yellow lines. Insets shown representative areas of neovascularization (NV). (F) Quantification of avascular (left) and neovascular tuft (right) area on the retinal flatmounts ( $n = 5$  pups per group; paired Mann-Whitney test \*  $p = 0.0312$  c-Ab versus anti-CSF1R avascular area, \*  $p = 0.0312$  c-Ab versus anti-CSF1R neovascular tufts). Scale bars = 1000  $\mu$ m.

**Supplemental Table 1:** Significantly differentially expressed genes between OIR-Norm and OIR-CIH retinas.

List of genes significantly down-regulated (top) or up-regulated (bottom) in OIR-CIH retinas compared to OIR-Norm retinas. Genes are ordered from largest to smallest fold change.

<b>Gene</b>	<b>log2(Fold Change)</b>	<b>P value</b>
<i>Mmp12</i>	-2.19546	2.47E-06
<i>Hmox1</i>	-2.17937	6.10E-76
<i>Edn2</i>	-2.16303	2.63E-35
<i>Bcl3</i>	-2.11643	1.89E-21
<i>Steap4</i>	-2.03947	2.28E-05
<i>Flnc</i>	-1.97922	0.000574
<i>Scube1</i>	-1.95972	2.40E-11
<i>Lad1</i>	-1.63299	2.77E-32
<i>Edn3</i>	-1.51173	0.000448
<i>Timp1</i>	-1.48226	1.58E-06
<i>Tnnt2</i>	-1.37407	1.62E-12
<i>Serpina3n</i>	-1.36266	1.54E-26
<i>Cd84</i>	-1.34968	4.4E-06
<i>Gm33148</i>	-1.34835	0.000536
<i>Socs3</i>	-1.33982	0.0001
<i>Cebpd</i>	-1.18778	1.58E-22
<i>Myh11</i>	-1.15113	0.000644
<i>Gfap</i>	-1.14415	2.13E-26
<i>Gna14</i>	-1.13474	0.000107
<i>Tgm2</i>	-1.13009	6.42E-10
<i>Pcolce</i>	-1.12208	3.49E-22
<i>C4b</i>	-1.11598	5.88E-29
<i>Itgb2</i>	-1.10869	4.84E-05
<i>Arhgap30</i>	-1.09396	0.000378
<i>Gm48237</i>	-1.06098	0.00072
<i>Fgf2</i>	-1.02972	1.08E-23
<i>Icam1</i>	-1.02534	0.000508
<i>Jak3</i>	-0.99286	1.19E-07
<i>Ptgfr</i>	-0.99034	7.27E-06
<i>Gm15983</i>	-0.97834	1.47E-15
<i>Ecell</i>	-0.92402	0.000201
<i>Spry3</i>	-0.91416	4.56E-12
<i>Antxr2</i>	-0.87876	1.01E-15
<i>Lyz2</i>	-0.86716	4.6E-07
<i>H19</i>	-0.86071	6.6E-07
<i>Slc7a11</i>	-0.85623	2.61E-08

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<i>Gm19667</i>	-0.85504	1.79E-07
<i>Gm20045</i>	-0.84859	0.000404
<i>Serping1</i>	-0.83281	1.18E-08
<i>Best3</i>	-0.82468	0.000811
<i>Spp1</i>	-0.82154	2.81E-08
<i>Fhad1</i>	-0.8206	2.61E-07
<i>Mob3c</i>	-0.8205	2.71E-09
<i>Kif14</i>	-0.81223	0.000397
<i>B230303O12Rik</i>	-0.7996	0.000182
<i>Tcf7</i>	-0.79754	0.000512
<i>2810032G03Rik</i>	-0.78735	0.000392
<i>Tnfrsf1b</i>	-0.78105	0.000245
<i>F5</i>	-0.7781	5.57E-05
<i>Ahnak</i>	-0.77099	2.53E-09
<i>Ddr2</i>	-0.76354	0.000123
<i>Sned1</i>	-0.75921	5.55E-10
<i>Lrrc2</i>	-0.752	5.07E-09
<i>Slc25a37</i>	-0.75087	3.33E-13
<i>Gm44037</i>	-0.73337	0.000654
<i>Vcan</i>	-0.72789	3.62E-06
<i>Cldn2</i>	-0.71944	0.000248
<i>Sh2d1a</i>	-0.71238	0.000407
<i>Mpeg1</i>	-0.71024	9.33E-09
<i>Gm15513</i>	-0.70878	0.000129
<i>Csf1</i>	-0.70338	7.35E-05
<i>Osmr</i>	-0.69593	3.65E-05
<i>Fcrls</i>	-0.68488	4.22E-06
<i>Scn7a</i>	-0.68074	3.37E-06
<i>Cx3cr1</i>	-0.64342	7.12E-05
<i>Gm20342</i>	-0.64274	0.000206
<i>Tnfrsf1a</i>	-0.63731	2.65E-05
<i>Lrrfip1</i>	-0.63443	9.22E-05
<i>Cp</i>	-0.62857	3.82E-14
<i>Stat3</i>	-0.62785	1.86E-11
<i>Fbln2</i>	-0.62562	0.000321
<i>E2f6</i>	-0.62409	2.24E-09
<i>Piezo1</i>	-0.61941	0.000558
<i>Ogn</i>	-0.61508	0.000244
<i>Arid5a</i>	-0.61281	0.000362
<i>Col4a6</i>	-0.60951	0.000308
<i>Marveld3</i>	-0.6045	0.000247
<i>Fgf2os</i>	-0.60432	0.000327
<i>Arpc1b</i>	-0.60113	1.06E-05

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<i>Tnip2</i>	-0.59794	3.27E-07
<i>Nr4a1</i>	-0.59197	1.59E-07
<i>Sbno2</i>	-0.58464	0.000018
<i>Nupr1</i>	-0.58277	0.000679
<i>A2m</i>	-0.5815	1.49E-05
<i>Col1a2</i>	-0.57627	0.000568
<i>Matn2</i>	-0.56825	0.000508
<i>Atp1a2</i>	-0.55361	7.23E-05
<i>Loxl4</i>	-0.55298	6.98E-06
<i>Myo10</i>	-0.54106	1.48E-09
<i>Pde3b</i>	-0.54083	2.68E-05
<i>Pprc1</i>	-0.53903	0.000011
<i>Notch1</i>	-0.53741	2.78E-06
<i>Pros1</i>	-0.52998	0.000051
<i>Eif4e3</i>	-0.52939	0.000413
<i>Klhl29</i>	-0.52778	1.9E-07
<i>Greb1</i>	-0.52628	0.00016
<i>Bcl6</i>	-0.5102	3.45E-05
<i>Lama5</i>	-0.50698	0.000229
<i>Bub1b</i>	-0.50344	1.79E-05
<i>Rfx2</i>	-0.50239	1.35E-05
<i>Jag1</i>	-0.50006	8.09E-10

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<b>Gene</b>	<b>log2(Fold Change)</b>	<b>P value</b>
<i>Asprv1</i>	1.396966	0.000405
<i>Upk3b</i>	1.334502	0.000197
<i>Col8a2</i>	1.213317	7.31E-15
<i>Arrdc3</i>	1.110585	3.83E-33
<i>Hmgb2</i>	0.971857	2.04E-13
<i>Gm26670</i>	0.961857	5.12E-11
<i>Tfap2e</i>	0.838723	8.83E-05
<i>Hist1h1c</i>	0.812708	9.37E-12
<i>Ciart</i>	0.79332	2.28E-09
<i>Bhlhe23</i>	0.749628	4.15E-11
<i>Id1</i>	0.726885	1.6E-08
<i>Wfikkn2</i>	0.716414	6.23E-06
<i>Ankrd37</i>	0.674222	6.94E-11
<i>Neurog2</i>	0.652274	2.65E-07
<i>Oasl2</i>	0.643976	2.66E-07
<i>Tifa</i>	0.628751	6.58E-06
<i>Insm1</i>	0.628095	1.30E-09
<i>Slc16a8</i>	0.62809	0.000473
<i>Id2</i>	0.62746	6.38E-07
<i>Gm27032</i>	0.62144	5.32E-06
<i>Ppp1r3c</i>	0.597896	6.84E-10
<i>Bsnd</i>	0.582821	0.000164
<i>Prdm13</i>	0.58005	1.81E-06
<i>Pim3</i>	0.569174	2.27E-09
<i>Selenbp1</i>	0.565297	1.32E-08
<i>2410006H16Rik</i>	0.560945	0.000261
<i>2810410L24Rik</i>	0.55609	0.00034
<i>Nr2e3</i>	0.552607	3.25E-08
<i>Pcsk9</i>	0.549653	0.000814
<i>Gm16551</i>	0.543908	2.29E-05
<i>Bhlhe40</i>	0.537619	1.03E-06
<i>Gm16958</i>	0.53288	0.000781
<i>Tfrc</i>	0.53074	1.94E-09
<i>Ubt1</i>	0.524596	1.46E-05
<i>Irf2bp2</i>	0.512755	6.26E-07
<i>2310039H08Rik</i>	0.510484	0.000493
<i>Sema7a</i>	0.503031	1.63E-07
<i>Irf7</i>	0.5022	3.14E-05
<i>Usp2</i>	0.501731	2.86E-08
<i>Nnat</i>	0.501629	1.17E-08
<i>Racgap1</i>	0.501533	7.65E-05

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# Annex

## Animal modelling

We used oxygen induced retinopathy (OIR) mice as our primary animal model. C57BL/6J mice at postnatal (PN) day 8 (PN8) with nursing mothers were kept in the cages (hyperoxia cages) for 5 days from PN8 to PN13. An oxygen concentrator connected to the cages and inputted oxygen into them to make O<sub>2</sub> concentration around 75% inside these hyperoxia cages (Supplemental Figure 1A). We had an oxygen detector that can detect the O<sub>2</sub> concentration inside the hyperoxia cages.

After 5-days exposure in hyperoxia, mouse pups exhibited phenotypes such as decreased body weight, vessel loss in central retinas (see details in Article-Results Figure 2B, 2E). At PN13, we removed mouse pups from the hyperoxia cages into normoxia cages and chronic intermittent hypoxia (CIH) cages (Supplemental Figure 1B). Normoxia cages and CIH cages are cages connected to a nitrogen concentrator. The N<sub>2</sub> concentrator inputted air into the normoxia cages and N<sub>2</sub> into the CIH cages. Therefore, the O<sub>2</sub> concentration maintained in normal level in normoxia cages as control but decreased in the CIH cages. A regulator controlled the nitrogen delivery, we set the CIH program to create an intermittent and chronic hypoxia environment inside the CIH cages (see details in Article-Methods and Article-Results Figure 1A, 2A). Each normoxia cage and CIH cage had an oxygen sensor inside for detecting the O<sub>2</sub> concentration at any time.

We kept the OIR mice in normoxia cages and CIH cages since PN13, analysed at different time points, such as PN14, PN16, PN18, PN21, PN24 and PN30. We have done immunohistochemistry for retinas flatmounts, flow cytometry for retinas dissociation, optical coherence tomography and electroretinography in living mouse pups, RNA sequencing and qPCR for retinas RNA extraction (see details in Article-Methods). Mice in CIH cages exhibited aggravated growth retardation, promoted photoreceptors degeneration, inhibited retinal revascularization, promoted pathological neovascularization, decreased retinal microglia population and down-regulated *CSF1* expression (see details in Article-Results). Our local

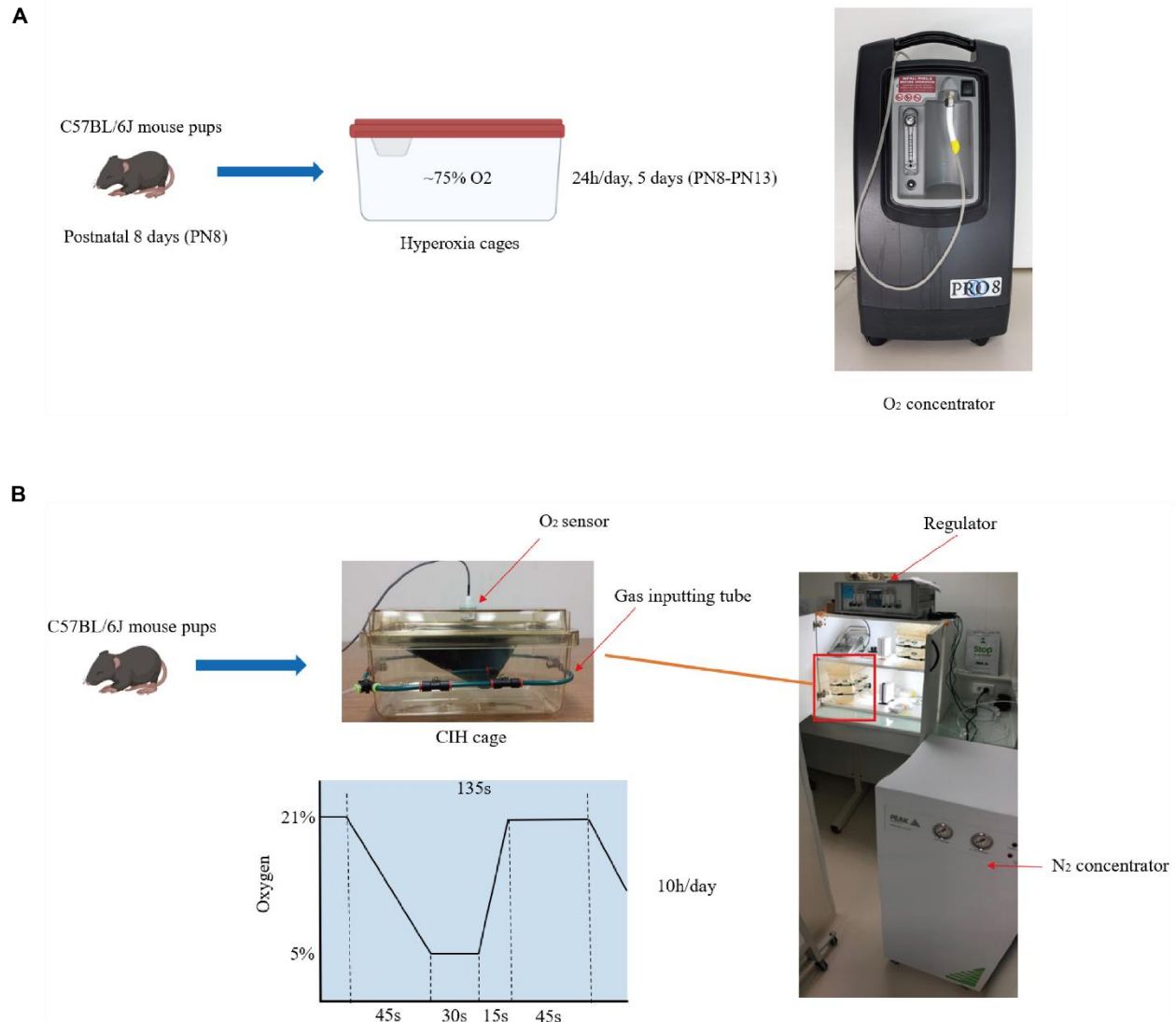
blocking of CSF1 receptor (CSF1R) verified the inhibitory effect of CIH on CSF1-CSF1R signalling (see details in Article-Results Figure 6).

### **Flow cytometry gating strategy**

For flow cytometry, we selected CD11b<sup>+</sup>CD45<sup>med</sup> retinal cells as retinal microglial cells (see details in Article-Methods-Flow cytometry). The gating strategy is shown in Supplemental Figure 2.

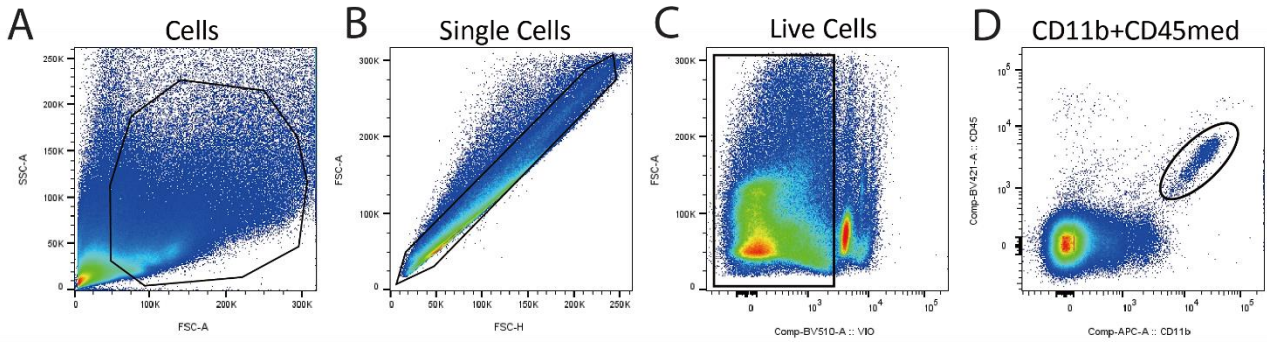
### **Gene Ontology (GO) analysis**

The GO analysis of our bulk RNA sequencing data reveals several key biological processes that are differentially regulated by CIH in ischemic retinas (the enrichment map in Supplemental Figure 3). The significant enrichment of immune response-related processes (such as “regulation of leukocyte mediated immunity,” “leukocyte activation involved in immune response,” and “adaptive immune response based on somatic recombination of immune receptors”) suggests a robust immune activity. The changes in extracellular matrix organization (such as “extracellular matrix organization,” “extracellular structure organization,” and “external encapsulating structure organization”) and cell differentiation processes (such as “neural precursor cell proliferation,” “neuroendocrine cell differentiation,” and “regulation of osteoblast differentiation”) indicate alterations in tissue structure and development. Additionally, the presence of processes related to negative regulation (such as “negative regulation of response to external stimulus,” “negative regulation of secretion,” and “negative regulation of secretion by cell”) highlights specific inhibitory processes that are prominent in the presence of CIH. This Go analysis provides a comprehensive overview of the functional changes affected by CIH in ischemic retinas and can guide further investigation into the underlying biological mechanisms.



**Supplemental Figure 1: Hyperoxia cages for OIR mouse and setup of CIH cages.**

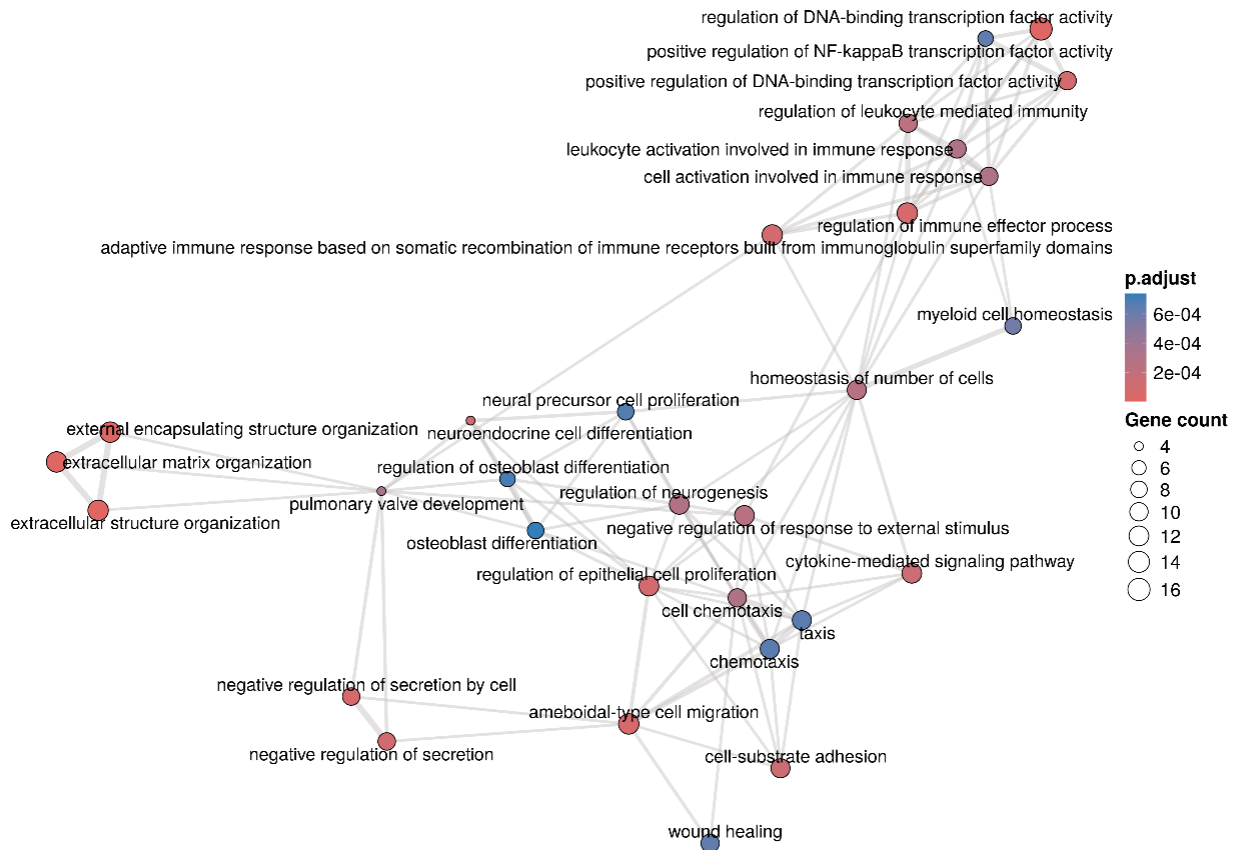
(A) Schematic of OIR mice modeling. The O<sub>2</sub> concentrator inputted oxygen into the hyperoxia cages to make O<sub>2</sub> concentration around 75% inside the cages. PN8 mouse pups with nursing mothers were kept in these cages for 5 days from PN8 to PN13. Hyperoxia were maintained for all the time in these 5 days. (B) Schematic of CIH setup. The N<sub>2</sub> concentrator inputted air or nitrogen into the normoxia cage or CIH cage. The O<sub>2</sub> concentration in these cages were controlled by the regulator with an O<sub>2</sub> sensor. A 135s cycle of CIH program was set by the regulator as shown, this CIH cycle is performed repeatedly for 10 hours per day during daytime.



**Supplemental Figure 2: Gating strategy of flow cytometry.**

(A) All cells were gated by FSC-A (x axis) and SSC-A (y axis), shown as the black circle. (B) Single cells were gated by FSC-H (x axis) and FSC-A (y axis), shown as the black box. (C) Live cells were gated by BV510-A (x axis) and FSC-A (y axis), shown as the black box. (D) CD45<sup>med</sup>CD11b<sup>+</sup> microglial cells were gated by APC-A (x axis) and BV421-A (y axis), shown as the black box.

**OIR-NORM-P18 vs. OIR-CIH-P18**





**Supplemental Figure 3: Enrichment map of differentially expressed genes detected in bulk RNA sequencing of OIR-Norm and OIR-CIH retinas.**

Each node represents a GO term, which is a functional category describing biological processes, molecular functions, or cellular components. The size of each node indicates the gene count associated with that GO term, with larger nodes representing more genes. Lines connecting the nodes represent the relationships between GO terms. Thicker edges suggest stronger relationships or higher degrees of overlap in the gene sets. Nodes are colored based on the adjusted p-value (p.adjust), which is a measure of the statistical significance of the enrichment for each GO term. The color gradient ranges from blue (less significant, higher p.adjust) to red (more significant, lower p.adjust). Terms colored in dark red represent highly significant GO terms with low p.adjust values, indicating strong enrichment.

# **Additional results**

The rodent laser trauma model, widely utilized to replicate choroidal neovascularization (CNV) in wet age-related macular degeneration (AMD), involves using laser photocoagulation to breach adult mice's Bruch's membrane. The laser stimulates the proliferation of new blood vessels from the choroid into the sub-retinal space. A recent report showed that patients with obstructive sleep apnea (OSA) have a significantly higher likelihood of being diagnosed with AMD, regardless of obesity (Keenan et al., 2017). CNV is the main characteristic of wet AMD. Therefore, we conducted chronic intermittent hypoxia (CIH) experiments on the CNV model (C57BL/6J mice, males, 3 months old) to investigate the effects of CIH on choroidal neovascularization. The CIH protocol, mirroring that of our OIR mouse model (5% hypoxia, 135s per cycle, 10h per day), was applied in three experiments. Mice underwent 14 days of CIH exposure without laser injuries (**Figure 9A**), mice underwent 15 days and 24 days of CIH with laser injuries at 14th day (**Figure 10A. 11A**). Our findings indicate that CIH elevated hematocrit levels at 1- and 10-days following laser injuries and slightly reduced choroidal NV area 10 days post-laser injuries. However, CIH did not impact retinal vessel density, nor did it change the population of choroidal macrophages and retinal microglial cell in the CNV model.

### **CIH increased hematocrit of the CNV mice**

After 15 and 24 days of CIH exposure, the hematocrit levels in the CIH group were significantly higher compared to the Normoxia group (**Figure 10B,11C**). In healthy mice, hematocrit ranges from 35% to 52% (O'Connell et al., 2015), our results are within this range. In hypoxia condition, mice can increase their hematocrit levels as an adaptation to hypoxia. This adaptation is a well-studied response to chronic hypoxia and can be initiated acutely through mechanisms such as sympathetically mediated splenic contraction. In this case, the chronic hypoxia induces erythropoietin, which increases the production of erythrocytes (Suresh et al., 2020). This process is part of the body's adaptation to survive under lower oxygen levels by increasing the number of red blood cells to enhance the oxygen-carrying capacity of the blood (Mayfield et al., 1994).

### **CIH had no impact on retinal vessel density and population of retinal microglia**

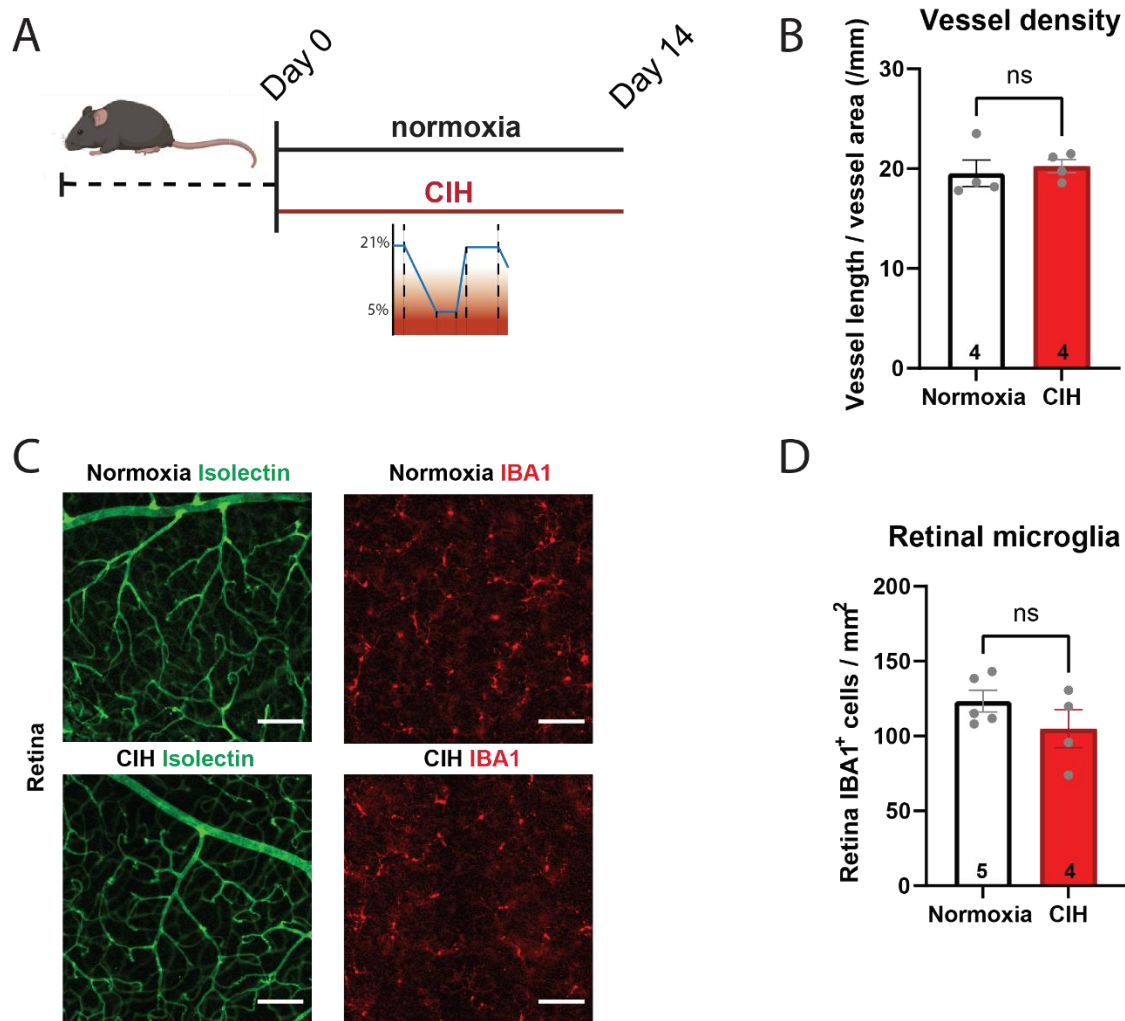
We observed no change of retinal vessel density (**Figure 9B, 9C, 10C, 10D**) and population of retinal microglial cells in all three experiments (**Figure 9D, 10C, 10E, 11F**). A well-developed retina can adapt to varying levels of oxygen through physiological adjustments, such as enhancing its oxygen-carrying capabilities or improving its function in conditions of low oxygen. (Lange and Bainbridge, 2012). The increase in hematocrit levels due to CIH confirmed this adaptive response. This adaptive capacity may shield normal retinas from CIH-induced impacts on vascular development or microglial function.

### **CIH decreased NV area and had no impact on population of choroidal macrophages in CNV mice**

10 days post-laser injuries, evident CNV was observed at the sites of choroidal laser impacts (**Figure 11B**). The NV areas in CIH group were significantly smaller than Normoxia group (**Figure 11C, 11D**). CIH did not influence population of macrophages around the laser impacts (**Figure 11E**), which may indicate that the decreased CNV areas were not due to the choroidal macrophages. The reason why choroidal macrophages are not affected by CIH may also be due to mice's adaptation to chronic hypoxia. The mechanisms underlying CIH-induced reduction in CNV areas remain unclear; however, it may be associated with the reported anti-angiogenic effects of CIH (Visniauskas et al., 2021), which we also observed in the ischemic retina of young pups.

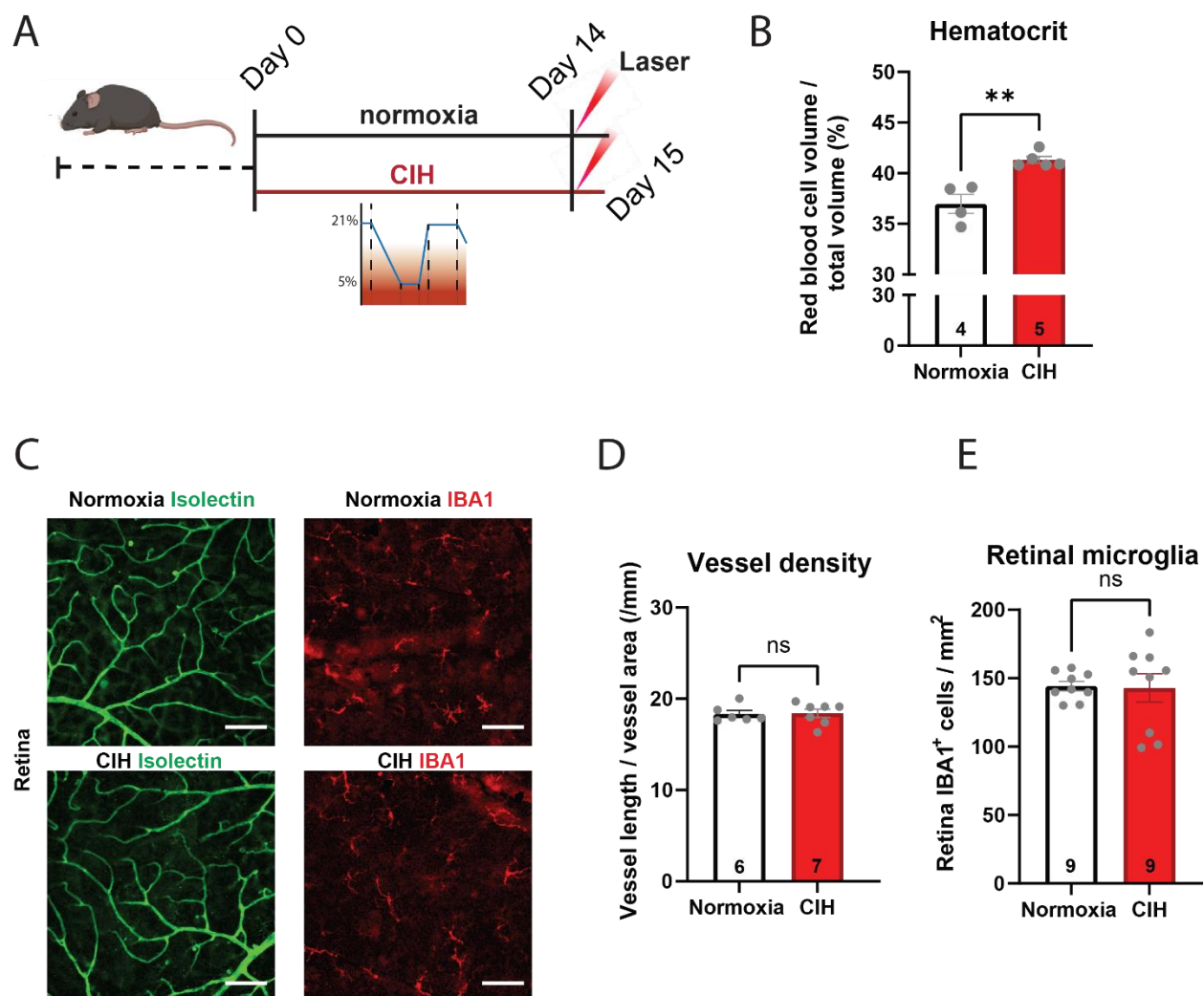
Initially, we hypothesized that CIH would exacerbate the pathological conditions in adult mice with CNV, but our findings did not support this. Instead, we observed that adult mice adapted to CIH by increasing their red blood cell number, thereby enhancing blood's oxygen-carrying capability. This adaptation to hypoxia allowed retinal vessels, microglia, and choroidal macrophages to resist the effects of CIH, similar to observations in the retinas of normal

newborn mice. Contrary to expectations, CIH slightly reduced choroidal neovascularization in CNV mice, though the exact mechanisms behind this effect are not yet understood. However, CIH did increase pathological retinal neovascularization in ischemic retinas. These observations in adult CNV mice contribute to a deeper understanding of how apnea and CIH might exacerbate ischemic proliferative retinopathies.



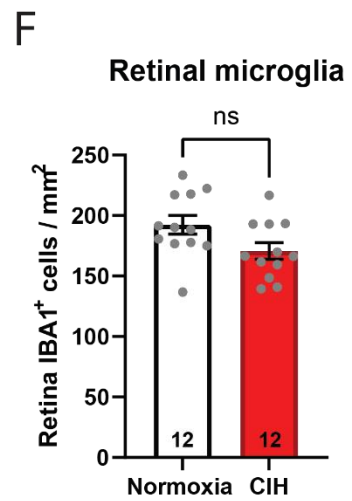
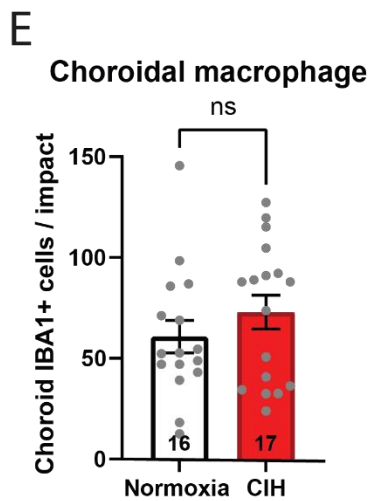
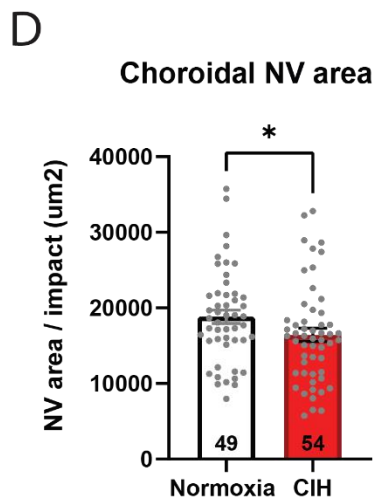
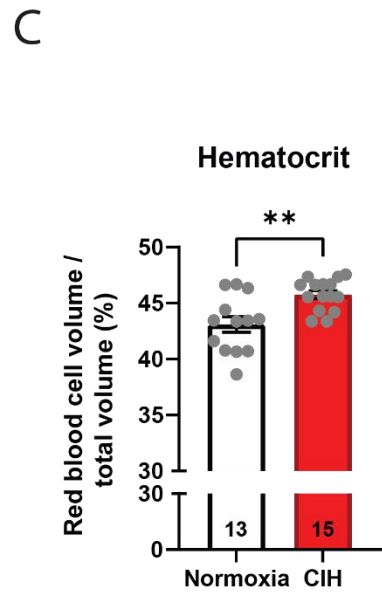
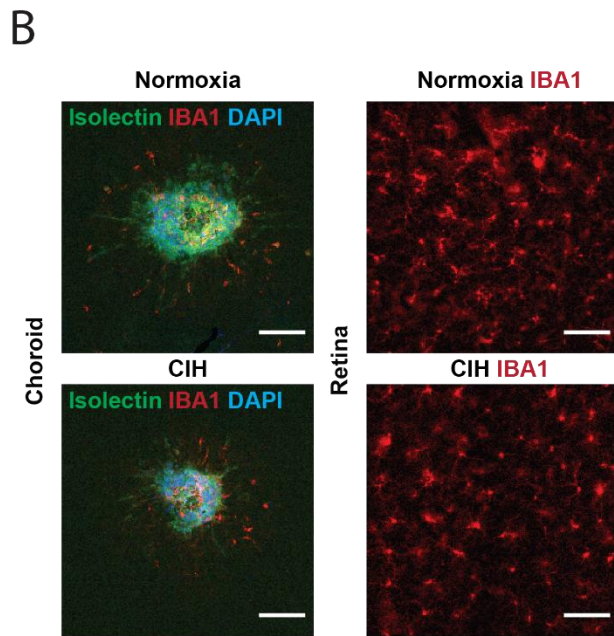
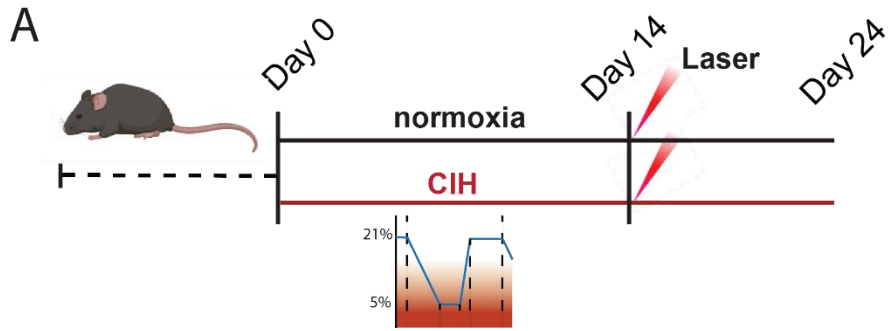
**Figure 9: 14-days CIH did not affect retinal vessel density or retinal microglial population**

(A) Schematic of the time course of normoxia and CIH conditions. (B) Quantification of retinal vessel density (mm vessel per mm<sup>2</sup> retinal area,  $n=4$  retinas per group). ns: not significant,  $p$ -values  $> 0.05$ . (C) Representative retinal flatmounts labeled with isolectin (green) to visualize vessels and IBA1 (red) to visualize microglial cells. Scale bars = 200 $\mu$ m. (D) Quantification of the number of retinal microglial cells ( $n= 4$ -5 retinas per group). ns: not significant,  $p$ -values  $> 0.05$ . All comparisons Mann-Whitney test; all values displayed as mean  $\pm$  SEM, individual points indicate values per retina.



**Figure 10: 15-days CIH increased hematocrit levels and did not affect retinal vessel density or retinal microglial population in mice 1 day post-laser injuries**

(A) Schematic of the time course of normoxia and CIH conditions. (B) Hematocrit levels of CNV mice ( $n=4-5$  mice per group). \*\*:  $0.001 < p\text{-value} < 0.01$ , individual points indicate values per mouse. (C) Representative retinal flatmounts labeled with isolectin (green) to visualize vessels and IBA1 (red) to visualize microglial cells. Scale bars =  $200\mu\text{m}$ . (D) Quantification of retinal vessel density (mm vessel per  $\text{mm}^2$  retinal area,  $n=6-7$  retinas per group). ns: not significant,  $p\text{-values} > 0.05$ , individual points indicate values per retina. (E) Quantification of the number of retinal microglial cells ( $n=9$  retinas per group). ns: not significant,  $p\text{-values} > 0.05$ , individual points indicate values per retina. All comparisons Mann-Whitney test; all values displayed as mean  $\pm$  SEM.



**Figure 11: 24-days CIH increased hematocrit levels, decreased CNV areas and did not affect choroidal macrophages or retinal microglial population in mice 10 days post-laser injuries**

(A) Schematic of the time course of normoxia and CIH conditions. (B) Left: Representative choroidal laser impacts flatmounts labeled with isolectin (green) to visualize vessels, IBA1 (red) to visualize macrophages, and DAPI (blue) to visualize nucleus. Right: Retinal flatmounts labeled with IBA1 (red) to visualize microglial cells, Scale bars = 200 $\mu$ m. (C) Hematocrit levels of CNV mice ( $n=13-15$  mice per group). \*\*:  $0.001 < p\text{-value} < 0.01$ , individual points indicate values per mouse. (D) Quantification of choroidal NV areas at per laser impact ( $n=49-54$  laser impacts per group). \*:  $0.01 < p\text{-values} < 0.05$ , individual points indicate values per choroidal laser impact. (E) Quantification of the number of choroidal macrophages at per choroidal laser impacts ( $n=16-17$  choroids per group). ns: not significant,  $p\text{-values} > 0.05$ , individual points indicate values per choroid. (F) Quantification of the number of retinal microglial cells ( $n= 12$  retinas per group). ns: not significant,  $p\text{-values} > 0.05$ , individual points indicate values per retina. All comparisons Mann-Whitney test; all values displayed as mean  $\pm$  SEM.



# Discussion

Retinopathy of prematurity (ROP) is a common ocular disorder in premature infants and chronic intermittent hypoxia (CIH) has been shown to be a risk factor for ROP (Fiore et al., 2010, Fiore et al., 2012, Martin et al., 2011, Kumar et al., 2023). We used OIR and CIH models to investigate the impacts of CIH on ischemic retina. We found that CIH inhibited the revascularization of ischemic retina and promotes pathological intravitreal neovascularization, dampened the reactive microglial expansion, reduced retinal *Csf1* transcription and microglial CSF1R expression.

### **Healthy Retinas Tolerate CIH Well**

Our results demonstrated that normal retinas (from both adult and newborn mice) can better withstand intermittent changes in oxygen levels, CIH alone has no impact on the healthy retinal vascular development or microglial cell populations. However, we found that the ischemic retinas are more vulnerable to oxygen fluctuations. The results of the increased hematocrit via CIH in adult mice indicated that a healthy retina, as opposed to one in an ischemic state, may adapt to fluctuating oxygen levels through physiological mechanisms, such as increasing oxygen transport capacity or enhancing functionality under low-oxygen conditions (Lange and Bainbridge, 2012). This adaptive capacity might prevent CIH from affecting vascular development or microglial activity in normal retinas, while ischemic retinas can still be impacted. Therefore, the OIR model is an ideal model for studying the effects of CIH on retinal angiogenesis.

### **The Angiogenic Function of CIH**

Our results showed that CIH significantly inhibits revascularization of the ischemic retina, indicating that CIH has anti-angiogenic properties. Previous research in mice has demonstrated that CIH exhibits anti-angiogenic effects in the left ventricle (Visniauskas et al., 2021). The anti-angiogenic properties might be related to endothelial cells dysfunction which is present in OSA

patients (Atkeson et al., 2009, Budhiraja et al., 2007, Foster et al., 2007, Atkeson et al., 2008). In this project we did not investigate endothelial cell dysfunction in the ischemic retina, so further research is necessary to understand the connection between retinal endothelial cell dysfunction and CIH.

Additionally, using RNA sequencing our study revealed that in P18 OIR pups exposed to CIH, the expression of endothelin-2 (*Edn2*) and endothelin-3 (*Edn3*) were down-regulated. Both *Edn2* and *Edn3* are members of the endothelin family, which also includes endothelin-1 (*Edn1*). This family of peptides is known for its potent vasoconstrictive properties, playing a key role in constricting blood vessels. Furthermore, they are involved in cell survival and angiogenesis. (Davenport et al., 2016, Kawanabe and Nauli, 2011, Kedzierski and Yanagisawa). There is a study showing that *Edn2* is induced by hypoxia in tumor cells (Grimshaw et al., 2002), which suggests that there could be an increase in endothelin-2 production in conditions of CIH. However, the specific relationship between CIH and *Edn2* is still unclear. Interestingly, obstructive sleep apnea (OSA) patients exhibit increased circulating levels of *Edn1*, and these levels are directly related to the severity of their apnea (Saarelainen et al., 1997, Trakada et al., 2001). In a rat CIH model, circulating levels of *Edn1* were also increased (Brunner et al., 2006, Kanagy et al., 2001). These findings of *Edn1* in systemic circulation contrast with our findings in the ischemic retina. This discrepancy may stem may be due to the different tissues studied and thus *Edns* could be different in different tissues. This suggests that *Edns* could be a significant topic for future research on the anti-angiogenic effects of CIH on ischemic retina. Future studies inhibiting and stimulation *Edn2* and *Edn3* will be important for unraveling the molecular mechanisms of the anti-angiogenic effects of CIH.

Interestingly, CIH can also have pro-angiogenic effects. Research conducted using rodent models indicates that CIH can lead to an increase of the blood vessel density in the cerebral cortex. (Bousslama et al., 2015, Kanaan et al., 2006, Morken et al., 2013, Yang et al., 2018), and CIH plays a protective role in cerebrovascular function via promoting angiogenesis in Alzheimer's disease (Manukhina et al., 2016). Similarly, CIH can protect cardiac vessels by reducing the production of reactive oxygen species (ROS) (Mallet et al., 2018). It remains unclear if decreased ROS production also drives the pro-angiogenic effects of CIH in the brain.

Regardless of the underlying molecular mechanism, this pro-angiogenic effect of CIH is not in agreement with our findings here, which demonstrate an inhibitory impact of CIH on ischemic retinal angiogenesis. ROS production is a key mechanism in the pathophysiology of ROP and diabetic retinopathy (DR), so modulating ROS is a potential avenue for either exacerbating or ameliorating the pathology. This process is triggered by the stabilization of the transcription factor hypoxia-induced factor 1 (HIF-1), leading to the increased expression of angiogenic mediators, such as vascular endothelial growth factor (VEGF) which are crucial in increasing abnormal blood vessel growth, a hallmark of both ROP and DR (Stevenson et al., 2010, Bartoli et al., 2009, Wilkinson-Berka et al., 2013). In our ischemic retina model for retinopathy, CIH exhibited the anti-angiogenic effects, contrasting with its role in the brain of other models. Our RNA sequencing analysis did not reveal any alterations in the expression of NADPH oxidase, an enzyme pivotal for ROS production, under CIH conditions. The influence of CIH on ROS production in ischemic retinopathies will be an important topic for future studies. Further research directly measuring ROS levels and NADPH activity would be beneficial for comprehensively understanding the impact of CIH.

### **The Impacts of CIH on Microglia**

We found that CIH decreased the reactive microglial cell population expansion in ischemic retinopathy, and we hypothesized that the decrease in microglial response may directly contribute to the inhibition of revascularization, since microglial cells play a role in guiding retinal vessel growth (Checchin et al., 2006). An *in vitro* study found that CIH can suppress the proliferation of microglia. This effect was attributed to the downregulation of cyclin D1 and cyclin E2 (crucial for cell cycle progression and cellular proliferation). And CIH increased inflammatory factors in microglia such as pro-inflammatory markers IL-6, TNF- $\alpha$ , and iNOS (Liu et al., 2016). This *in vitro* finding mentioned align with our own observations.

However, it is important to note that there are also studies exhibiting varying effects of CIH on microglia. In an *in vitro* study using an oxygen-glucose deprivation (OGD)–reoxygenation model, it was observed that CIH conferred protective effects on microglia from the OGD and

reoxygenation stress. Also, CIH induced the M2 phenotype of microglia, which is generally associated with anti-inflammatory functions and tissue repair, under OGD and reoxygenation conditions. Additionally, the study noted a reduction in the generation of ROS by microglia in the presence of CIH (Tantingco and Ryou, 2020). Another in vivo study in mice revealed an increase in microglial cell density in the dorsal hippocampus following 6 and 24 weeks of CIH exposure (Sapin et al. 2015). Like its impact on angiogenesis, CIH exhibits both inhibitory and protective effects on microglial cells, notably the function of reducing ROS. This may also indicate that microglia are related to retinal angiogenesis.

The varying effects of CIH observed in different studies may be attributed to diverse experimental designs and conditions. For instance, the study reported an increase in brain microglia density only after relatively prolonged exposure (6 and 24 weeks) (Sapin et al. 2015), whereas we only had a maximum of 17 days of CIH exposure. In our experiment, no significant differences in neovascularization, avascularization, or microglia number were observed between the OIR-CIH and OIR-normoxia groups after 17 days of CIH.

We found that CIH reduced retinal *Csf1* transcription and microglial, but not endothelial cell, CSF1R expression, and the results of our local CSF1R blocking reducing microglial numbers similarly to CIH. This explains how CIH decreased the reactive microglial cell population expansion. When activated by CSF1, CSF1R undergoes dimerization and auto-phosphorylation, leading to the activation of various signaling pathways like PI3K/Akt, JNK, and ERK1/2, which regulate microglia proliferation and survival (Zelante et al., 2012, V Chitu et al., 2016). However, to our knowledge, this is the first time CIH has been shown to modulate *Csf1* and *Csf1r* expression. It's important to note that while we demonstrated CIH does not affect endothelial cells via the CSF1/CSF1R pathway, it remains unknown if other mechanisms exist that CIH could impact endothelial cells to influence retinal angiogenesis in ischemic retina.

Our study revealed no significant changes in MHCII levels in microglia within normal retinas due to CIH. The expression of MHCII molecules in microglial cells can be an indicator of their activation state. Interestingly, in the ischemic retinas, the most notable differences at P24 were observed in revascularization and neovascularization, while the number of microglia did not exhibit such significant changes by CIH. The reduced activation of microglial cells may lead to

their diminished role in promoting angiogenesis. Future studies in MHCII of ischemic retinas may provide evidence for this.

### **The Impacts of CIH on Inflammation**

Numerous studies have investigated the relationship between CIH and inflammation, with animal research demonstrating that CIH can induce inflammation in the lungs, liver, and adipose tissue (da Rosa et al., 2012, Murphy et al., 2017, Gileles-Hillel et al., 2017). However, our RNA sequencing revealed that following CIH there was a down-regulation of the expression of several pro-inflammatory genes, including TNF receptor superfamily member 1A and 1B (*Tnfrsf1a* and *Tnfrsf1b*), interleukin 6 cytokine family signal transducer (*Il6st*), intercellular adhesion molecule 1 (*Icam1*) and matrix metalloproteinase 12 (*Mmp12*). *Tnfrsf1a* and *Tnfrsf1b* are both receptors for TNF- $\alpha$ . IL6ST is an important receptor subunit for transmitting IL6 signaling. ICAM1 plays a crucial role in leukocyte adherence to the vascular endothelium during inflammation. MMP12, belonging to the matrix metalloproteinases family, is involved in degrading extracellular matrix proteins and tissue remodeling in inflammatory processes.

Research indicates that inflammation contributes to the onset and progression of ischemic retinopathies, including DR and ROP. Notably, inflammatory factors such as cytokines and chemokines, crucial in inflammatory responses, contribute to pathological angiogenesis associated with these conditions (Liou, 2010, Latini et al., 2019, Aouiss et al., 2019, Fevereiro-Martins et al., 2022). Therefore, an increase in inflammation would be expected with CIH, especially considering our observation that CIH leads to promoted neovascularization. However, it is interesting that CIH seems to reduce inflammation in our ischemic retina according to the RNA sequencing results. It is important to note that our RNA sequencing data was collected at P18, while CIH was observed to promote NV only after P24. This temporal discrepancy in disease stages might influence the inflammatory states observed. Therefore, conducting RNA sequencing analysis at and beyond P24 will be crucial for a clearer understanding of the inflammatory responses associated with CIH. Also, we previously mentioned when we discussed CIH and microglia that in vitro CIH can promote the M2 phenotype microglia, which is known

for its anti-inflammatory functions (Tantingco and Ryou, 2020). Although we have not yet examined how CIH affects microglial polarization, it's important to recognize the contrast between the pro-inflammatory M1 phenotype and the anti-inflammatory M2 phenotype of microglia. Future studies should consider exploring the M1/M2 phenotypes and investigating direct evidence of inflammatory factors to better understand the inflammatory response of the ischemic retina under CIH conditions.

In summary, there is a correlation between fluctuation in infants' blood oxygen saturation and ROP, as well as a link between hypoxemia and DR (York et al., 2004, Saito et al., 1993, Anderson et al., 2004, Banerjee et al., 2013). Here, we identified a mechanistic explanation of how CIH, a critical feature of apnea, can aggravate ischemic proliferative retinopathies. Our work emphasizes the need to monitor and treat apnea in DR and ROP patients to prevent severe, vision-threatening conditions. Recent studies highlight the extensive use of anti-VEGF therapies in advanced neovascular complications treatment (Sapieha et al., 2010; Hellstrom et al., 2013; Antonetti et al., 2021). Our findings suggest that CIH affects ischemic retinal angiogenesis by inhibiting the CSF1/CSF1R pathway, potentially pointing towards alternative treatment strategies beyond anti-VEGF for ischemic retinopathies such as ROP and DR.

# Conclusion



Sleep apnea that leads to chronic intermittent hypoxia (CIH), is a risk factor for ischemic retinopathies such as diabetic retinopathy (DR) and retinopathy of prematurity (ROP), but the mechanisms involved are unknown. Unraveling the link between CIH and ischemic retinopathies is crucial for a deeper understanding and effective management of these conditions. Our work used the oxygen induced retinopathy (OIR) mouse model to investigate the impact of CIH on ischemic proliferative retinopathy.

First, we found that CIH alone does not impact healthy mouse pups' body weight, normal retinal vascular development, or microglial cell populations. However, CIH following OIR induction decelerated the weight gain of pups. In the ischemic retinas, CIH promoted photoreceptor degeneration, significantly inhibited revascularization and promoted pathological neovascularization.

CIH reduced retinal *Csf1* transcription and microglial *Csf1r* expression. This suppression of the CSF1/CSF1R pathway in microglial cells resulted in altered retinal microglial cell populations, specifically delaying the expansion of reactive microglial cells in the ischemic retina. The CIH-induced suppression of retinal microglial cells led to inhibited physiological vascular development, manifesting as reduced revascularization, and promoted pathological intravitreal neovascularization in the ischemic retina. The inhibited physiological vascular development and increased pathological neovascularization demonstrated that CIH aggravated ischemic proliferative retinopathies. Additionally, *Csf1r* expression was unchanged on endothelial cells, suggesting that the effects of decreased CSF1/CSF1R signaling are not mediated by loss of CSF1R activation on endothelial cells.

Collectively, these results offer a detailed understanding of the mechanisms by which apnea and CIH exacerbate ischemic proliferative retinopathies. This highlights the critical need for regular monitoring and treatment of apnea in conditions such as DR and ROP to mitigate the risk of severe, vision-threatening complications.

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