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Blood-Brain Barrier Disruption: A Common Driver of Central Nervous System Diseases

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**Title: *Blood-brain barrier (BBB) disruption: A common driver of Central Nervous System (CNS) diseases***

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**ABSTRACT**

The brain is endowed with a unique cellular composition and organization, embedded within a vascular network and isolated from the circulating blood by a specialized frontier, the so-called blood-brain barrier (BBB), which is necessary for its proper function. Recent reports have shown that increments in the permeability of the blood vessels facilitates the entry of toxic components and immune cells to the brain parenchyma and alters the phenotype of the supporting astrocytes. All of these might contribute to the progression of different pathologies such as brain cancers or neurodegenerative diseases. Although it is well known that BBB breakdown occurs due to pericyte malfunctioning or to the lack of stability of the blood vessels, its participation in the diverse neural diseases needs further elucidation. This review summarizes what it is known about BBB structure and function and how its instability might trigger or promote neuronal degeneration and glioma progression, with a special focus on the role of pericytes as key modulators of the vasculature. Moreover, we will discuss some recent reports that highlights the participation of the BBB alterations in glioma growth. This pan-disease analysis might shed some light into these otherwise untreatable diseases and help to design better therapeutic approaches.

**Keywords:** BBB, blood-brain barrier; NVU, neurovascular unit; pericytes, neurodegeneration; glioma; Alzheimer's disease.

## 1. Introduction

The brain is the most unique and complex biological system. It is composed of specialized cells, such as neurons, astrocytes, oligodendocytes, and microglia, fed by an intricate vascular network. The blood vessels also serve perfusion-independent roles as they modulate stem cell survival and function, both in physiological (BjornssonApostolopoulouTian and Temple 2015; Licht and Keshet 2015) as well as in pathological conditions (Quail and Joyce 2017). Moreover, the brain vasculature possesses unique properties, named the blood-brain barrier (BBB), whose function is defined by the neurovascular unit (NVU). The NVU is embedded in the parenchyma of the brain and it is constituted by endothelial cells (EC), pericytes, astrocytes, vascular smooth muscle cells (VSMCs), myeloid cells and excitatory and inhibitory neurons (Iadecola 2017). The NVU is capable of controlling the vascular blood flow to ensure a rapid increase in the flow rate and the delivery of oxygen to activated brain areas (Kisler and others 2017). This structure presents tight junctions between endothelial cells to generate a barrier for the passage of ions and metabolites (including essential nutrients and oxygen) that allows a correct balance for the synaptic coordination of neurons (Abbott and others 2010). This function protects the neuronal cells from sudden changes of neurotransmitters such as norepinephrine and glutamate, whose blood concentrations can increase significantly in response to stress or even after a meal (Bernacki and others 2008). At the same time, the BBB clears out toxins from the brain, insulating this tissue from any harm that may be present in the periphery, and limits the entrance of red blood cells and leukocytes into the brain. By this means, a very well-regulated environment is created and maintained, which is optimal for proper neuronal functioning, synaptic transmission, synaptic remodeling, angiogenesis, and neurogenesis in the adult brain.

Multiple central nervous system (CNS) diseases such as stroke, traumatic brain injury (TBI), multiple sclerosis (MS) and Alzheimer's disease develop with dysfunction of the BBB, which correlates with the progression and some of the characteristic features of these pathologies (Liebner and others 2018). Although the mechanisms for these associations are not completely understood it has been shown that BBB breakdown is an early event in the aging brain and favors a toxic environment for neurons (Montagne and others 2015). Moreover, an increasing body of evidence shows that in many of these pathologies the brain microenvironment has to face with the process of astrogliosis, which could play a deleterious role for the recovery of the BBB (Pekny and others 2016).

Brain tumors, particularly gliomas serve as paradigmatic example of a neuropathology associated with vascular alterations and BBB dysfunction. According to its capacity to disrupt the surrounding brain we can differentiate between low-grade glioma (grade I and II) and lower-grade gliomas (LGG) (grade II and III of the WHO (World Health Organization) classification) and glioblastomas (GBM) (grade IV gliomas), which have a worse prognosis and induce profound changes of the CNS vasculature (Gargini and others 2020). Glioma cells develop their own mechanisms to corrupt the adjacent tissue: they control the composition of the extracellular matrix (ECM) and the function of the endothelium and the immune

system in order to acquire a growing advantage. The disruption of the homeostatic intercellular communication leads to changes in the whole microenvironment, including the disruption of the BBB, which favours the progression of gliomas (Gargini and others 2020; Quail and Joyce 2017).

This review aims to illuminate the function of the NVU and the BBB under physiological and pathological conditions. We will highlight recent advances in the knowledge about the cellular composition of the brain vasculature, with special emphasis on the function of the pericytes. We will discuss changes on the pericyte function during aging and in different Central Nervous System (CNS) diseases, such as gliomas and neurodegenerative processes, with a special focus on Alzheimer's disease. We will also analyze how the brain milieu is modified in the presence of glioma cells, focusing on the possible mechanisms of BBB disruption in these tumors.

## **2. The Blood-Brain Barrier (BBB) in health**

### **2.1 BBB structure**

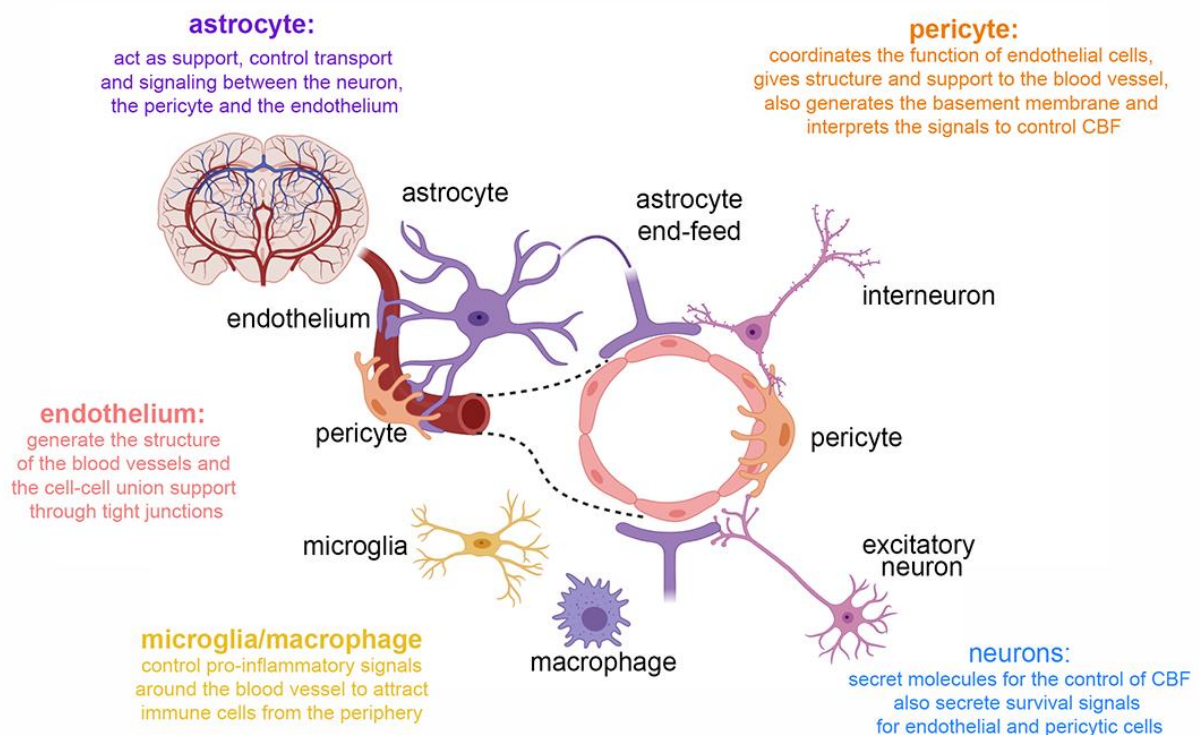
Structurally, the NVU is made of ECs that form the tightly sealed wall of all cerebral vessels (**Fig 1**). There is ample evidence about the specific physical and chemical features of the brain endothelium that contribute to its barrier properties and distinguish it from the endothelium of peripheral tissues. The endothelial cell-to-cell contacts limit the passage of blood to the brain and the accumulation of practically all molecules except from some small and lipophilic ones (Pardridge 2007b). Tight junctions contain the transmembrane proteins claudins, occludins, and junction adhesion molecules, acting in concert to close off interconnecting endothelial cells (HuberEgleton and Davis 2001). Adherent junctions are formed by homophilic interactions between vascular endothelial (VE-) cadherins, to seal adjacent ECs (Dejana and Giampietro 2012), or neural (N-) cadherins, which mediate their association with pericytes (Li and others 2011).

Approximately 98% of the neurotherapeutic drugs (recombinant peptides, proteins, anti-sense-agents and genetic vectors) are excluded from the brain through specific transport mechanisms in the ECs (Pardridge 2007a). These include ABC (ATP Binding Cassette) or SLC (solute carrier) transporters. ABC molecules primarily act as pump transporters, using energy to expel toxic molecules and limit their entry into the brain. The SLC transporters (such as GLUT-1 and LAT1), capture substrates that are necessary for the proper functioning of the brain such as glucose or amino acids.

ECs are surrounded by the basement membrane, mainly composed of laminin, fibronectin, collagen IV and heparan sulfate (Farkas and Luiten 2001). In addition, adhesion molecules are expressed by the ECs, pericytes and astrocytes and its functions are to physically support the cells of the NVU. It can also help regulate the selective function of the endothelium by expressing some polarized signaling molecules such as integrins. Recently, the  $\beta$ 1-integrin and its binding to basement membrane proteins have been shown to regulate the tight junctions structure and therefore the BBB integrity (Izawa and others 2018). Moreover, components of the basement membrane seem to prevent immune cells from entering into the brain parenchyma (Sixt and others 2001). In agreement with these data, it has been described how changes in the molecular composition of the extracellular matrix generate an increase in

the permeability of the BBB in pathological situations (Zhao and others 2015). Therefore, the basement membrane plays an important role in the functional integrity of the BBB, similar to other components of the NVU.

Pericytes cover a major part of the endothelial surface of capillaries and venules, especially in the brain where the pericyte-to-endothelium ratio is 1:3, compared with the 1:100 ratios in striated muscles, for example (von Tell and others 2006) (**Fig. 1**). Pericytes contain various cytoskeletal and contractile proteins, such as alpha-smooth muscle actin ( $\alpha$ SMA); desmin; vimentin; nestin and myosin, which allow them to function as muscle-like cells and regulate the cerebral blood flow (CBF). Moreover, they express multiple membrane proteins such as PDGFR $\beta$  (platelet-derived growth factor receptor  $\beta$ ), CD146, NG2, aminopeptidase A, and CD13, RGS5 (regulator of G protein signaling 5), and glycoproteins like MUC18 and CD248 (RuckerWynder and Thomas 2000; von TellArmulik and Betsholtz 2006). Pericytes predominantly maintain the vascular integrity, regulating the permeability of the BBB. Several reports have described an increase in vascular leakiness when the pericyte coverage diminishes (Armulik and others 2010; Ben-Zvi and others 2014; Lindblom and others 2003; Tallquist and others 2003). Pericytes can modulate this permeability through direct polarization of the astrocyte end-feet (Armulik and others 2010) or through the release of signaling factors that affect EC tight junctions. EC secrete molecules such as angiopoietin-1 (ANGPT1), transforming growth factor beta 1 (TGF- $\beta$ 1) and platelet-derived growth factor BB (PDGF-BB) to direct the migration of pericytes towards new vessels in order to stabilize the vascular wall (Ribatti and others 2011). At the same time, pericytes control the cell cycle of EC, as well as directly contribute to the formation of the basement membrane (Bergers and Song 2005). Pericytes also communicate with other NVU cells and enables precise control over CBF and BBB permeability, grating pericytes a central role within this unit (Obermeier and others 2013).



**Figure 1.** Diagram of the components of the NVU and their main functions.

The basement membrane of the NVU interacts directly with the “astrocyte end-feet” (**Fig. 1**), which cover approximately 90% of the neurovasculature (MathiisenLehreDanbolt and Ottersen 2010) and plays a crucial role in the function of the BBB, translating the nutritional and metabolic needs of neurons into changes in the blood flow (Abbott and others 2006; Harder and others 2002)(see below). Astrocytes secrete molecules (growth factors, cytokines, and neurotransmitters) that act directly not only on neurons, but also on ECs and other components of the NVU. The induction and maintenance of several of the properties of the BBB, including the expression of tight junctions or the polarization of endothelial membrane transporters depend on the association between astrocytes and ECs (Abbott 2002; Lee and others 2003; Wolburg and others 2009). Communication through the gap-junction subunit proteins, connexins 30 and 43, defines the network of astrocytes and allows the supply of metabolites and glucose from blood vessels to distal neurons in an efficient way (Rouach and others 2008). This intercellular communication between astrocytes has strong implications in the establishment of the neurovascular coupling (Giaume and others 2010).

In addition, the expression of cell adhesion molecules like Intercellular Adhesion Molecule 1 (ICAM-1) and Vascular Cell Adhesion Molecule 1 (VCAM-1) in the endothelium, which participate in leukocyte adhesion and infiltration into the CNS, is regulated by chemokines and inflammatory cytokines produced by astrocytes (Muller 2014). Moreover, several proteins like aquaporin-4 (AQP4) and the potassium channel Kir4.1 are localized at the end foot membrane to regulate ionic concentration at the NVU (Amiry-Moghaddam and others 2004).

Microglia are the resident myeloid cells, and they are considered as part of the NVU because of their perivascular localization and their capacity to survey the entrance of blood cells (**Fig. 1**). They present antigens and secrete signals that guide the transmigration of leukocytes from the blood to the brain, controlling the levels of inflammation in the CNS environment (Nimmerjahn and others 2005). Moreover, they modulate angiogenesis and the opening of the BBB in basal conditions. On the other hand, microglia become activated in a variety of neuroinflammatory conditions associated with the functional impairment of the BBB. Under these conditions, these cells produce inflammatory cytokines that further increase the vascular leakiness through downregulation of paracellular tight-junction proteins or by secretion of cytokines that further promote the entrance of leukocytes, including macrophages (Shigemoto-Mogami and others 2018). Thus, the role of microglia in the regulation of the NVU appears to be dual: depending on the environmental conditions they may have a protective or deleterious function on the BBB stability (Haruwaka and others 2019).

The main mission of the BBB is to provide an ideal working environment for neurons. For this reason, neurons must be considered an integral part of the NVU, since barrier function and dysfunction have an immediate impact on the neuronal network (Giaume and others 2010). Oligodendrocytes have been shown to be key in the establishment, maturation and function of the BBB during development (Bergers and Song 2005). Moreover, oligodendrocyte lineage cells can also be recruited by ECs, secreting soluble factors, such as TGF- $\beta$ 1 that increases BBB stability by upregulating tight junction

proteins (Seo and others 2014). But more recently, it has been observed that an aberrant interaction between oligodendrocytes and the cells of the neurovascular unit, which affects the junctions of pericytes-endothelium and astrocytes-endothelium in multiple sclerosis lesions and generates the disruption of the blood-brain barrier (Niu and others 2019).

## 2.2 BBB function: Regulation of the Cerebral Blood flow (CBF)

The brain needs a large amount of energy for its operation. In fact, the CBF represents a 20% of that generated by the heart and if this flow is interrupted, even for a few minutes, irreversible neuronal damage is generated (Girouard and Iadecola 2006). The mammalian brain has developed a unique mechanism for the regional control of CBF known as the neurovascular coupling, controlled by the close connection of pericytes, astrocytes and neurons (Alarcon-Martinez and others 2020; Iadecola 2017). The orchestrated regulation of the NVU ensures rapid control of the increase and decrease of O<sub>2</sub> and nutrients in the different brain areas depending on the neuronal activity (Iadecola 2017; Moskowitz and others 2010; Sweeney and others 2019). When this functional connectivity is interrupted, for example during stroke, there is a loss of pericyte coverage and a reduction of the neuronal activity. This process is observed as well in the early stages of other neurological disorders such as Alzheimer's disease and Amyotrophic lateral sclerosis (ALS) (Iadecola 2017; Moskowitz and others 2010).

The CBF is regulated by the contractile function of vascular smooth muscle cells (VSMCs) in arterioles (MacVicar and Newman 2015) and pericytes in capillaries (Kisler and others 2017; Peppiatt and others 2006), which respond to signals secreted by neurons, astrocytes and ECs. In astrocytes, Ca<sup>2+</sup> elevation evoked by the glutamate released from neurons, generate the secretion of molecules from their end-feet that induce the hyperpolarization and relaxation of VSMCs (Zonta and others 2003). Those include prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and epoxyeicosatetraenoic acids (EETs) (MacVicar and Newman 2015). By contrast, the phospholipase A<sub>2</sub>-arachidonic acid (AA) pathway and the astrocyte production of 20-hydroxyeicosatetraenoic acid (20-HETE) induces depolarization and contraction of VSMCs through its interaction with its receptor P2X (Mulligan and MacVicar 2004). Notably, capillary dilatations mediated by pericytes seem to be regulated by a different mechanism that involves ATP secretion (by neurons or other cells). The ATP binds to the astrocyte channel P2XR1, increasing intracellular calcium and activating the phospholipase D<sub>2</sub> (PLD<sub>2</sub>)-cyclooxygenase 1(COX1) pathway and promoting the release of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) that induce the relaxation of pericytes through activation of the prostaglandin E receptor 4 (EP4R) in the pericytes (Mishra and others 2016). Furthermore, a novel mechanism has been described recently that explains the synchronization of microvascular dynamics through nanotubes-like processes connecting pericytes at separated capillary systems. These structures coordinate the formation of Ca<sup>2+</sup> waves to regulate blood flow in a synchronized way (Alarcon-Martinez and others 2020; Sweeney and others 2016). It has also been observed that the gap-junctions are regulators of the BBB permeate through the regulation of Ca + 2 in endothelial cells (De Bock and others 2011).

Apart from astrocytes, the endothelium can also modulate the diameter of the blood vessels in response to shear stress (the frictional blood flow force acting on the wall of ECs). Shear stress induce AA, activation of the nitric oxide synthase (eNOS) and liberation of nitric oxide (NO), which contribute to VMSC and pericyte relaxation (Attwell and others 2010; Chen and others 2014; Hillman 2014). Moreover, shear stress on red blood cells (RBC) induce ATP release from RBCs themselves, which through their receptor binding in the endothelial cells activates phospholipase C (PLC) or phospholipase A2 (PLA2) and results in the production of EETs and PGI2, both contributing to VSMC relaxation via cAMP (Hillman 2014; Ralevic and Dunn 2015). Nevertheless, liberation of ECs-derived hyperpolarizing factor (EDHF) can trigger VSMC relaxation too. In addition, recent studies have already shown a direct neuron-mediated regulation of VSMC, mostly through NO production and neurotransmitters, although more information is needed to determine the exact function (Iadecola 2004).

### **3. The BBB disruption**

BBB breakdown, due to disruption of the tight junctions and/or the loss of pericytes, changes the blood flow and the permeability of the vessels. It alters the bidirectional transport of molecules between the blood and the brain, directly affecting nutrient supply and faulty clearance of toxic molecules and cells. These processes may initiate and/or accelerate a "vicious circle", resulting in progressive synaptic and neuronal dysfunction. Proper interaction between ECs and pericytes and astrocytes is required for the maintenance of the BBB, these are mediated by the canonical Wnt- $\beta$ -catenin signaling pathway. This pathway controls BBB formation and stability via the action of ligands (Wnt7a/7b, Norrin), receptors (Fzd4, Frizzled 4), coreceptors (Lrp5/6, low-density lipoprotein receptor-related protein 5/6), coactivators (Tspan-12, tetraspanin-12 (Tspan-12); Grp124, G-protein coupled receptor 124) and the downstream target,  $\beta$ -catenin, itself (Liebner and others 2018). Genetic loss of these signals results in a compromised BBB integrity (Wang and others 2012; Chang and others 2017). By contrast, overexpression of the ligands or constitutive activation of the pathway can protect from BBB breakdown by stabilizing the junctions between the endothelium or with pericytes and astrocytes (Chang and others 2017; Chen and others 2015). In addition, several inflammatory factors, free radicals and even vascular endothelial growth factor alpha (VEGF $\alpha$ ), released by cells of the NVU, can modify the endothelium and destabilize the BBB (Almutairi and others 2016). Moreover, BBB disruption causes secondary damage by oedema and inflammation (Anderson and Cranford 1979; Bralet and others 1979; Hzecka 1996). Another consequence is the appearance of astrogliosis, which is defined by an increase in the number of astrocytes, with a hypertrophic cytoskeleton and an increased expression of GFAP, commonly linked to inflammatory processes (Pekny and others 2016). The enhanced astrocytic proliferation and the secretion of several factors by reactive glial cells can lead to aggravation of the BBB disruption and further induce leukocyte transmigration (Michinaga and Koyama 2019). Notably, the astrogliosis phenomena has been observed as well in neurodegenerative diseases and gliomas (Dossi and others 2018; Quail and Joyce 2017), where it can aggravate the neuronal loss.

### 3.1 Techniques to characterize the integrity of the BBB

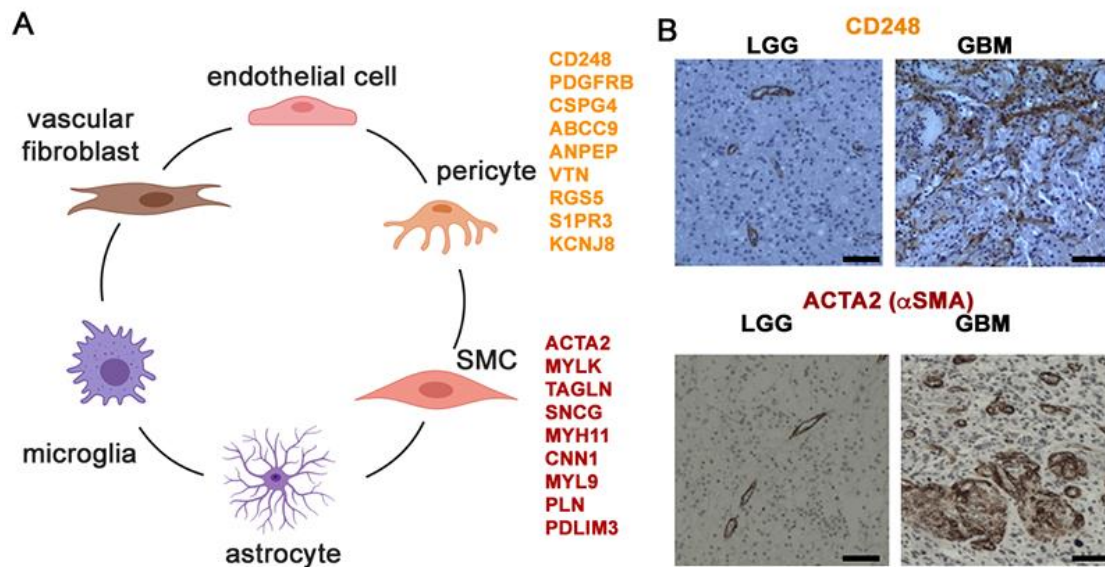
The composition and the stability of the BBB can be studied by immunohistochemical using the common markers for endothelium (CD34, CD31, endomucin), pericytes ( $\alpha$ SMA, NG2, CD248 or desmin), and astrocyte end-feet (AQP4 and GFAP) (Cheng and others 2013b; Gargini and others 2020; Liebner and others 2018). Moreover, distribution of endothelial junctions' molecules like claudin5, occludin and VE-cadherin can be measured, as well as changes in the pericyte coverage or immunoglobulin G (IgG) and albumin extravasation (Engelhardt and Liebner 2014; Park and others 2016).

Apart from these conventional techniques, several in vivo image methodologies have been developed to measure the integrity of the BBB and the CBF. One of the most well-known is the magnetic resonance imaging (MRI), used to determine the extravasation and subsequent tissue accumulation of intravenously injected paramagnetic contrast agent, typically gadolinium (Gd). Detection of signal enhancement on post-contrast images allows the identification of regions with a leaky BBB. In addition to this qualitative assessment, BBB permeability can be quantified when MR images are serially acquired before, during and after injection of a contrast agent. Thus, the efflux rate of contrast agent from blood plasma into the tissue extravascular extracellular space and permeability-surface area product can be calculated (Sourbron and Buckley 2013). Moreover, magnetic resonance spectroscopy (MRS) allows the evaluation of changes in concentrations of various metabolites caused by BBB disruption.

Other advanced imaging technologies have been developed to be used in laboratory animal models, including extravasation of contrast agents with different sizes and pharmacodynamics properties, which are used to measure the BBB pore dimensions (Liebner and others 2018). Moreover, intravital fluorescence imaging allows the determination of blood flow and vascular permeability, together with the detection of the cellular and molecular mechanisms that can generate the BBB disruption. As an example, a recent study using this technique has determined the central role of pericytes in BBB-disruption through activation of matrix metalloproteinase 9 (MMP9) (Underly and others 2017).

The recent dissection of vascular-associated cells using the transcriptional profiling technique (Single cell-RNA-seq) has unraveled the different cell populations of the entire vasculature of the mouse brain, associated with specific locations and/or specialized functions. Six types of endothelial cells (EC1, 2, 3, vEC, capEC, aEC), two populations of perivascular fibroblasts (FB1, FB2), in addition to the standard pericytes as well as three different types of smooth muscle cells (SMC: vSMC, aaSMC, aSMC) were described (He and others 2018; Vanlandewijck and others 2018). This study has highlighted the complexity of the mesenchymal cells that cover the vasculature in the different zones of the brain. Moreover, it has suggested a series of specific cell markers and short genetic signatures (**Fig. 2A**) that can help to understand the function of these pericytes in the normal brain and in pathological conditions. In gliomas, for example, different subsets of NG2 positive cells have been described (Girolamo and others 2013), and markers of pericytes (CD248) and SMCs ( $\alpha$ SMA) accumulate in the vicinity of tumor vessels during glioma progression (**Fig. 2B**) (Gargini and others 2020b; Segura-Collar and others). However, the specific identity of these pericytes, as well as their spatial and temporal distribution in gliomas or in other

brain pathologies, is still unknown. Future transcriptomic and proteomic analysis would be needed to understand their precise contribution to the stability of the BBB and which of them should be targeted to prevent the disruption of the vasculature during the evolution of the different CNS diseases (see below).



**Figure 2.** Regulatory cells in the BBB. (A) Cells involved in the control of the vascular network of the brain: endothelial cells, pericytes, SMC (smooth muscle cell), astrocyte, microglia, vascular fibroblast. The transcriptomic signature of pericytes and SMC is shown on the right. (B) Immunohistochemistry of CD248 and  $\alpha$ SMA on sections from lower-grade (LGG) and higher-grade (GBM) gliomas from patient's samples. Scale bar 100  $\mu$ m.

#### 4. The BBB disruption in pathological conditions

As we have previously mentioned, an increase in the permeability of the BBB has been observed after ischemic infarction or brain trauma. In MS, the BBB dysfunction is considered a hallmark of its pathophysiology and contributes to the development of the disease (Minagar and Alexander 2003). More recently, it has been observed that an aberrant interaction between oligodendrocyte precursor cells and the cells of the neurovascular unit, which affects the junctions of pericytes-endothelium and astrocytes-endothelium in active MS lesions, contributes to the disruption of the BBB (Niu and others 2019). Moreover, alterations in the BBB have been observed in neurodegenerative diseases like Parkinson's disease, ALS or dementia (Daneman and Prat 2015; Iadecola 2017; Kisler and others 2017), but also in brain disorders associated with autism, epilepsy, or schizophrenia (Palmer 2010). Conditions interfering with brain vasculature, such as diabetes, obesity and hypertension, can induce to BBB breakdown as well, fueling the development of neurodegenerative conditions (Carvalho and Moreira 2018; Zlokovic 2011). In relation to changes in the CBF, there have been reports of regional alterations in Huntington's disease, (Hasselbalch and others 1992), Parkinson (Derejko and others 2001) and ALS. The mechanisms that govern these vascular modifications are still unknown but in Alzheimer's disease it is debated whether a reduced CBF reflects diminished demand because of advanced neurodegeneration or if cerebral hypoperfusion contributes to dementia.

Recently, molecular changes associated with BBB disruption have been defined as a hallmark of various psychiatric disorders, mainly associated with the down-regulation of adhesion molecules such as

claudin-5, claudin-12 or ZO-1 in endothelial cells (Greene and others 2020). Especially, this type of dysregulation seems to show a dose-dependent response to favor the pathological condition in the case of schizophrenia (Greene and others 2020). The structure of the BBB is not static and undergoes changes in levels of permeability by fine regulation associated with sleep and circadian rhythm (Cuddapah and others 2019; Pan and Kastin 2017). This change favors the movement of molecules and liquid through the BBB, thus allowing the cleansing of metabolites in the brain but more studies are needed to establish all the processes where the BBB seems to be key to the proper functioning of the brain.

#### 4.1 Dysregulation of the BBB in Alzheimer's disease

Alterations of the BBB function in Alzheimer patients were described in the early 90's, associated with other vascular factors (Blennow and others 1990). Only in recent years has it begun to be considered of clinical relevance for the diagnostic imaging of the pathology (Iturria-Medina and others 2016; Sweeney and others 2019). Moreover, the presence of soluble PDFGR $\beta$  in the cerebrospinal fluid (CSF), which is a marker of pericyte injury and BBB dysfunction, has been proposed as an early biomarker (Nation and others 2019). Even as stated above, there could be a connection between BBB dysfunction and sleep disorders observed in patients with neurodegeneration, although studies that address this issue are lacking (Pan and Kastin 2017)

The two-hit vascular hypothesis has been postulated to explain Alzheimer's disease evolution, the A $\beta$ -independent (hit 1) and the A $\beta$ -dependent (hit 2), which can interact and converge in the impairment of blood vessels (Sagare and others 2013; Sweeney and others 2019; Zlokovic 2011). It has been suggested that the reduction and dysregulation of the CBF and the BBB dysfunction, which occurs in parallel or even earlier to the accumulation of A $\beta$  in the brain, leads to a vascular-mediated interruption of the neuronal function (hit 1). Moreover, it also leads to the impairment of A $\beta$  clearance, leading to increased accumulation of the A $\beta$  peptides (hit 2), which further contribute to the vascular and neuronal defects (Cao and others 2019; Montagne and others 2020; Nation and others 2019; Park and others 2020; Uddin and others 2020).

In relation with the first hit, the analysis of a large cohort of individuals (aged 55 years and older) showed that the decrease in CBF velocity (hypoperfusion) precedes cognitive deterioration and neurodegeneration-related hippocampal atrophy (Ruitenberg and others 2005). Along the same lines, several authors have shown that changes in the MRI and reduction of the CBF, linked to vascular dysregulation, are the initial events that lead to cognitive impairments, before they detect changes in the standard biomarkers, such as A $\beta$  and tau deposition (Fernández-Klett and others 2010; Iturria-Medina and others 2016). Moreover, it has been shown that older people who carry the  $\epsilon$ 4 variant of the apolipoprotein E (APOE4) gene show a reduced CBF compared to older non-carriers, suggesting a vascular contribution to their increased vulnerability (Filippini and others 2011; Suri and others 2015). More recently, the breakdown of the BBB in the hippocampus and medial temporal lobe has been detected specifically in individuals bearing APOE4 (with the  $\epsilon$ 3/ $\epsilon$ 4 or  $\epsilon$ 4/ $\epsilon$ 4 alleles), before the onset of cognitive deficiencies (Montagne and others 2020). These individuals also show higher levels of soluble

PDGFR $\beta$  in the CSF (Montagne and others 2020). As a possible explanation for these observations, expression of APOE4 has been shown to activate the proinflammatory CypA-nuclear factor- $\kappa$ B-matrix-metalloproteinase-9 pathway in pericytes, leading to defects in the integrity of the BBB and reduced CBF (Bell and others 2012).

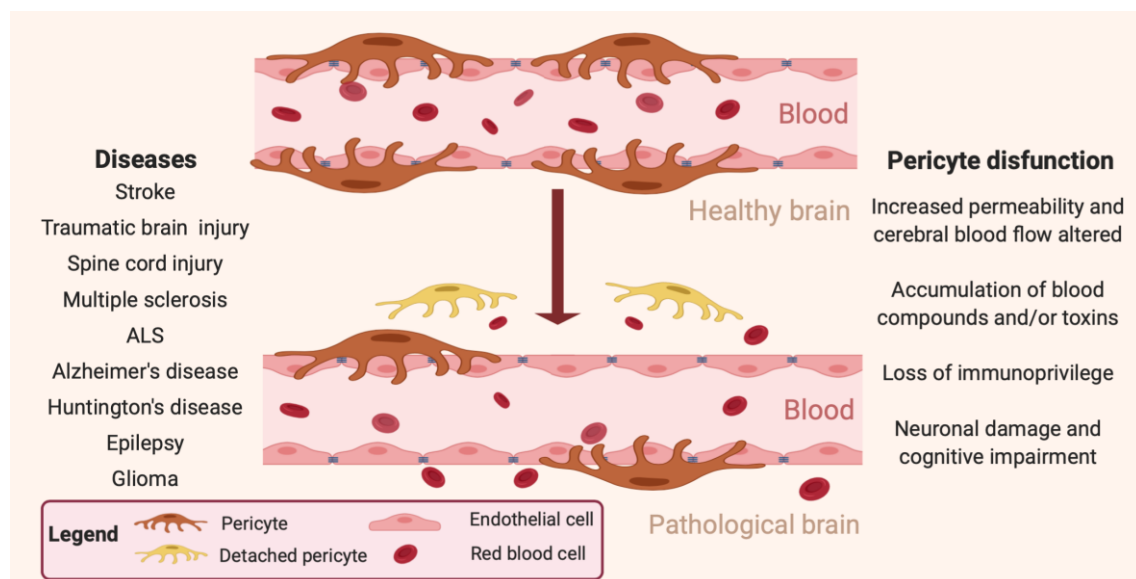
Regarding the second hit of the Alzheimer's disease hypothesis, the function of different components of the NVU is affected by A $\beta$ /Tau-dependent mechanisms (Iadecola 2017; Nation and others 2019; Sweeney and others 2016). The analysis of transgenic mouse models with amyloid precursor protein (APP) (Tg2576, APP Swedish mutation), or Tau (rTg4510, TauS301L or PS19, TauP301S) alterations has demonstrated that these genetic alterations reduce CBF by different mechanisms. Thus, the Tg2576 mouse shows low CBF levels due to a reduction in endothelium-dependent vasodilators (such as bradykinin or acetylcholine) or increased response to vasoconstrictors that act on VSMCs (U46619, analog of thromboxane A2) (Iadecola and others 1999). It has been shown that A $\beta$  binds to cells in the vessel wall that express the receptor for advanced glycation end products (RAGE), which results in the transport of A $\beta$  across the BBB and the expression of proinflammatory cytokines that induces the vasoconstriction (Deane and others 2003). This mechanism is responsible for the reduction of CBF and can be manipulated by the use of inhibitors against RAGE, which attenuates neurodegeneration in a mouse model of Alzheimer's disease (Deane and others 2012). The mechanism by which alterations in Tau affect the vasculature have been less studied, but changes in the diameter and in the morphology of the blood vessels (Bennett and others 2018), as well as BBB breakdown (Blair and others 2015) have been observed in transgenic mice, even in the absence of neurodegeneration. Moreover, an inverse correlation between the levels of soluble and insoluble Tau and APOE4, and the levels of tight junction proteins have been observed in the brains of AD patients (Liu and others 2020). In relation with these observations, a recent study has shown that overexpression of mutant Tau induces a failure in the neurovascular coupling due to a deficiency in the production of the vasodilator nitric oxide during glutamatergic synaptic (Park and others 2020). This could explain the vascular dysfunction observed in tauopathies before the onset of cognitive impairments.

#### 4.2 The role of pericyte dysfunction in Central Nervous System (CNS) diseases

Multiple genetic defects in different cells of the NVU can cause the disruption of the BBB and the development of severe CNS diseases (Zhao and others 2015). However, given the unique position of pericytes in the NVU, integrating neuronal and astrocytic signals with those derived from the peripheral blood and the endothelium, they are seen as key players in the dysfunction of the BBB. Seminal works described how the reduction in the number of pericytes due to decreased PDGFR $\beta$  levels, increases the vascular permeability and allows the influx toxic components for the brain (Armulik and others 2010; Daneman and others 2010).

Altered pericytic function has been associated with stroke, Alzheimer's disease, traumatic brain injury, migraine, epilepsy, spine cord injury, Huntington's disease, ALS and MS (**Fig. 3**) (Brown and others 2019; Cheng and others 2018; Uemura and others 2020). In these pathological conditions, the dysfunctional pericyte can induce the degradation of the basement membrane or alter the NVU

coordination, leading to BBB instability (Sweeney and others 2016). In addition, brain pericytes can regulate the inflammatory response due to their phagocytic and antigen-presenting capacity or through the interaction with microglial cells (Rustenhoven and others 2017).



**Figure 3.** Pericytes and BBB disruption. The pericytes exert a series of structural and coordinating functions in the blood vessels of the brain. The lack of function of these cells is observed in multiple CNS diseases, associated with a wide variety of alterations in neuronal and immune cell functions. ALS: amyotrophic lateral sclerosis.

An increase in the permeability of the BBB has been associated with cognitive decline during the aging of the brain of elderly individuals (Farrall and Wardlaw 2009; Yang and others 2020). The analysis of adult viable pericyte-deficient mice showed that the loss of these pericytes reduced the CBF, both in resting conditions and in response to brain activation, and impaired the BBB stability, leading to secondary neurodegeneration (Bell and others 2010). The neuronal damage seems to be mediated in part by the accumulation of blood compounds, such as thrombin or iron-binding proteins (Bell and others 2010), a process already observed in the brain of Alzheimer patients (Akiyama and others 1992; Grundke-Iqbal and others 1990). More recently, the analysis of a new model of acute pericyte loss of function (using the PDGFR $\beta$  and the Cspg4 (chondroitin sulfate proteoglycan-4) promoters) has shown a severe BBB breakdown and a rapid neuronal loss associated with a reduced secretion of pericyte-derived pleiotrophin, a survival factor for neurons (Nikolakopoulou and others 2019). Moreover, BBB impairments have been linked to activation of TGF $\beta$  signaling in astrocytes and cognitive impairments in aging human and mouse brains (Senatorov and others 2019). This could be a general mechanism to disturb the structure of the NVU in several pathologies as TGF $\beta$  overproduction affects the functionality of pericytes and VSMCs (Kato and others 2020).

In Alzheimer's disease, the A $\beta$  peptide shows frequent accumulation in the blood vessel (Zlokovic and others 1993) and has the capacity to induce vasoconstriction (Thomas and others 1996), paralleled by a decrease in pericyte number and coverage (Sengillo and others 2013). Moreover, the analysis of a mouse model that combined adult pericyte-loss (Pdgfr $\beta^{+/-}$ ) with the overexpression of the A $\beta$  peptide

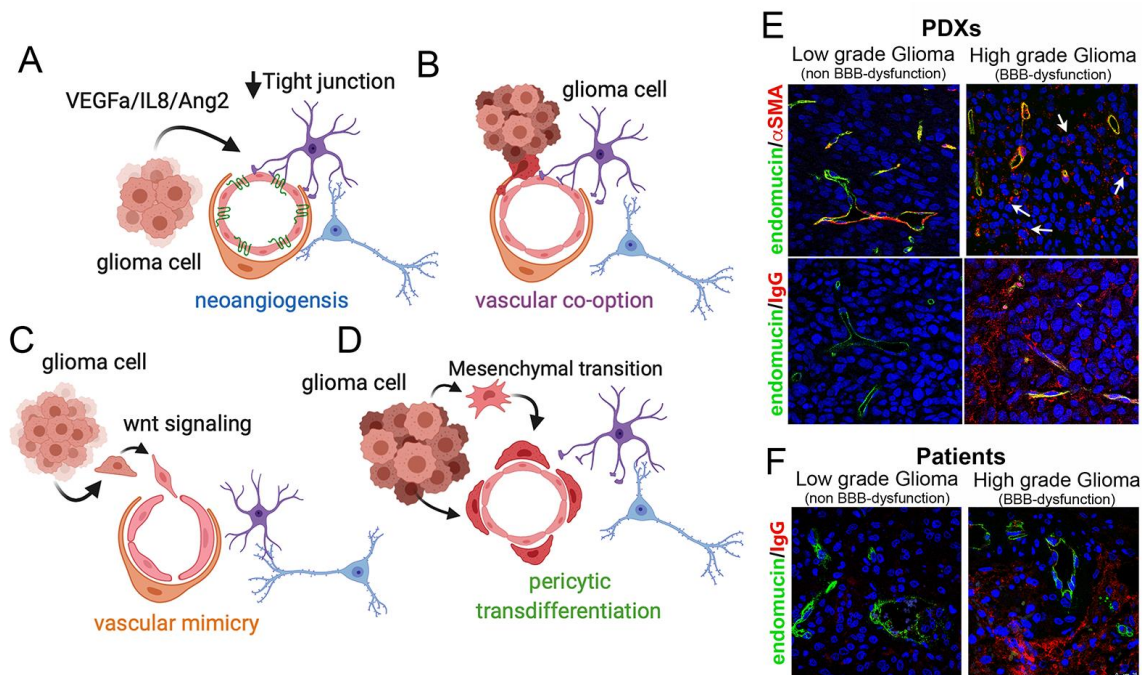
(APP<sup>sw/0</sup>) showed an accelerated  $\beta$ -amyloidosis and the development of tau pathology and early neuronal loss, which was absent in the APP<sup>sw/0</sup> mice (Sagare and others 2013), suggesting that aberrant pericytic function could represent a therapeutic target in Alzheimer's disease to control both the vascular and the neuronal degeneration.

VMSCs have also been proposed to participate in the hypercontractile vasculature and the reduction of the CBF observed in Alzheimer's disease. These cells express high levels of serum response factor (SRF) and myocardin (MYOCD), two transcription factors that regulate the expression of contractile proteins, both in Alzheimer patients and in mouse models (Tg-SwDI mice and Tg2576 mice) (Chow and others 2007). Although these changes seem to be independent of A $\beta$ , it is likely that these changes act synergistically with these peptides to accelerate the CBF decrease (Chow and others 2007) and the reduced A $\beta$  clearance, favoring the progression of the disease (Bell and others 2009).

#### 4.3 Vascular processes associated with BBB disruption in glioma pathology

Glioma cells govern the perivascular niche directly and indirectly, promoting the appearance of a florid and aberrant vasculature, which then, in turn, fuels the growth of highly aggressive tumors (Louis and others 2016). Studies in mouse glioma models and human samples suggest that there are at least four distinct mechanisms of tumor vascularization, which affect the NVU and can eventually lead to BBB leakage (**Fig. 4A-D**). Furthermore, it seems that these processes interact extensively, with potential overlap among them. One of the first vascular changes observed in gliomas is called neoangiogenesis. It is induced by molecules such as VEGF $\alpha$ , ANGPT2, and interleukin 8 (IL-8), which are secreted by tumor cells to stimulate the formation of new vessels from pre-existing ones (**Fig. 4A**) (Brat and others 2005; Carmeliet and Jain 2011; Hardee and Zagzag 2012a; Jain and others 2007; Saharinen and others 2017; Scholz and others 2016). An antibody against VEGF $\alpha$  (Bevacizumab) has been used in several clinical trials to stop glioma growth. Although no increase in the overall survival of the patients was observed, it has been widely used because it improves the patient's clinical status, reducing the complications related to inflammation and edema (Lu-Emerson and others 2015). As an alternative to angiogenesis, tumor cells can move towards the pre-existing blood vessels (vessel co-option) to gain access to nutrients, facilitating the invasion of the brain parenchyma by gliomas (**Fig. 4B**) (Hardee and Zagzag 2012b). Notably, this mechanism has been proposed as one of the possible mechanisms of resistance to bevacizumab as it does not depend on VEGF $\alpha$  (Seano and Jain 2020). Apart from the indirect effect of tumor vessels, glioma cells can transdifferentiate into endothelial (vascular mimicry) (**Fig. 4C**) or pericytic cells (**Fig. 4D**) due to their high plasticity. (Cheng and others 2013a; Ricci-Vitiani and others 2010; Wang and others 2010). We and others have observed that the majority of the vascular pericytes in aggressive gliomas are derived from tumor cells (Cheng and others 2013a; Gargini and others 2020a). The tumor-to-pericyte process has been related to TGF $\beta$  signaling (Cheng and others 2013a). Moreover, it can be driven by the NF $\kappa$ B-TAZ pathway in response to EGFR (epidermal growth factor receptor) activation and implies the acquisition of mesenchymal features, including the secretion of pro-angiogenic factors (Gargini and others 2020a). Although the consequences and mechanism of glioma cell transformation to vascular cells have not been

further explored, this is inherently associated with the acquisition of poor prognosis, which is a requirement for high-grade progression (Gargini and others 2020a). Furthermore, the elimination of glioma-derived pericytes seems to favor treatment with traditional therapies. (Gargini and others 2020; Zhou and others 2017).



**Figure 4.** Glioma effects on the NVU. Diagrams of the components of the NVU and how they are affected by the four different tumor processes that control vascular growth and glioma progression: (A) neoangiogenesis, (B) vascular co-option, (C) vascular mimicry (endothelial transdifferentiation) and (D) pericytic transdifferentiation. Representative tumor tissue images of PDXs (patient-derived-xenografts) (E) and human samples (F) from lower-grade gliomas (no NVU alteration and no BBB disruption) and high-grade gliomas (NVU alteration and BBB disruption).

Glioma progression from low to high-grade tumors correlate with the appearance of an aberrant vasculature through some of the processes described above (Fig. 4A-D) and is associated with increased leakiness of the BBB. This feature can be easily measured by IgG extravasation, both in the patient's tumors and also in the mouse models derived from isolated glioma cells (patient-derived-xenografts, PDX), with a slight increase in endomucin expression (Fig. 4E-F) (Sarkaria and others 2018). Indeed, microvascular proliferation, hypoxic areas and disruption of the BBB are considered a hallmark of the aggressiveness of gliomas, since it generates vasogenic edema, the main cause of mortality in these patients (Anderson and others 2008; Gargini and others 2020s; Herting and others 2019; Wolburg and others 2012). In relation with this, the possibility of rational normalization of the vasculature has been postulated as the main strategy for brain tumors (MartinSeano and Jain 2019). It has been shown, for example, that anti-Ang2/Tie1 or anti-Vegf/Ang-2 inhibitors are able to normalize the tumor vasculature, favoring a normal functioning of the tumor microenvironment that makes it susceptible to other therapies (Klopper and others 2016; Park and others 2016; Peterson and others 2016).

BBB disruption during glioma evolution can be mediated by the over-expression of VEGF $\alpha$  or ANGPT2, which are capable of generating instability in the NVU, increasing the permeability of the BBB (Argaw and others 2012; Argaw and others 2009; Scholz and others 2016). Gliomas have also been reported to weaken the BBB through the reduction of endothelial junction levels through the secretion of

matrix regulators (Rascher and others 2002; Wolburg and others 2012). The authors used a deficient mouse model of *gpr124* that weakens the structure of the NVU by reducing the expression of tight junction proteins, which generates an increase in the aggressiveness of the GL261 mouse glioma model and facilitates vascular alterations in gliomas (Chang and others 2017). In relation with the tumor-derived-pericytes, it has been proposed that their elimination disrupts the tight junctions and increased the vascular permeability, favoring the entrance of chemotherapy (Zhou and others 2017). By contrast, depletion of CD248 expression in tumor cells normalizes the vasculature in gliomas with a highly disrupted vasculature (Gargini and others 2020a), suggesting that they could have positive and negative function in the stabilization of the BBB. Alternatively, inter or intra-tumor heterogeneity in the pericyte-SMC content could explain these discrepancies.

#### 4.4 Crosstalk between glioma cells and neurons

Glioma-associated neurodegeneration has been related with the premature death of the patients and with the cognitive and motor dysfunctions that are frequently observed prior to surgical resection, especially in GBM patients (van Kessel and others 2017). Some of these disturbances (like those related to language and visuospatial functioning) could be directly linked to the appearance of the tumors in specific brain domains. However, gliomas, except for some childhood-related subtypes, do not seem to have a preferred location (Sturm and others 2012) and their tumor site is not linked to a differential prognosis in adult patients (Awad and others 2017). Moreover, many patients present defects in widely distributed functions such as executive functioning and memory (van Kessel and others 2017). These results suggest that the strong neuronal impact of aggressive gliomas is not restricted to the embedded and surrounding neurons. Instead, high-grade gliomas seem to cause a more delocalized and broader neuronal degeneration, probably linked to alterations of the NVU and the subsequent BBB leakage.

In agreement with this hypothesis, normalization of the glioma vasculature and growth slowdown after treatment with sunitinib, a pan-RTK (receptor tyrosine kinase) inhibitor, has been shown to alleviate the tumor-induced neurodegeneration (Hatipoglu and others 2015). This example and other drugs such as cediranib (PDGFR inhibitor) and bevasizumab (anti-VEGF antibody), highlighting the strong connection between the vascular and neuronal components of the glioma microenvironment.

### 5. Concluding remarks

The correct role of the healthy brain depends on its semi-isolation from the rest of the body, a function that is exerted by the BBB. The evidences reviewed here strengthens the idea that alterations of the BBB by the deficient function of any of its components, particularly pericytes, associate with neuronal aging and with a wide variety of brain pathologies, from neurodegenerative diseases to brain tumors (Sweeney and others 2019). Further elucidation of the process of BBB disruption in these pathologies will be pivotal to understand their physiological evolution, which could help to improve their diagnosis but also to develop more effective treatments. Furthermore, we propose that pan-disease approaches could lead to more meaningful discoveries, particularly for diseases of the brain. In that sense, we suggest that the

various therapeutic strategies that aim to normalize the vessels of gliomas could be applied to several CNS diseases, as they share common vascular gene signatures. In that sense, some molecules have been shown to reduce neurodegeneration by restoring the BBB, such as rapamycin or 3K3A-activated protein C (APC) (Lazic and others 2019; Van Skike and others 2018). Thus, compounds that normalize the vasculature could function as rejuvenators of the function of the BBB, reducing or slowing the progression of different CNS diseases. And the other way around, molecules used to reduce the vascular abnormalities in the aging brain could be repurposed to reduce glioma progression.

### **Conflicts of interest**

All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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### **Ethics approval**

Glioma tissues were obtained after patient’s written consent and with the approval of the Ethical Committee at Hospital 12 de Octubre (Madrid, Spain) (CEI 14/023). Animal experiments were reviewed and approved by the Research Ethics and Animal Welfare Committee at our institution (Instituto de Salud Carlos III, Madrid) (PROEX 244/14 and 02/16), in agreement with the European Union and national directives.

### **Authors' contributions**

Designing research studies: BSC, PMM, PSG and RG; Acquiring data: BSC, AHL, and RG; Writing-Original Draft: BSC, PSG and RG; Writing-Review & Editing: BSC, PMM, AHL, PSG and RG; Funding Acquisition: RG, AHL and PSG; Supervision: RG and PSG.

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