The roles of Transient Receptor Potential (TRP) channels and aberrant calcium signalling in blood-retinal barrier dysfunction

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Abstract

The inner blood-retinal barrier (iBRB) protects the retinal vasculature from the peripheral circulation. Endothelial cells (ECs) are the core component of the iBRB; their close apposition and linkage via tight junctions limit the passage of fluids, proteins, and cells from the bloodstream to the parenchyma. Dysfunction of the iBRB is a hallmark of many retinal disorders. Vascular endothelial growth factor (VEGF) has been identified as the primary driver leading to a dysfunctional iBRB, thereby becoming the main target for therapy. However, a complete understanding of the molecular mechanisms underlying iBRB dysfunction is elusive and alternative therapeutic targets remain unexplored.

Calcium is a universal cellular messenger whose homeostasis is dysregulated in many pathological disorders. Among the extensive components of the cellular calcium signalling toolkit, cation-selective transient receptor potential (TRP) channels are broadly involved in cell physiology and disease and, therefore, are widely studied as possible targets for therapy. Albeit that TRP channels have been discovered in the photoreceptors of *Drosophila* and have been studied in the neuroretina, not much is known about their expression and function in the iBRB. Within this article, we discuss the structure and functions of the iBRB with a particular focus on calcium signalling in retinal ECs and highlight the potential of TRP channels as new targets for retinal diseases.

Introduction

At least 2.2 billion people worldwide suffer from visual impairment. Most retinal diseases, including proliferative diabetic retinopathy, diabetic macular oedema, wet age-related macular degeneration and retinal vein occlusion, share standard clinical features, including retinal oedema, ischemia and neovascularisation, which reflect BRB dysfunction[1]. The BRB supplies the retina with oxygen and nutrients and comprises the outer BRB (oBRB) and the inner BRB

(iBRB), with the latter being made of specialised endothelial cells (ECs), which limit both paracellular and transcellular permeability[1,2].

The vascular endothelial growth factor (VEGF) is the main driver of iBRB dysfunction, as its expression rises in pathological conditions, leading to increased EC migration and proliferation, angiogenesis, extracellular matrix degeneration and vascular permeability[3,4]. Indeed, intravitreal injections of anti-VEGFs are the main therapies for retinal disorders. However, up to 40% of patients do not respond to anti-VEGFs, and among the patients who do respond, many develop resistance or suffer heavy side effects, which reduce their compliance rate. Therefore, recent research is looking at finding alternative therapeutic strategies.

Calcium (Ca^{2+}) is a universal cellular messenger, practically involved in all aspects of cell and developmental biology. Preclinical and clinical evidence shows that Ca^{2+} signalling is dysregulated in many pathological disorders[5,6]. Transient receptor potential (TRP) proteins are multifunctional signalling cation channels involved in many aspects of cell physiology. Dysregulation of TRP channels is a hallmark of numerous diseases, ranging from neurological and psychiatric disorders to diabetes and cancer, rendering these channels attractive therapeutic targets[7,8]. Recent evidence has started to uncover the role of TRP channels in iBRB physiology and disease.

Within this review, we first discuss the structure and function of the iBRB, focusing specifically on endothelial dysfunction. We then present evidence supporting the role of calcium signalling in iBRB dysfunction, pointing at TRP channels as emerging targets for retina diseases and highlighting the urgent need for further investigation.

The neuroretina

Animals have evolved the ability to sense and decode their environment through the development of sensory systems. Vision is the most utilised sense in many vertebrate species for gathering and processing environmental information and interacting with other living organisms[9]. The eye comprises several tissues, with the retina being the critical component that converts light into electrical signals and transmits these signals to the brain for visual recognition[10]. To exert its function, the retina requires large amounts of oxygen and metabolites; indeed, it is one of the body's tissues with the highest metabolic demand[11-16].

The retina is an architectural masterpiece, containing a variety of cell types, including five types of neurons (ganglion, bipolar, amacrine, and horizontal cells and rod and cone

photoreceptors)[17], retinal pigment epithelial cells, Müller cells, microglia, and endothelial cells (ECs), which associate with smooth muscle cells, pericytes and astrocytes[18]. This myriad of cell types is organised into multiple functional layers, whose organisation and structure are shown in Figure 1.

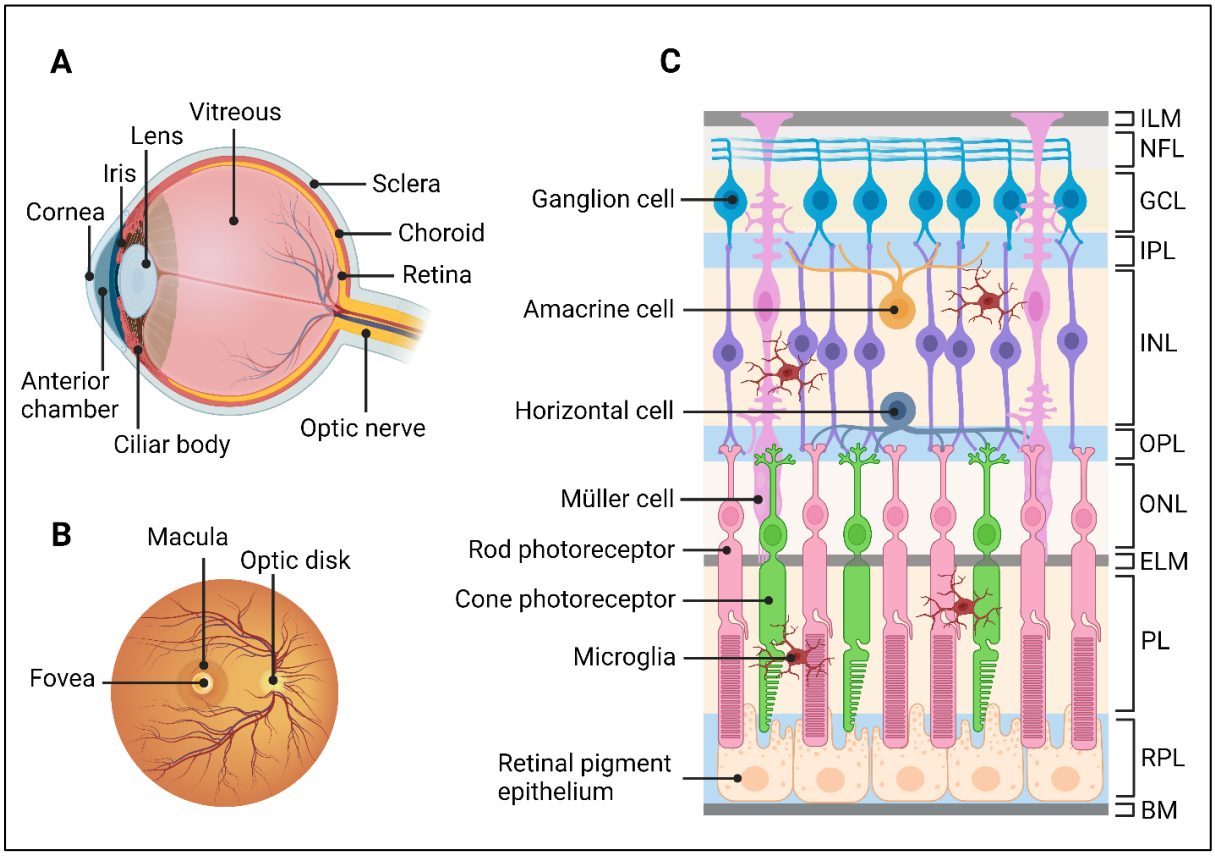

Figure 1. Schematic organisation of retinal layers. (A-B) Schematic of the human eye **(A)** and retina **(B)**. **(C)** Representation of the retinal layers, which, from the innermost (top) to the outermost (bottom), can be classified as follows: internal limiting membrane (ILM), which structurally separates the vitreous from the retina and contains extensions of Müller cells; nerve fibre layer (NFL), which is formed by ganglion cell unmyelinated axons; ganglion cell layer (GCL), composed of retinal ganglion cells; inner plexiform layer (IPL), where bipolar cells make synapses with ganglion and amacrine cells; inner nuclear layer (INL), which is composed of cell bodies of Muller cells in addition to the bodies of horizontal, bipolar and amacrine cells; outer plexiform layer (OPL), a thin layer where photoreceptors make synaptic contact with horizontal and bipolar cells; outer nuclear layer (ONL), which contains the soma of the photoreceptors; external limiting membrane (ELM), where adherens junctions connect photoreceptors to Muller cells; photoreceptor layer (PL), which is composed of the inner and outer segments of the photoreceptors; retinal pigment layer (RPL), which is made of epithelial cells that separate the retina from the choroid and Bruch's membrane (BM). Created with BioRender.com.

In humans and other primates, the centre of the retina, called the macula, is specialised for detail and colour vision and in particular the fovea, located at the centre of the macula, provides the highest resolution for daytime vision[19]. The five types of retina neurons are

interconnected via two different pathways, the vertical and the horizontal pathways, which allow a visual stimulus to travel through the retina and reach the brain[9].

The vertical pathway begins with the photoreceptors, which convert photons into electrical signals and transmit them to the retinal ganglion cells (RGCs) via the intermediate bipolar cells for additional processing. Both rods and cones hyperpolarize in response to light but differ in shapes, composition of the outer segment, sensitivity to light and convergence to the RGCs[20]. Rods are thinner and longer than cones, with rods averaging 2 μm x 50 μm, against 4 μm x 40 μm for cones.

Rods are responsible for vision under low-light conditions, called scotopic vision; they can respond to a single photon and are saturated by high luminosity. Their outer segment contains only one kind of pigment, named rhodopsin, which confers black-and-white vision and whose insufficiency results in night blindness. There are about 90 to 120 million rods in the human retina located primarily in the extra fovea region. Several rods connect to the same bipolar cell, resulting in low visual acuity[20].

Cones, on the other hand, have a lower degree of convergence, and in the fovea, one cone is connected to one RGC, thereby delivering high-acuity vision[9]. Cones are sensitive to high-intensity light and are mostly used for day vision. The retina of humans and other primates have three types of cones, termed short-, medium- and long-wavelength sensitive cones, which express three different opsins with different spectral sensitivity, namely, OPN1SW, OPN1MW, and OPN1LW, respectively. Consequently, cones are responsible for colour or photopic vision [21,22]. There are about 5 to 7 million cones in the human retina, concentrated in and around the fovea. As a result of the distribution of the photoreceptors, the central part of the retina provides high resolution and colour perception in bright light conditions, whereas the peripheral region is responsible for motion sensitivity and lower acuity in dimly light environments[9]. Both types of photoreceptors send signals to bipolar cells via glutamate release[23].

Bipolar cells are interneurons that connect photoreceptors to the RGCs, thereby linking the outer retina to the inner retina. They present two long processes; one interacts with photoreceptors and horizontal cells, whilst the other connects with amacrine cells and RGCs[17]. The human retina contains one type of rod bipolar cell and 12 different types of cone bipolar cells[23]. The circuitry regulated by bipolar cells is the key component of the process of visual signal transmission and elaboration. Indeed, the architecture of the IPL allows bipolar cells to branch at different planes of the IPL, each containing a specific set of different amacrine and

ganglion cells. Bipolar cells differ in the number of synapses, sets of neurotransmitter receptors, and calcium-binding proteins. These distinctive features allow different modalities of intracellular signalling[17]. For instance, glutamate is the neurotransmitter released by all photoreceptor synapses. However, some bipolar cells express metabotropic glutamate receptors, whose binding of glutamate leads to cell hyperpolarisation and, therefore, inactivation; hence these cells will be active when the light is on (on-center cells), and glutamate release by the photoreceptors decreases. Other bipolar cells instead express ionotropic glutamate receptors, and therefore, they depolarise and activate in response to glutamate, hence when the light is off (off-center cells)[24,25].

At the OPL and IPL levels, the vertical pathway is integrated into the horizontal pathway, which includes two types of interneurons, namely horizontal and amacrine cells. Horizontal cells are GABAergic interneurons that interact with bipolar cells and send inhibitory feedback to the photoreceptors. They are classified into axon-bearing horizontal cells (A-type) and axon-less horizontal cells (B-type)[26]. Amacrine cells are GABAergic or glycinergic inhibitory interneurons that receive inputs from the bipolar cells and send feedforward signals to the RGCs to mediate their response to light[27]. They also send feedback signals to bipolar cells and inhibitory signals to other amacrine cells. All information gathered and processed by both pathways is then transmitted as a train of spikes to the brain by the RGCs, whose axons form the optic nerve and converge into the optic disc to leave the eye and reach the visual cortex.

The blood-retinal barrier

Besides the photoreceptor layer, which is avascular, the remaining components of the retina are supplied with oxygen and nutrients by an intricate network of blood vessels. The retinal vasculature is protected from peripheral circulation by the so-called blood-retinal barrier, which consists of the outer blood-retinal barrier (oBRB) and the inner blood-retinal barrier (iBRB)[1,28-30]. The oBRB includes the choroid, Bruch's membranes, and the RPE. An exhaustive description of the oBRB structure and functions can be found in other publications[31-35]. The iBRB, which is the focus of this review, is composed of ECs with features that make retinal vessels relatively impermeable. The retinal vasculature derives from the central retinal artery and infiltrates the retina at four distinct plexuses (**Fig. 2**). The superficial vascular plexus includes the nerve fiber layer plexus, which is the most superficial of the plexuses and pervades the NFL, and the ganglion cell layer plexus, which perfuses the GCL and the IPL. Deeper into the retina lies the capillary plexus, which includes the intermediate capillary plexus at the IPL/INL border and the deep capillary plexus between the INL and the OPL[36-38] (**Fig. 2**).

Cellular components of the iBRB

The iBRB is not simply an impermeable physical entity but rather represents the unique properties of the ECs linked by tight junctions, which restrict the passage of fluid, molecules, and cells between the bloodstream and the parenchyma[1]. ECs lie on a basement membrane wherein pericytes are embedded. Pericytes are phagocytic cells on the abluminal side of capillaries, which modulate EC proliferation, and vessel growth and remodelling, thereby regulating angiogenesis. Pericytes possess contractile apparatus and regulate blood flow, which is crucial for maintaining the iBRB[39-41]. On post-capillary venules, pericytes express several chemokines, cytokines, and adhesion molecules, thereby regulating leukocyte transmigration. Astrocytes can also control blood flow and modulate the integrity of the iBRB by releasing pro- and anti-inflammatory cytokines and trophic factors. Astrocyte endfeet surround ECs leading to a tighter barrier[42,43]. Finally, microglia are macrophages that release proinflammatory factors and clear cellular and metabolic debris[44,45]. ECs, mural cells (smooth muscle cells and pericytes), astrocytes, microglia, and neurons form the neurovascular unit (NVU) (**Fig. 2**). The correct interaction of all these components is vital to maintain a healthy and functional barrier, which in turn is essential to retain retinal environmental homeostasis and dynamically coordinate local blood flow to meet metabolic demands.

Numerous pathological features, such as altered oxygen levels and an increase of inflammatory cytokines, chemokines, and leukocyte adhesion molecules, impair the communication among the NVU components, thereby disrupting the architecture of retinal microvasculature, and aggravating inflammation and pathological processes. For example, the interaction between EC and pericytes via gap junctions, peg-and-socket interactions and paracrine signalling factors is essential for the stability and effectiveness of the NVU[46]. Indeed, insufficient pericyte recruitment leads to a lack of structural support, which causes retinal oedema; pericyte drop-out is a typical early feature of retinal diseases such as diabetic retinopathy[47]. The communication between ECs and pericytes via several signalling pathways, including PDGFB/PDGFR-β signalling, TGF-β/TGFβR2 signalling, as well as Notch pathway and Ang1/Tie2 signalling, is required for angiogenesis[48-50]. Pericytes are also in contact with the glia, which physically connects vessels to neurons to modulate neurotransmission[42]. Among glial cells, astrocytes maintain neuron health by controlling the release of neurotransmitters, such as glutamate or adenosine. Importantly, astrocytes optimise the interstitial space for synaptic transmission by controlling water and ionic homeostasis via calcium signalling, potassium and chloride channels and aquaporin4[51,52]. Astrocytes contact both neurons and blood vessels, thereby regulating blood vessel diameters and blood flow, in a process known as neurovascular coupling[53-55]. During the development of the retinal microvessels, the interactive signalling among ECs, pericytes and astrocytes coordinates the

increased expression of tight junction proteins required for BRB function[56]. Finally, the basement membrane is another crucial component to ensure the integrity of the NVU; indeed, basement membrane thickening disables the interaction of the ECs with the other components of the NVU and is an early feature of retinal diseases[57].

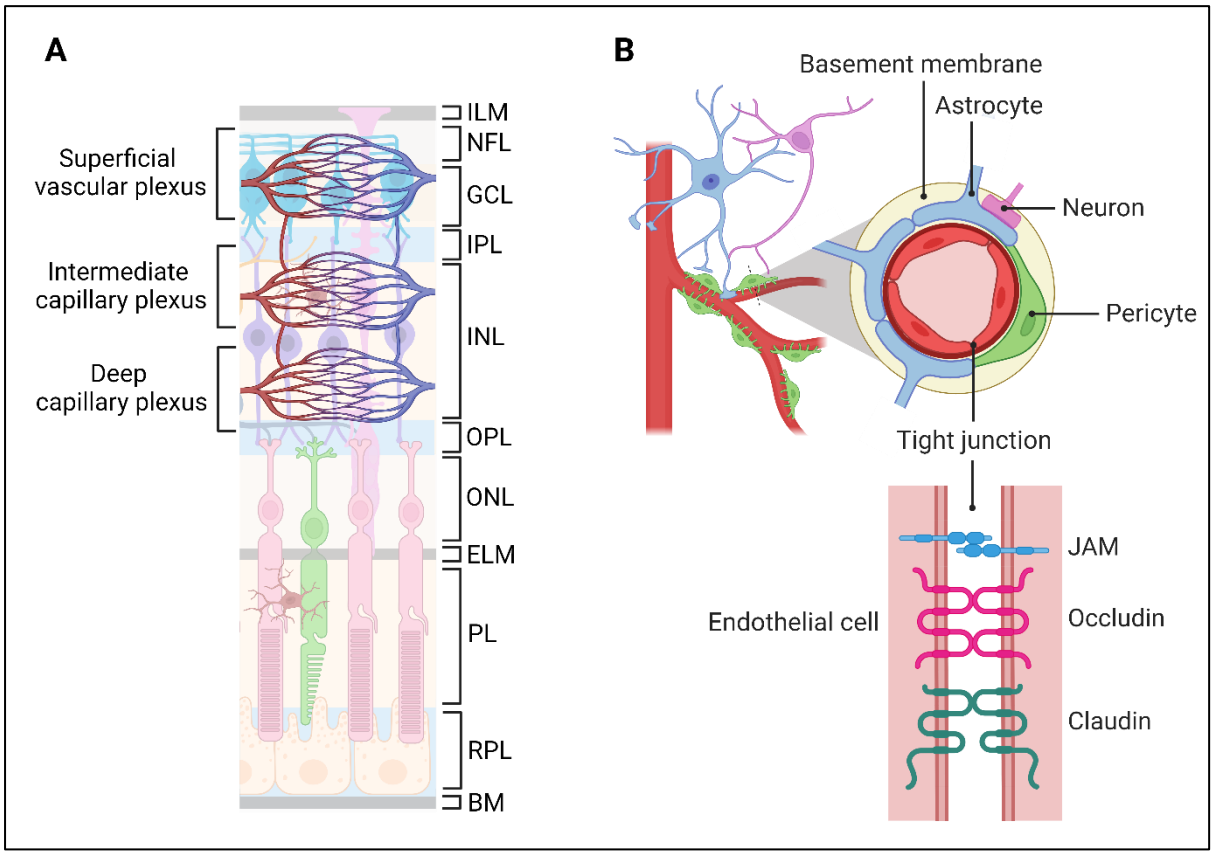

Figure 2. Structural components of the neurovascular unit. (A) Alignment of the retina layers with the vascular plexuses. **(B)** The cellular components of the vascular unit and molecular components of the tight junctions. Created with BioRender.com.

Retinal endothelial cells

ECs are the core of the iBRB; they are highly specialized to limit both paracellular and transcellular permeability. Indeed, whilst in all tissues, vascular endothelial cadherin (VE-cad) and catenins form adherens junctions to hold ECs together, in the brain and the retina, levels of tight junctions seal adjacent ECs together to create a barrier with a high electrical resistance, thereby preventing paracellular permeability and maintaining apicobasal polarity[58]. Tight junctions comprise claudin family proteins, the MARVEL family transmembrane proteins such as occludin, tricellulin, and MarvelD3 and junctional adhesion molecules (JAMs; **Fig. 2**). Claudins are likely the most important proteins for tight junction properties; their structure includes four transmembrane regions linked by two extracellular loops, with two C-terminal intracellular domains that are tethered to the actin cytoskeleton by zonula occludens (ZO)

proteins. Twenty-seven claudin family members have been identified in mammals, with claudin-5 being the most expressed in retina ECs[58,59]. Importantly, whilst claudin-5 knockout mice show size-selective leakage of the blood-neural barriers and die at birth, knock-down of claudin-5 in the adult mouse is accompanied by an increase in claudin-1 expression, which is normally weakly expressed, suggesting a compensatory role of claudin-1 under pathological conditions[60]. Among the proteins of the MARVEL family, occludin has been shown to regulate adhesion properties between cells and interact with the scaffolding proteins ZO1-3. However, occludin knockout mice develop viable tight barriers; therefore, other proteins seem more important for tight junction development and function[61,62]. Interacting with occludins through the PDZ-binding motif of their C-terminal domain are the JAMs, which are members of the immunoglobulin superfamily and contribute to tight junction assembling and function. Finally, ZOs are scaffolding proteins with cytoplasmic domains that anchor the membrane proteins to the cytoskeleton, forming multi-protein platforms and signalplexes[63-65].

The integrity of the iBRB is critical for homeostasis and neural protection throughout life. While preserving or replenishing barrier properties is one important focus of the research that aims to tackle retinal disorders, drug delivery research aims to find an effective mechanism to cross the barrier to allow drugs to reach the nervous system.

Transcellular permeability is also restricted at the iBRB due to the low level of caveolin-mediated transcytosis and lack of fenestrae. Plasmalemma vesicle-associated protein (PLVAP), which is important for fenestrae formation, is downregulated in the iBRB and coincides with the overexpression of the transporter MFSD2A which regulates lipid composition, thereby limiting caveolin-dependent transcytosis[66-68].

Several transporters are highly expressed in retinal ECs and can be classified as efflux and solute transporters. Efflux transporters utilise ATP hydrolysis to transport molecules up their concentration gradient from the parenchyma to the blood. Among them, MDR1/P-glycoprotein (PGP) and the ATP-binding cassette superfamily G member 2 (ABCG2), often referred to as breast cancer resistance protein, are the most expressed efflux transporters in the iBRB and limit the access of xenobiotics and exogenous molecules such as steroids[69]. Solute transporters such as GLUT1 and MCT1 carry important nutrients including glucose, amino acids, and fatty acids down their concentration gradient, whereas others provide receptor-mediated vesicular transport, such as the transferrin receptor and low-density lipoprotein receptors[70,71].

ECs express lymphocyte adhesion molecules that bind leukocytes, allowing the leukocytes to enter the tissues and initiate immune responses. When the iBRB is in a healthy physiological state, ECs express low levels of lymphocyte adhesion molecules, limiting the passage of leukocytes through the iBRB to keep its immune privilege[72].

Dysfunction of the iBRB

The integrity of the iBRB is necessary to preserve a dry retinal environment. Impairment of the iBRB can cause the accumulation of fluids and solutes in the parenchyma, leading to retinal oedema. In a healthy eye, intraocular pressure, which is important to maintain the eye shape, pushes water into the retina and is balanced by the choroidal osmotic pressure, which draws water into the vasculature, thereby maintaining the retina in place and safely relatively dry[73,74]. When the intraocular pressure increases, water percolates through the retina and is actively removed by the RPE. However, when the iBRB loses its functional integrity, water, and also proteins, will leak into retinal extracellular space, and when they reach the ELM, adherens junctions will prevent them from passing through, thus attracting more water, and contributing to oedema formation[1,74]. Muller cells and aquaporin-4 channels work to reabsorb fluids[75,76], but when these mechanisms are overwhelmed, BRB dysfunction is inevitable[73].

The breakdown of the intercellular junctions is the most common mechanism that leads to iBRB dysfunction[77] with consequent vasogenic oedema, which happens much more frequently than cytotoxic oedema[78], initiated by cell damage, or intracellular swelling, where the breakdown of the iBRB is a secondary event[79,80]. In many retinal diseases, for example, age-related macular degeneration or diabetic retinopathy, expression of tight junction and adherens junction proteins decreases[2-4], thereby leading to vascular hyperpermeability. Increased expression of pro-inflammatory mediators, such as VEGF, TNFα and plasma kallikrein[81,82], contribute to iBRB breakdown via inflammation and angiogenesis, which in turn lead to the growth of new fragile blood vessels, which are leaky and contribute to oedema in a positive feedback loop. Chemokines, instead, recruit monocytes, which secrete pro-inflammatory factors, contributing to leukocyte infiltration. The glycocalyx would normally prevent leukocyte adhesion, but pathological conditions, such as hyperglycaemia, lead to the upregulation of leukocyte adhesion molecules and activation of matrix metalloproteinases, impairing the integrity of the glycocalyx and allowing leukocytes to adhere to the ECs and cross the barrier[83].

Some of the most frequent retinal diseases such as proliferative diabetic retinopathy, diabetic macular oedema, age-related macular degeneration, and retinal vein occlusion, are characterized by iBRB dysfunction with consequent vascular oedema **(Fig. 3)** and aberrant

angiogenesis. The vascular endothelial growth factor (VEGF) is the primary contributor to retinal permeability and angiogenesis[3,4,77,84]. Indeed, intravitreal injections of anti-VEGFs are the main therapy for the aforementioned disorders[85]. The main driver for VEGF expression is hypoxia which triggers hypoxia inducible factor-1 (HIF-1) signalling. In normoxic conditions, HIF-1α hydroxylation by the prolyl-4-hydroxylase, leads to its interaction with E3 ligase with consequent HIF-1α degradation by the ubiquitin system. Additionally, factor inhibiting HIF-1, impairs the binding between HIF-1α and its co-receptor p300/CBP. When the oxygen level decreases, both prolyl-4-hydroxylase and factor inhibiting HIF-1 are downregulated, thereby freeing HIF-1α, which migrates to the nucleus to bind HIF-1β. The complex thus formed binds to the hypoxia response element (HRE) on the VEGF gene promoter, thereby activating VEGF expression[86]. Despite its pathological effects, VEGF acts also as a survival and neuroprotective factor and its expression is necessary for the development and the maintenance of a healthy iBRB[87]. Consistently, anti-VEGFs, which target all VEGF functions indiscriminately, trigger a variety of side effects[88], which, together with the side effects due to the intravitreal injection per se, include increased intraocular pressure and intraocular inflammation, infectious endophthalmitis, ocular haemorrhage formation of protein aggregates, RPE tears, choriocapillary atrophy and thinning, photoreceptor degeneration, and more rarely retinal artery occlusion and retinal detachment[89]. Additional standard treatments include pan-retinal photocoagulation, for proliferative diabetic retinopathy, and laser photocoagulation or steroids for macular oedema, with vitreoretinal surgery left as the last option for the worst cases[1].

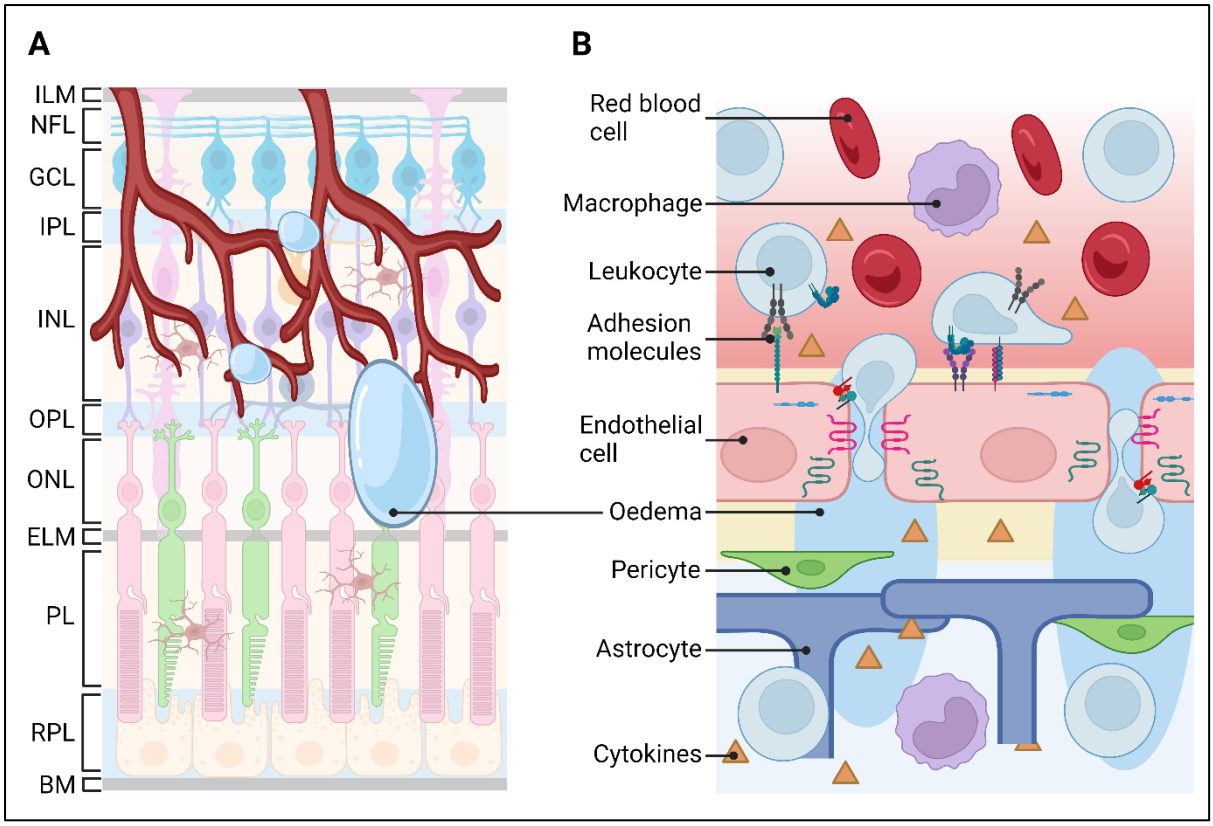

Figure 3. Retinal oedema is the consequence of endothelial dysfunction. (A) Leaky blood vessels lead to oedema formation in the retinal layers. (B) Schematic representation of the characteristics of endothelial dysfunction, including the opening of the intercellular junctions, leukocyte adhesion to endothelial cells and consequent transmigration, release of cytokines, pericyte and astrocyte detachment and oedema formation.

Overview of endothelial Ca^{2+} signalling

Being located at the interface between circulating blood and surrounding tissues, vascular ECs are exposed to diverse chemical and physical stimuli, including mediators in the blood, mechanical forces exerted by the bloodstream, and neurotransmitters released by perivascular nerves. ECs transduce the incoming inputs through an increase in the intracellular calcium concentration ($[Ca^{2+}]_i$) with precise spatiotemporal features to recruit specific Ca^{2+}-dependent effectors and thereby elicit the most appropriate vascular response [90-92]. Endothelial Ca^{2+} signals finely tune virtually all vascular functions, including vascular resistance and blood pressure, vascular permeability, lymphocyte and leukocyte trafficking, thrombus formation, angiogenesis and vasculogenesis [90-93].

Endothelial Ca^{2+} signals are shaped by intracellular Ca^{2+} release and extracellular Ca^{2+} entry

The ability to serve as an intricate sensory system that senses and integrates the multiple inputs converging on both their luminal and basolateral membranes is conferred to ECs by a

variety of G_q-protein coupled receptors (G_qPCRs), tyrosine-kinase receptors (TKRs) and ion channels that are located on the plasma membrane **(Fig. 4)** and lead to an increase in $[Ca^{2+}]_i$ [90-93]. G_qPCRs and TKRs are, respectively, activated by neurohumoral mediators and growth factors and activate two distinct isoforms of phospholipase C (PLC), namely PLCβ and PLCγ [91,93]. PLC, in turn, cleaves a minor phospholipid component of the plasma membrane, phosphatidylinositol 4,5-bisphosphate (PIP_2), into the second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) [91,93]. IP_3-induces Ca^{2+} release from the endoplasmic reticulum (ER), which accounts for ~75% of the intracellular Ca^{2+} pool in ECs, with the remaining 25% being primarily located in mitochondria [94]. Vascular ECs express all three IP_3R isoforms [95], while ryanodine receptors (RyRs) were mainly detected in cultured endothelial cells and are unlikely to shape endothelial Ca^{2+} signals in naïve vessels [96]. Recent evidence highlighted the emerging role of endolysosomal (EL) vesicles in the endothelial Ca^{2+} response to chemical cues [97]. It has been suggested that NAADP-induced EL Ca^{2+} mobilization through two-pore channels (TPCs) could trigger ER Ca^{2+} release through IP_3Rs upon stimulation of either G_qPCRs [98,99] or TKRs [84,100] in ECs from several vascular beds.

The endothelial Ca^{2+} response to chemical stimulation is supported by extracellular Ca^{2+} entry across the plasma membrane through multiple Ca^{2+} entry pathways. IP_3-induced reduction of ER Ca^{2+} concentration activates STIM1 to engage several store-operated Ca^{2+} entry channels, which consist of different pore-forming subunits and present different biophysical features [101]: Orai1 hexamers, which mediate the Ca^{2+}-release activated Ca^{2+} current (I_{CRAC}) [102,103]; members of the transient receptor potential channel family (TRP; specifically TRPC1/TRPC4 heterotetramers), which mediate the non-selective store-operated current (I_{SOC}) [104-106]; and Orai1/TRPC1/TRPC4 heterotetramers, which mediate the moderately Ca^{2+}-selective I_{CRAC}-like current [107-109]. SOCE is likely to play a major role in mediating Ca^{2+} entry in ECs from large vessels [101,108,110], while second messenger-operated TRP channels sustain Ca^{2+} influx in resistance-sized arteries and arterioles [92]. These second messenger-operated TRP channels include: TRPC3, which is gated by DAG [111,112], TRPC4, a polymodal channel that can also be activated by the inhibitory Gαi protein [113], and TRPV4, another polymodal channel that can be activated by arachidonic acid [114,115] and cytochrome P450 (CYP) metabolites of arachidonic acid, such as 5'-6'-epoxyeicosatrienoic acid (EET) [116] and 14'-15'-EET [117], IP_3 binding to a cytosolic COOH-terminal domain [118], and protein kinase C (PKC)-dependent phosphorylation [119]. Ca^{2+}-permeable ionotropic receptors, such as purinergic P2X receptors [120], may also support Ca^{2+} entry in vascular ECs. Interestingly, a recent series of studies showed that N-methyl-D-aspartate (NMDA) receptors are expressed in brain microvascular ECs and mediate local Ca^{2+} entry in response to somatosensory stimulation and glutamate application [121-123].

Finally, vascular ECs may perceive mechanical stimuli and transduce them into appropriate Ca^{2+} signals through several mechanosensitive channels. These include: TRPV4, which can be activated both by laminar shear stress [124] and a reduction in intravascular pressure [125]; TRPP1/TRPP2 complexes [126,127] and Piezo channels [128,129], which are also activated by an increase in intravascular flow.

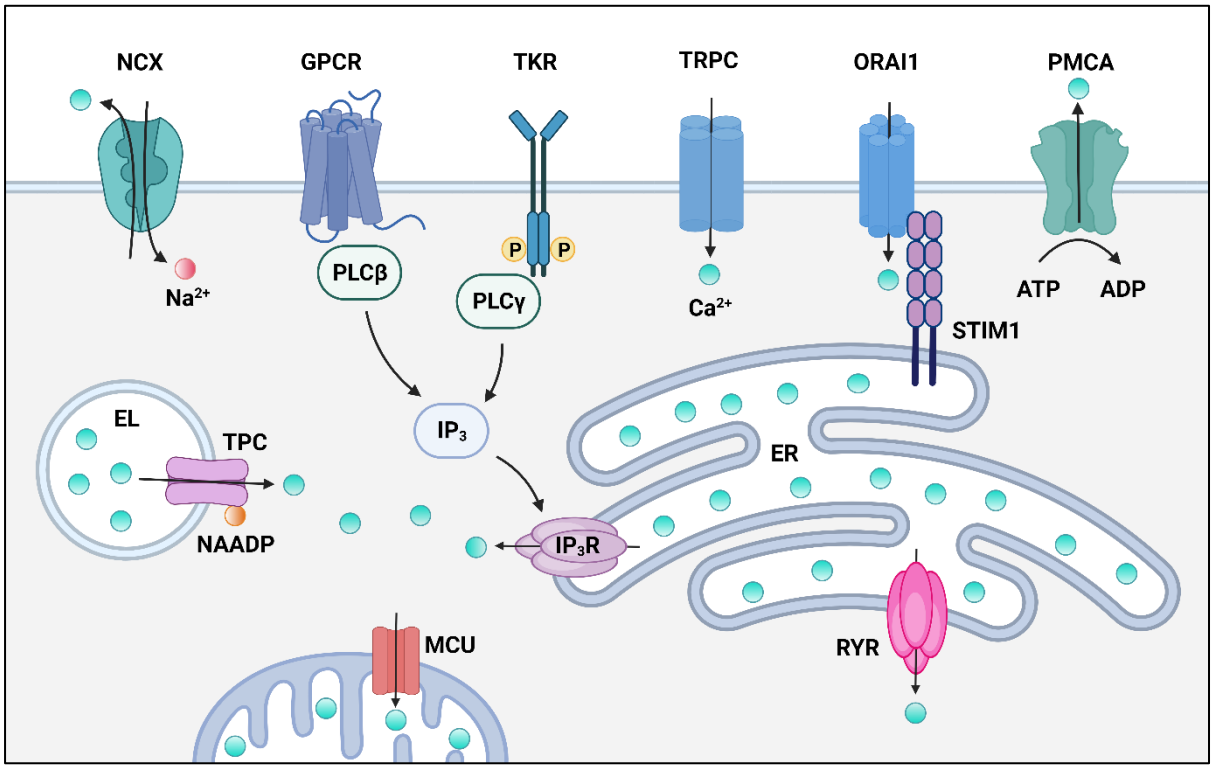

Figure 4. Calcium signalling toolkit in endothelial cells. ECs express both G-protein-coupled receptors (GPCRs) and tyrosine kinase receptors (TKRs) in the plasma membrane. Both receptors, when activated, initiate phospholipase PLCβ and PLCγ respectively, resulting in the production of the second messenger inositol 1,4,5-trisphosphate (IP_3). IP_3 binds to its receptor (IP_3R) on the endoplasmic reticulum (ER) membrane resulting in its depletion. A decrease in the ER Ca^{2+} concentration is detected by the Stromal Interaction Molecule 1 STIM1, which in turn activates ORAI1, triggering the store-operated calcium entry (SOCE). Depletion of the ER is also one of the numerous stimuli that can activate the Transient Receptor Potential (TRP) Channels located on the plasma membrane. ECs also express ryanodine receptors (RYRs) which move Ca^{2+} out of the ER and the mitochondrial calcium uniporter (MCU) which transport Ca^{2+} into the mitochondria. The Na^+/Ca^{2+} exchanger (NCX) and the calcium-ATPase (PMCA) restore basal cytoplasmic Ca^{2+} concentration by pushing Ca^{2+} out of the cell. Finally, NAADP induces Ca^{2+} mobilization form the endolysosomes (ELs) through two-pore channels (TPCs).

Subcellular architecture and intercellular propagation of endothelial Ca^{2+} signals

The dynamic interplay between IP_3-induced ER Ca^{2+} release, TPC-mediated EL Ca^{2+} mobilization, and the Ca^{2+} entry through the multiple pathways outlined above results in a variety of Ca^{2+} signals, which range from spatially-restricted Ca^{2+} microdomains to global elevations in $[Ca^{2+}]_i$ that may spread to adjacent ECs as intercellular Ca^{2+} waves [90-92,130]. Local Ca^{2+} release events through clusters of IP_3Rs, known as Ca^{2+} pulsars, may spontaneously occur at myo-endothelial projections (MEPs) in resistance-sized arteries and arterioles due to tonic PLC activation [131]. As compared to Ca^{2+} puffs, which are elementary IP_3R-mediated ER Ca^{2+} release events also reported in other cell types[132-134], endothelial Ca^{2+} pulsars present slower rise time and longer duration and do not spread beyond MEPs: however, local Ca^{2+} pulsars are crucial to regulate blood pressure since they are selectively coupled to small- and intermediated-conductance K^+ channels (IK_{Ca}/SK_{Ca}) to stimulate endothelium-dependent hyperpolarization (EDH). EDH may electrotonically spread to overlying vascular smooth muscle cells (VSMCs) through myo-endothelial gap junctions (MEGJs) and thereby decrease VSMC contractility via electrotonic spread [131]. A distinct mode of local Ca^{2+} signalling elicited at MEPs upon IP_3R activation is represented by Ca^{2+} wavelets, which are activated upon sympathetic stimulation of VSMC α1-adrenergic receptors and present longer duration and wider spatial spread as compared to Ca^{2+} puffs [135]. α1-adrenergic receptor signalling results in the flux of IP_3 and Ca^{2+} from VSMCs to endothelial cells through MEGJs, thereby leading to repetitive localized Ca^{2+} transients that recruit IK_{Ca} channels to moderate VSMC contractility via a mechanism known as myoendothelial feedback [135].

Unitary Ca^{2+} entry events, known as Ca^{2+} sparklets, can be mediated in vascular endothelial cells by the opening of a few Ca^{2+}-permeable channels on the plasma membrane, such as TRPV4 [124,136], TRPV3 [137], and TRPA1 [138]. Endothelial Ca^{2+} sparklets primarily occur at MEPs and are also selectively coupled to IK_{Ca}/SK_{Ca} channels, thereby reducing vascular resistance and increasing blood supply to downstream capillaries [92]. Local Ca^{2+} signals were also reported at the forefront of migrating ECs upon PLC-dependent engagement of the SOCE machinery [139]. Stimulation of G_qPCRs in naïve ECs causes a dose-dependent increase in sub-cellular Ca^{2+} activity, ranging from repetitive Ca^{2+} puffs to regenerative Ca^{2+} waves that present either a biphasic or an oscillatory pattern and may then spread along the vascular intima through inter-endothelial gap-junctions [140-144]. Studies conducted on rodent cremasteric arteries showed that the focal application of acetylcholine induced a local increase in $[Ca^{2+}]_i$ that triggered intercellular Ca^{2+} waves spreading for over 1 mm along the arterioles at a speed of 116 μm/s [143]. In mouse brain capillary ECs, somatosensory stimulation triggers of hierarchy of local Ca^{2+} release events driven by IP_3R activation and sustained by TRPV4 and Orai1 [145].

These endothelial Ca^{2+} signals, which recruit endothelial nitric oxide synthase (eNOS) to relax local pericytes and redirect blood flow to the activated capillary [145], could be triggered by synaptically-released glutamate acting on the endothelial Group 1 metabotropic glutamate receptors [146,147]. The inter-endothelial communication of the Ca^{2+} signal evoked by focal endothelial cell stimulation enables coordination of the vascular response and induces either vasodilation [143] or an increase in vascular permeability [148] depending on the activating signal.

The analysis of large populations of naïve ECs revealed that neighboring ECs associate into clusters that are sensitive to the same agonist, e.g., acetylcholine or ATP, but the sensitivity to different ligands can vary among spatially-separated EC clusters [149]. McCarron and colleagues suggested that vascular endothelium is organized in predefined networks that are configured to use short-cuts to transmit the information encoded within the Ca^{2+} waves from the sensory site to more distant locations [150]. The sub-cellular organization of the Ca^{2+} signals arising from the activation of TKRs is yet to be fully understood [139]. However, studies conducted on zebrafish revealed that vascular endothelial growth factor (VEGF) stimulated intracellular Ca^{2+} oscillations to induce both migration in tip cells and proliferation in stalk cells budding from dorsal aorta [151,152]. VEGF-induced repetitive Ca^{2+} waves in the endothelial lineage are triggered by IP_3-induced Ca^{2+} release and NAADP-evoked Ca^{2+} mobilization from EL organelles and sustained over time by SOCE activation [84,152-154].

The wealth of studies conducted to dissect the underlying machinery and the multifaced roles of endothelial Ca^{2+} dynamics in the vascular system laid the foundation to understand how endothelial Ca^{2+} signals are generated at the iBRB and how they regulate retinal vasculature.

Ca^{2+} signalling at the iBRB

The mechanisms underlying endothelial Ca^{2+} signalling at the blood-brain barrier (BBB)[155,156] and their primary contribution to modulate vascular permeability[155,157], perceive neuronal activity[122], and regulate cerebral blood flow[146,158] have recently been object of intense investigation. Conversely, only scarce information is available regarding the composition of the endothelial Ca^{2+} toolkit in the iBRB. The expression of IP_3R1 protein has been reported[159], while functional RyRs are likely to be absent and TPC expression is yet to be investigated. Similarly, only pharmacological evidence suggests that SOCE is expressed and mediated by the interaction between STIM1 and Orai1[160]. Currently, the largest, but still preliminary, body of information available on the endothelial Ca^{2+} toolkit in the iBRB regards the expression and role of TRP channels.

TRP channels in the iBRB

The expression and function of TRP channels in ECs have been previously studied and for a broad summary we refer the reader to a previous article[161]. Briefly, ECs express most of the TRP channels isoforms, with expression of particular channels changing based on the vascular bed considered[162]. TRP channels regulate vascular tone, angiogenesis and vascular permeability. Vascular tone has been shown to depend on TRPP1/TRPP2 complex[163,164] and TRPV4[165]. With regard to angiogenesis, TRPV4 is well known to regulated EC proliferation, migration and angiogenesis[166]. TRPV4 expression is dramatically increased in breast tumor-derived ECs where it mediates arachidonic acid-induced cell migration via actin remodelling[167]. A proangiogenic role has also been proved for TRPV1 via the production of nitric oxide *in vitro* and *in vivo*[168,169]. Switching to TRPC channels, TRPC1 have been shown to play a pro-angiogenic role in zebrafish[170], whereas TRPC3, TRPC4, TRPC5 and TRPC6 have been involved in the process of tube formation in HUVECs[111,171,172]. Finally, TRPM2[173], TRPM4[174], and TRPM7[175] have also been shown to induce pro-angiogenic pathways and TRPA1 mediates simvastatin-induced angiogenesis[176]. Many TRP channels have been investigated in the permeability pathways. For example, TRPC1 and TRPC4 drive TNFα-[177] or thrombin-induced[104] permeability respectively, whereas activation of TRPC6 and TRPV4 induce permeability in the lung[117,178].

The good understanding of TRP channel function in phototransduction, particularly in *Drosophila*, is in stark contrast to the poor understanding of TRP expression and function in the retinal vasculature. Nevertheless, the limited knowledge available suggests that TRP channels play crucial roles in the retinal vasculature in both physiological and pathological conditions. All TRP channels have been detected by RT-PCR in the whole mouse retina[179,180]. So far, TRPC1, TRPC3, TRPC4, and TRPC6 expression have been detected in human retinal ECs, whereas TRPV1 and TRPV4 have been detected in human retinal ECs, primary bovine retinal ECs, and intact retinal vessels in mice and rats. Rat retinal VSMCs instead express TRPC1, TRPM7, TRPV1, TRPV2, TRPV4, and TRPP1.

TRP channels in iBRB physiology and disease

Blood flow regulation

Changes in retinal blood flow are known to occur before the early clinical stages of retinal diseases such as diabetic retinopathy[181-183]. The ability of the retina to maintain constant blood flow despite fluctuations in artery or intraocular pressure is known as retinal pressure autoregulation[184]. It has been suggested that impaired pressure autoregulation causes retinal damage in diabetes by increasing retinal capillary pressures and inflicting shear-induced EC damage[185]. The myogenic response of the retinal arterioles, which is triggered by the stretch-

dependent activation of TRPV2 channels on the retinal vascular smooth muscle cells (VSMCs), mediates the autoregulation of retinal blood flow[186]. Rats with early diabetes show an impaired myogenic reactivity of retinal arterioles, which is associated with the downregulation of TRPV2 in VSMCs, and its inability to be activated by stretch. Expression of TRPV2 is also decreased in human retinal VSMCs from diabetic donors. *In vivo*, non-diabetic TRPV2 heterozygous rats of at least 3 months of age lack of the myogenic reaction in the retinal arterioles. Regardless of not having diabetes, these rats show features of diabetic retinopathy such as vascular permeability, increased formation of acellular capillaries, spontaneous neovascular tufts in the superficial plexus, Müller cell gliosis, increased presence of microglia and upregulation of inflammatory factors[187]. Overall, this evidence suggests that the reduced myogenic responsiveness of retinal arterioles in diabetes is caused by a loss of TRPV2 function which alone might be one of the triggers in the onset of diabetic retinopathy.

Permeability

Vascular hyperpermeability is one of the key features of iBRB dysfunction, and it is regulated by the exchange of paracrine signals between the cells of the NVU[188]. Ca^{2+} entry is a triggering upstream event of permeability[189], but the contribution of TRP channels to such an event in the retinal vasculature hasn't been fully elucidated yet.

An important role in retinal vascular permeability seems to be played by TRPV4, which in human retinal vascular ECs is primarily localised in the plasma membrane. When activated, it induces a non-selective cation current, which is associated with a reduction of EC monolayer impedance and therefore increased permeability[190]. Activation of TRPV4 reduces VE-Cadherin and β-catenin colocalization, increases degradation of the tight junction protein occludin and disrupts cortical F-actin[190]. *In vivo*, pharmacological activation of TRPV4 induces vascular permeability in the retina of WT mice, which vasoinhibins, peptides with anti-vasodilatatory, and anti-vasopermeability properties, can downregulate by interfering with the TRPV4/Ca^{2+}/NO/ cytoskeletal reorganization cascade[191]. In mouse models of retinal vein occlusion[192] and in streptozotocin-induced (STZ) diabetic rats[191,193] TRPV4 participates in the onset of retinal oedema. However, controversies exist, as a study by Monaghan et al., proved that in bovine retinal vascular ECs, exposed to high glucose for 72h and in the retinal vessels of STZ-rats after 3 months of diabetes, there is a decrease in the expression of TRPV4[194]. This is not surprising as the role of TRPV4 in vascular permeability has been a matter of debate for almost twenty years. Controversial results depend on the different cell and animal model used[190]; indeed, TRPV4 appear to protect[195,196] or disrupt[197,198] the endothelial barrier in different vascular beds or based on the level of activation of the channel. Moreover, in the

retina an additional complication is due to the expression of TRPV4 in different cells of the NVU[199-201].

Angiogenesis

Numerous physiological and pathologic processes depend critically on angiogenesis. In retinal illnesses, such as proliferative diabetic retinopathy, AMD, and retinopathy of prematurity, aberrant angiogenesis within the eye contributes to visual impairment[1]. Angiogenesis requires the activation, migration, and proliferation of ECs, which is followed by the development of proper vascular tubes. An important intracellular signalling mechanism involved in the control of angiogenesis Ca^{2+} signalling. When Ca^{2+} signalling is blocked, EC proliferation, motility, and tubulogenesis are inhibited *in vitro*, and angiogenesis is suppressed *in vivo*.

Retinal ECs can detect changes in blood glucose. Excessive hyperglycaemia (HG) is a key factor in promoting the development of diabetic retinopathy because it induces retinal ECs proliferation and migration. Under HG conditions TRPC1 and TRPC6 expression increases in human retinal ECs[202], together with VEGF expression, which in turn is downregulated by the treatment of human retinal ECs with SKF 96365, an inhibitor of SOCE, TRPCs and voltage-gated channels. SKF 96365 also impairs human retinal ECs proliferation, migration, and tube formation, induced by HG. *In vivo* Trpc1/4/5/6 quadruple knockout mice did not show pericyte loss or vasoregression in mice subjected to 30 weeks of hyperglycaemia as a model for diabetes. These mice were also protected by the thinning of the retinal layer caused by STZ-induced hyperglycaemia[203]. However, TRPC5 deletion alone affects vascular recovery in the OIR model, which, in turn, suggests that endogenous TRPC5 plays a role in preventing vasculopathy exacerbation in ischemic retinas. Indeed, in contrast to WT mouse retinas, TRPC5-/- mouse retinas displayed increased avascular and neurovascular tufts[204].

Consistently, transfection of human retinal ECs with siTRPC4 inhibited VEGF-induced proliferation and tubulogenesis, together with the activation of the downstream mediators p38, ERK and AKT and in a mouse model of OIR, intravitreal injection of siRNA against TRPC4 prevented neovascularization[205].

As mentioned above, in human retinal ECs, TRPV4 is expressed, functional, and mechanosensitive. As for its role in permeability, TRPV4 role in angiogenesis is a matter of debate. *In vitro*, TRPV4 activation stimulates human retina EC migration and tubulogenesis[206]. However, when TRPV4 associates with TRPV1 to form heteromeric channels, its pharmacological blockage prevents tubulogenesis *in vitro*, surprisingly, without affecting retinal EC proliferation or migration. Furthermore, TRPV1/TRPV4 channels do not mediate the Ca^{2+}

signalling induced by VEGF and the consequent VEGF-induced angiogenesis *in vitro*[207]. *In vivo* the situation is not much clearer as TRPV4 seems to contribute to retinal stability and vessel maturation, but also to pathological angiogenesis. Indeed, its genetic deletion increases pathological neovascularization in response to oxygen-induced retinopathy (OIR), but it has no effect on post-natal developmental angiogenesis. In comparison to WT mice, retinal vasculature from knocked-out animals for TRPV4 that underwent OIR show lower pericyte coverage and neovascular tufts, indicating that TRPV4 absence can worsen pathological angiogenesis[208]. However, in a different study TRPV4 and TRPV1 antagonists significantly impair pathologic retinal angiogenesis in an OIR model, suggesting an active role of TRPV4 in inducing pathological angiogenesis.

Overall, these preliminary results suggest that much work still needs to be done to determine the role of TRP channels in iBRB dysfunction. However, although the role of TRP channels in controlling the integrity of the iBRB remains largely unexplored, evidence clearly shows their potential as regulator of iBRB properties.

TRP channels as therapeutic targets

The evidence discussed here highlights the role of TRP channels as regulator of iBRB properties, thus TRP channels role in retinal vascular endothelium should be investigated further. Indeed, different facts point to TRP channels as a plausible target for iBRB dysfunction. First, despite TRP channels not having properly been studied in the iBRB, a large amount of research explored their expression and functions in other organs and in multi-organ pathologies such as diabetes[209,210] and cancer[211-213]. Indeed, a role for TRP channels have been found in the pathology of cardiac, pulmonary, urinary, dermatologic and neurological disorders, with mutation of TRP channels, or TRP channelopathies, being identified as the cause of some human hereditary diseases[8]. Even in the eye for example, TRPA1, TRPM8 and TRPV1 have been considered as targets for retinal dry eye disease[214-217]; TRPA1 and TRPV1 have been also associated with allergic conjunctivitis[218-220]. TRPA1, TRPV1, TRPV4 and TRPC6 have been found playing a role in glaucoma[221-223], whereas TRPM8 has been associated with deregulated tear fluid and corneal inflammation. Finally, TRPM1 has been associated with congenital stationary night blindness and TRPV1 and TRPM7 with retinoblastoma. When considering TRP channels as a therapeutic target, their location is a huge advantage, because by being expressed mainly on the plasma membrane, they are easily accessible; however, given their diverse roles in cell functions, a challenge has arisen in term of specificity, as difficulties have been encountered when trying to find drugs to target specific TRP functions, without eliciting unacceptable side effects. Therefore, investigating

their downstream pathways to highlight similarities and differences among the different channels is the next logical step.

Second, several studies performed in different cell types, including ECs, show that VEGF, which as discussed above is one of the main drivers of iBRB dysfunction, exerts its functions by activating Ca^{2+} entry through different isoforms of TRP channels, such as TRPC3, TRPC6[111,224], TRPM2[173], and TRPV1[225].

Third, studies performed by Olesen showed that different permeability-inducing factors exert their function in ECs from pial microvessels by increasing $[Ca^{2+}]_i$[226,227]. Consistently more recent research showed how histamine, thrombin and bradykinin induce permeability in the BBB via activation of TRP channels[228,229]. Therefore, targeting TRP channels might also impair those compensatory mechanisms, such as increased expression of other inflammatory and permeability mediators, elicited by impairment of the VEGF signalling.

Conclusion

A delicate balance of fluid, molecule, and solute levels is essential to maintain retina health and clear vision. The iBRB is essential in delivering oxygen and nutrients to the retina layers while restricting the passage of fluid and maintaining a relative dry environment. However, pathological events, such as excessive release of VEGF can lead to the breakdown of the iBRB with consequent oedema and loss of vision. The effects of VEGF are driven by an increase of the intracellular Ca^{2+} concentration. TRP channels are components of the Ca^{2+} toolkit whose dysregulation is a key feature of many diseases. Recent evidence suggests that TRP channels are involved in the regulation of iBRB properties, such as vascular tone, permeability, and angiogenesis. Preventing Ca^{2+} entry via TRP channels was proven to have a protective effect of the iBRB and prevent diabetes-associated angiogenesis and hyperpermeability. It is highly plausible that TRP channels, and their downstream pathways, in the iBRB might provide novel targets for prevention of eye diseases.

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