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Peri(vascular)cytes identity in the brain

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Keywords:	pericytes, brain, pericyte plasticity, Alzheimer disease, stroke, CADASIL

Peri(vascular)cytes identity in the brain

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Running Title: Brain pericytes – current state

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Pericytes are multifunctional cells, and critical elements of the neurovascular unit (NVU) for regulating cerebral blood flow and blood-brain barrier (BBB) integrity, as well as angiogenesis, wound repair and neuroinflammation. Pericytes from distinct peripheral tissues may have various properties, and may differ from those in the brain. Pericyte deficiency has been acknowledged in various central nervous system (CNS) diseases including multiple sclerosis, diabetic retinopathy, neonatal intraventricular hemorrhage, and neurodegenerative

disorders. Despite their importance, their developmental origins and phenotypic diversity are incompletely understood. However, besides pericytes, other perivascular cells have been described in the brain, including fibroblasts, macrophages, microglia, adventitial cells, and vascular smooth muscle cells. Identifying pericytes among other perivascular cell types and deciphering their specific role in the brain vasculature remains a challenge. This review focuses on the most recent studies regarding pericyte identification, differentiation, and involvement in pathological states of the CNS.

Keywords: pericytes, brain, pericyte plasticity, Alzheimer disease, stroke, CADASIL

Introduction

Pericytes are perivascular cells with long cell processes surrounding the microvessel wall on the abluminal side, in any organ. However, other cell types are also present in the perivascular space, such as smooth muscle cells, adventitial cells, fibroblasts and macrophages (Dias Moura Prazeres et al., 2017). Pericytes are found in close association with the endothelial cells through physical contacts and paracrine signaling, sharing their basal lamina. Additionally, they stabilize blood vessels and participate in angiogenesis, vascular remodeling, permeability, and control the access of immune cells into the tissue (Stark *et al.*, 2013; Dias Moura Prazeres *et al.*, 2017; Cheng *et al.*, 2018) in both health and disease.

In the brain, pericytes represent a key component of the neurovascular unit (NVU), together with neurons, astrocytes, endothelial cells, extracellular matrix elements, and the structural and molecular interactions between them (Insert Figure 1 here, created with BioRender.com).

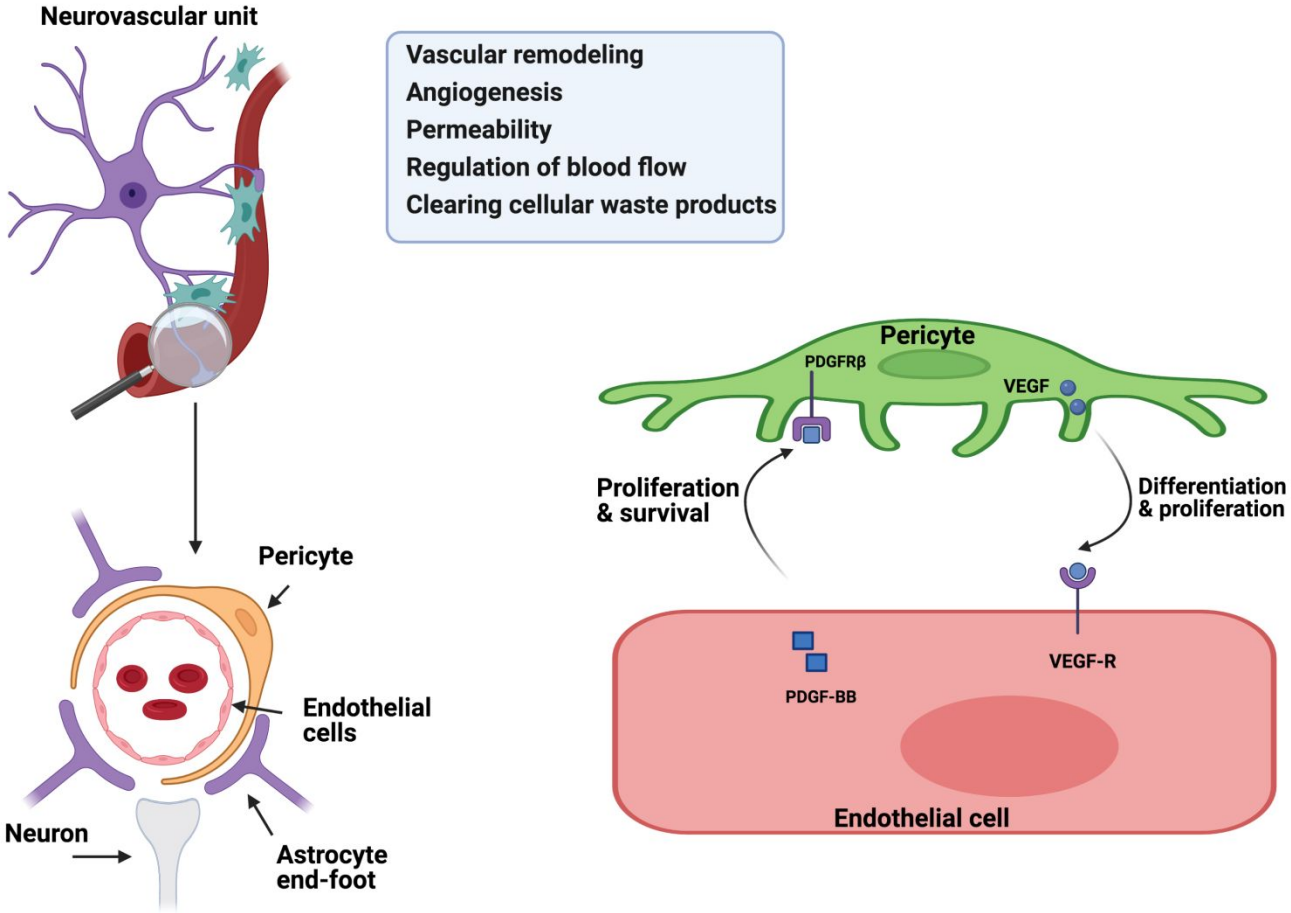


Figure 1. Pericytes and the neurovascular unit. Pericytes regulate the blood-brain barrier and play an important role in vascular ~~remodelling~~remodeling, regulation of blood flow, angiogenesis and vascular permeability. Created using BioRender.com.

The NVU is responsible for detecting the composition of the microenvironment in the brain and triggering vasodilation or vasoconstriction reactions (Muoio, Persson and Sendeski, 2014; Herland *et al.*, 2020), a phenomenon known as neurovascular coupling. There is a strong relationship between local neural activity and subsequent **changes in cerebral blood flow** (Pasley and Freeman, 2008). Moreover, pericytes participate in the **formation and maintenance of the blood-brain barrier** (BBB) (Bandopadhyay *et al.*, 2001; Armulik *et al.*, 2010a), and they regulate its function (Daneman, Zhou, Kebede, *et al.*, 2010; Thanabalasundaram *et al.*, 2010). Brain pericytes have also been reported to display phagocytic activity by clearing cellular waste products (Santos *et al.*, 2019).

Neural vasculature has the highest density of pericytes, with estimates ranging between one pericyte to every 1 to 3 endothelial cells (Armulik, Genové and Betsholtz, 2011) and a decrease in pericyte number and increased vascular permeability have been reported in central nervous system (CNS) disorders, such as multiple sclerosis, diabetic retinopathy, stroke, traumatic brain injury, migraine, epilepsy, spinal cord injury, and neurodegenerative diseases (Montagne *et al.*, 2015; Cheng *et al.*, 2018; Yamazaki and Y. S. Mukouyama, 2018). Known for their quiescent nature, pericytes are also targeted in both physiological and pathological vascular remodeling processes (Berthiaume, Hartmann, *et al.*, 2018).

Based on their capacities of self-renewal and differentiation into multiple lineages, various studies concluded that pericytes behave as **multipotent stem cells** in various organs, including the human brain. Most recently, a vast array of microenvironmental signals have been reported to regulate different pericyte functions, but their impact on quiescence, self-renewal or differentiation has not been entirely assessed (Nwadozi, Rudnicki and Haas, 2020).

Summary statement: Pericytes are a heterogeneous cell population in terms of **origin, distribution, morphology, phenotype, and function**. Additionally, multiple phenotypes/different sub-types have been described (Dias Moura Prazeres *et al.*, 2017), which makes their accurate identification very challenging. However, deciphering their

identity could open new perspectives for their role in various types of diseases (Cheng *et al.*, 2018).

Identification and diversity

To date, transmission electron microscopy (TEM) alone holds unequivocal criteria for pericyte identification. However, such techniques do not allow functional assessment (Yamazaki and Y. Mukouyama, 2018) .

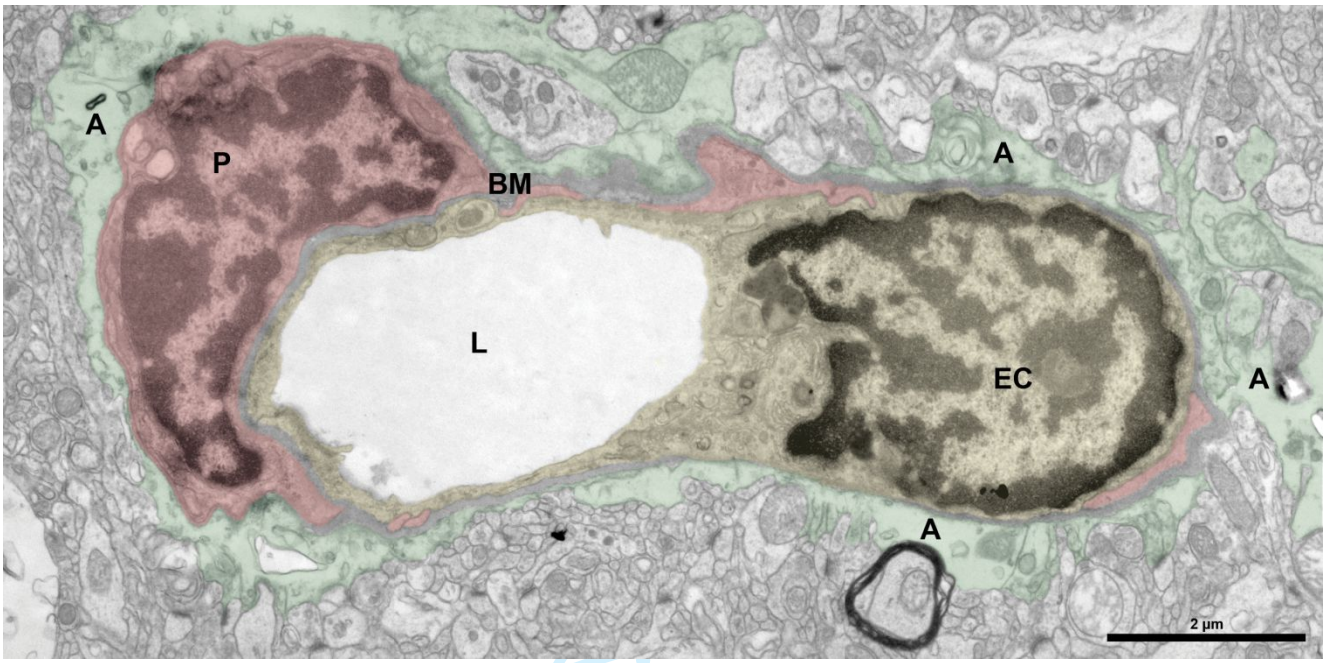
There is no single molecular marker known to be unique to pericytes, which indisputably and exclusively labels the whole population, regardless of organ and tissue distribution. Thus, a combination of general criteria is commonly used to define pericyte populations, such as perivascular localization, morphology, the expression of at least one or preferably a combination of molecular markers (Armulik, Genové and Betsholtz, 2011; Holm, Heumann and Augustin, 2018; Nwadozi, Rudnicki and Haas, 2020), along with the absence of endothelial and glial cell markers (Cheng *et al.*, 2018).

Ultrastructural characterization

TEM unravels multiple features unique to pericytes but only in terms of tissue distribution and morphology. Starting with the late '50s, pericytes have been described in various organs – cerebral cortex included – as perivascular cells, with long and branching cell processes extending along and embracing the microvessels, covered by a basal lamina that is continuous with the basal lamina of the endothelial cells. They establish close connections with the endothelial cells, such as “peg and socket" contacts, and occluding junctions toward the edges of the cell processes. Moreover, pericytes contain all common organelles, including a complete set of protein-producing organelles and dense bands of actin and myosin filaments distributed throughout the cytoplasm. In brain pericytes, the lysosomal compartment is also very well represented (Sims, 1986).

[Insert Figure 2. here]

Figure 2. Neurovascular unit in the mouse brain. The pericyte (P) is enclosed by the endothelial cell (EC) basement membrane (BM), which separates it from the surrounding astrocytes (A). The pericyte extends long cell projections embracing the endothelial cell. Digitally colored transmission electron micrograph.



Origin

Pericyte diversity starts with their origin. Some developmental biology studies showed that there are multiple sources for the pericytes residing in a specific organ (Dias Moura Prazeres *et al.*, 2017; Yamazaki *et al.*, 2017) . For CNS pericytes, these sources are highly heterogenous, from mesodermal (Etchevers *et al.*, 2001) to neuroectodermal (Korn, Christ and Kurz, 2002), and hematopoietic (Dias Moura Prazeres *et al.*, 2017; Yamamoto *et al.*, 2017).

Structural and molecular diversity

~~**Molecular marker expression could be the most reasonable approach for cellular identification. However, to date, there is no molecular marker known to be unique to pericytes or at least to be shared among all known sub-populations.**~~

Phenotypic heterogeneity

The most commonly used molecular markers are platelet-derived growth factor receptor beta (PDGFRβ) (Lindahl *et al.*, 1997) (Renner *et al.*, 2003), alanyl aminopeptidase N (CD13) (Kunz *et al.*, 1994), the proteoglycan neuron-glial antigen 2 (NG2) (Ozerdem *et al.*, 2001) (Stark *et al.*, 2013), desmin (Nehls, Denzer and Drenckhahn, 1992) and alpha-smooth muscle actin (α-SMA) (Nehls and Drenckhahn, 1991) ~~(doi: 10.1083/jcb.113.1.147)~~. However, some studies discuss sub-populations of pericytes that do not express NG2 (Stark *et al.*, 2013) or α-SMA, which is only used to define a contractile sub-type (Nehls and Drenckhahn, 1993; Cheng *et al.*, 2018) (Table 1).

More specific markers, such as the ATP-sensitive/inward-rectifying potassium channel 6.1 (Kir 6.1) (Bondjers *et al.*, 2006), which was reported as being restricted to pericytes within the CNS, did not gain trust over the years. Very recently, vitronectin (VTN) emerged as a potentially more specific marker for pericytes in the subventricular zone (SVZ), where adult neurogenesis occurs, but only half of the pericytes were immunoreactive (Jia *et al.*, 2019).

Moreover, even the most common markers are not entirely specific for pericytes, as they were also detected in other cell types. For example, PDGFRβ was detected in fibroblasts (Soderblom *et al.*, 2013), while NG2 may be expressed by macrophages and vascular smooth muscle cells (VSMCs) (Persidsky *et al.*, 2015; Yotsumoto *et al.*, 2015). RGS5 is a key regulator of pericyte detachment and plays a role in the transition from a perivascular to a parenchymal phenotype. In the chronic ~~period~~phase after stroke, RGS5 could be used to restore the pericyte response, preserve the vasculature, and prevent vascular leakage (Roth *et al.*, 2019)

Additionally, the expression of these molecular markers varies during growth and development, and different pathological conditions (Armulik, Genové and Betsholtz, 2011).

Novel molecular markers, such as Gli1 (Kramann *et al.*, 2015) and Tbx18 (Guimarães-Camboa *et al.*, 2017), were recently proposed. However, Gli-1 positive perivascular cells were not tested in brain tissue, but only in organs such as kidney, lung, liver, or heart, where they represent only a proportion of the PDGFRβ positive perivascular cells. Tbx18, a transcription factor that selectively marks pericytes in multiple organs, was also reported in brain pericytes. However, VSMCs were also labeled positive for Tbx18, which further muddles the precise identification of pericytes (Dias Moura Prazeres *et al.*, 2017; Cheng *et al.*, 2018).

Table 1 ***Pericyte molecular markers***

Marker symbol	Marker name	Reference
PDGFRβ	platelet-derived growth factor receptor beta	(Lindahl *et al.*, 1997), (Renner *et al.*, 2003), (Göritz *et al.*, 2011)
CD13	alanyl aminopeptidase N	(Kunz *et al.*, 1994)
NG2/ CSPG4	proteoglycan neuron-glial antigen 2	(Ozerdem *et al.*, 2001), (Stark *et al.*, 2013)
des	desmin	(Nehls, Denzer and Drenckhahn, 1992)
α-SMA	alpha-smooth muscle actin	(Nehls and Drenckhahn, 1993), (Cheng *et al.*, 2018)
RGS5	G protein signaling 5	(Bondjers *et al.*, 2003)
Kir6.1	potassium inwardly-rectifying channel subfamily J member 8	(Bondjers *et al.*, 2006)
ABCC9	ATP-binding cassette transporter subfamily C member 9	(Bondjers *et al.*, 2006)
SUR2	sulfonylurea receptor 2	(Bondjers *et al.*, 2003)
DLK1	delta-like non-canonical Notch ligand 1	(Bondjers *et al.*, 2006)
vim	vimentin	(Bandopadhyay *et al.*, 2001)
CFTR/MRP	ATP binding cassette, subfamily C	(Bondjers *et al.*, 2006)
Myh11	myosin heavy chain 11	(Asada *et al.*, 2017)
CD146		(Chen *et al.*, 2017)
CD133		(Graumann *et al.*, 2010)
Tem1	endosialin	(Christian *et al.*, 2008), (Cheng *et al.*, 2018)
Tbx18	T-box transcription factor 18	(Guimarães-Camboa *et al.*, 2017), (Cheng *et al.*, 2018)
VTN	vitronectin	(Jia *et al.*, 2019)
GLAST	sodium-dependent glutamate/ aspartate transporter 1	(Göritz *et al.*, 2011)
Gli1	glioma-associated oncogene	(Kramann *et al.*, 2015)
Nes	nestin	(Göritz *et al.*, 2011), (Birbrair *et al.*, 2013)
LepR	leptin receptor	(Kunisaki *et al.*, 2013)

Microscopy approach

Based on **localization and morphology**, pericytes were classified into three types: **pre-capillary, mid-capillary, and post-capillary** (Nehls and Drenckhahn, 1991). Mid-capillary pericytes are elongated, spindle-shaped, whereas pre- and post-capillary pericytes are shorter, stellate-shaped and have a variable expression of α-SMA (Nehls and Drenckhahn, 1991).

More recently, by using ***two-photon imaging*** *and* ***confocal microscopy*** on cortex from adult transgenic mice, Grant *et al.* highlighted the structural diversity of pericytes at this level based on morphology, vascular territory, and α-SMA expression (Grant *et al.*, 2019) ~~(doi: 10.1177/0271678X17732229)~~. They provided names for three types of pericytes, based on the appearance of their processes: ensheathing, mesh, and thin-strand pericytes. ***Ensheathing pericytes*** cover much of the vessel surface at the arteriole-capillary junction and are believed to be contractile; ***mesh pericytes***, which are located on the capillary and post-capillary venule, have short processes that are longitudinal and wrap around the vessel; and ***thin-strand pericytes*** are found in the intermediate part of the capillary, displaying the *bump-on-a-log* morphology with thin processes running along the vessel (Attwell *et al.*, 2016; Berthiaume, Hartmann, *et al.*, 2018; Lachlan S Brown *et al.*, 2019).

When comparing immunofluorescence images of α-SMA expression in pericytes, the fluorescent signal was higher in ensheathing pericytes, and undetectable in mesh and thin-strand pericytes.. Nevertheless, neither of these trends reached statistical significance, as there was substantial overlap in the range of microvessel diameters for each pericyte type. The authors concluded that microvessel caliber alone cannot define the pericyte subpopulation (Grant *et al.*, 2019). Pericytes are surely heterogeneous and the precise role of each sub-population in NVU function is still unknown.

The current focus on **pericyte identification** based on **new state-of-the-art *omics* technologies** enabled a better understanding of the unexpected roles of pericytes in brain health and disease. However, human brain pericytes have not been completely described at both proteome or transcriptome levels.

Proteomic approach

In addition to immunocytochemistry, proteomics offers the opportunity to identify and quantify many proteins and to explore how they correlate and interact with each other in biological networks.

In 2011, Chun *et al* analyzed the proteome of mouse brain microvessel membranes and basal lamina, using a chromatography-based proteomic technique called multidimensional protein identification technology (**MudPIT**) to identify transmembrane proteins in pericytes (Atp1a2, Atp1b2, Slc12a2, Atp2a3, Atp13a5, Abcc9, and Abca9) (Cahoy *et al.*, 2008; Daneman, Zhou, Agalliu, *et al.*, 2010; Chun *et al.*, 2011). Since it was observed that membrane fractionation of microvessels recovers both transmembrane proteins and associated extracellular matrix (ECM) proteins, some pericyte markers, such as solute carrier family 1 member 2 (Slc1a2), neutral amino acid transporter A (Slc4a4), and laminin α2 have been reconsidered (Chun *et al.*, 2011).

One of the most abundant transmembrane transporter/channel proteins was Atp1a2, a protein encoded by genes detected in both isolated pericytes (Daneman, Zhou, Agalliu, *et al.*, 2010) and astrocytes (Cahoy *et al.*, 2008). It was also observed that PDGFRβ was downregulated in pericytes, and in addition CD13 and NG2 were not included in the generated proteomic database (Chun *et al.*, 2011).

Furthermore, **untargeted mass spectrometry** was used to **characterize the cellular proteomes** from whole cell lysates (Herland *et al.*, 2020). More than 2000 proteins were identified, with a large number of proteins that were unique for each cell type. The analysis showed that the proteome of the astrocytes was more **similar to that of pericytes than to either neural cells or endothelium proteome.** In addition, pericyte proteomics demonstrated the presence of NG2 (Herland *et al.*, 2020).

PDGFRβ regulates brain angiogenesis and blood vessel stability. Pericyte damage and BBB breakdown can be evaluated by elevated **soluble PDGFRβ (sPDGFRβ) levels in cerebrospinal fluid (CSF),** which is an early biomarker of human cognitive impairment (Sagare *et al.*, 2015). Pericytes represent the main source of shedded sPDGFRβ in CSF, so it has become a pericyte-specific marker of injury in both human and animal models. A new sPDGFRβ immunoassay to calculate sPDGFRβ in human CSF was recently validated on the **Meso Scale Discovery** electrochemiluminescence platform for the study of brain pericytes

and microvascular damage in relation to cognition, in conditions associated with neurovascular and cognitive dysfunction (Sweeney *et al.*, 2020).

Transcriptome assessment

Single-cell RNA sequencing has emerged as a powerful tool in defining the brain pericyte transcriptomic profile. In this context, a recent mouse transcriptomics study led to the discovery of potential new pericyte explicit markers (Vanlandewijck *et al.*, 2018), (He *et al.*, 2016). Closed analysis of the 1,088 transcriptomes of the pericyte cluster revealed a gradual arteriovenous transcriptional zonation in endothelial cells, but surprisingly not in the case of brain pericytes (Vanlandewijck *et al.*, 2018). This observation raises the question of whether signaling from nearby cells could influence pericyte morphology along the arteriovenous axis.
The current focus on **pericyte gene expression profile** represents an important step forward in defining pericyte roles in brain pathophysiology. A study conducted by Song et al in 2020 compared brain microvessel transcriptome datasets, obtained from laser capture microdissection (LCM) preparations, with whole brain datasets. Single-cell RNA sequencing proved that **SLC12A7 gene** was strongly and selectively expressed by human brain pericytes. Compared to existing brain endothelial cells datasets, a number of genes were revealed to be enriched in the studied LCM datasets – SLC6A12, SLC12A7, PRELP, NDUFA4L2, GEM, FRZB, LRRC32, EHD2, FOXL1, COLEC12, KIAA0040, PTGDR2, C1QTNF1, FHL5, SMOC2, LPL. They could be potentially significant as **specific pericyte-expressed genes** (Song *et al.*, 2020).
A transcriptome analysis of brain mural cells – pericytes and VSMCs (He *et al.*, 2016) reported a “surprisingly limited overlap” in the gene expression profile, the cell populations having only three core transcripts in common, of which two are involved in actomyosin contraction (Vanlandewijck *et al.*, 2018). Interestingly, a previous study of the same group revealed almost no α-SMA expression in pericytes, although α-SMA has been reported by numerous immunohistochemical studies (Hill *et al.*, 2015; Attwell *et al.*, 2016; Alarcon-Martinez *et al.*, 2018), a feature attributed mostly to pre-capillary arteriolar pericytes (Hill *et al.*, 2015). Moreover, videos provided by different studies (Peppiatt *et al.*, 2006; Hall *et al.*, 2014) have shown that pericytes contract and relax, emphasizing these discrepancies, which

probably reflect limitations in methods of pericyte isolation or a quick down-regulation of α-SMA expression during transcriptomic analyses (Cheng *et al.*, 2018; Grant *et al.*, 2019).

A study conducted by Daneman R *et al*. analyzed the mouse BBB transcriptome by using Affymetrix microarrays in order to compare the mRNA expression of specific markers for neurons, astrocytes, oligodendrocytes, microglia and pericytes/VSMCs (PDGFRβ, Abcc9, and Kcnj8) (Daneman, Zhou, Agalliu, *et al.*, 2010).

The endothelial and pericyte transcriptome datasets were the starting points in generating predictions of cell-cell interactions at molecular level, critical for vascular contraction, remodeling, differentiation, and permeability. Out of 50 pericyte enriched genes, 40 were predicted to encode transmembrane or secreted proteins potentially involved in endothelial-pericyte signaling (Daneman, Zhou, Agalliu, *et al.*, 2010) (doi:10.1371/journal.pone.0013741). For example, PDGF-BB secreted by endothelial cells regulates the proliferation and survival of brain pericytes via PDGFRβ, the most constant pericyte surface marker (Gaceb *et al.*, 2018), as evidenced by the deficiency of PDGFB in pericytes or PDGFRβ deficient mice (Lindahl *et al.*, 1998). Angiotensin I converting enzyme 2 (ACE2) was reported to be overexpressed by pericytes, thus being potentially involved in generating ligands for agtrl1 receptors on endothelial cells; the ligand-receptor complexes are important players in brain angiogenesis and BBB stability (Daneman, Zhou, Agalliu, *et al.*, 2010).

To date, transcriptome studies have reported contradictory results and further studies are still needed to precisely define the mRNA and protein expression profile of brain pericytes in both normal and pathological conditions (Cheng *et al.*, 2018).

Genetic engineering approach

New research tools, such as **advanced transgenic mouse technology,** are being applied to answer the new questions raised and to better understand the defining roles and characteristics of pericytes (Lachlan S Brown *et al.*, 2019).

However, most of the current approaches in pericyte identification in **mouse models** rely on transgenic promoter expression based on NG2 or PDGFRβ and both reporters label all mural cells, both pericytes and VSMC. Thus, the use of some models requires careful reflection due to the absence of a unique marker for pericytes. **Genetic mouse models,** like NG2-

dsRed, NG2-eGFP, NG2-EYFP and NG2/PDGFRβ-tdTomato, which label different groups of pericytes, can be used to study pericytes in physiological and pathological conditions (Schallek *et al.*, 2013; Zehendner, Wedler and Luhmann, 2013; Özen *et al.*, 2014; Hartmann *et al.*, 2015; Hill *et al.*, 2015). Furthermore, in 2017, a new mouse line expressing tamoxifen-inducible Cre-recombinase under the control of the PDGFRβ promoter (PDGFRβ-P2A-CreER^{T2}) was developed and it was used as a genetic tool for a deeper understanding of the role of pericytes in angiogenesis (Cuervo *et al.*, 2017). Other pericyte-deficient mice, like Pdgfb$^{ret/ret}$ mice (Lindblom *et al.*, 2003), *Pdgfrβ*$^{+/-}$ or *Pdgf* hypomorph mice (with a 20–50% cutback of pericytes) (Armulik *et al.*, 2010b; Bell *et al.*, 2010; Daneman, Zhou, Kebede, *et al.*, 2010), have been developed to investigate the consequence of pericyte deterioration on neurovascular function (Cheng *et al.*, 2018).

Recently, Damisah *et al.* observed that a fluorescent Nissl dye – NeuroTrace 500/525 – labels brain capillary pericytes with specificity, permitting high-resolution light microscopy imaging in the live animal, and thus opening a new perspective for studying pericyte behavior in normal and diseased brain (Damisah *et al.*, 2017). However, it is not clear yet whether NeuroTracer labels all pericytes or only the non-contractile ones (Cheng *et al.*, 2018).

A study published in 2019 by Roth and colleagues ~~published a study in 2019 that~~ showed that switching from a parenchymal pericyte to a perivascular phenotype has vascular remodeling repercussions in the chronic phase following stroke. RGS5-KO mice had significantly more perivascular pericytes, which resulted in increased pericyte coverage, as well as better vascular density and length (Roth *et al.*, 2019)

Secretome assessment

The secretory capacity of brain pericytes is currently explored as a potential therapeutic regenerative approach in different pathologies. In response to various stimuli, a heterogeneous secretome is released, consisting of a plethora of pro- and anti-inflammatory cytokines/chemokines, suggesting that pericytes could play important roles in modulation of inflammation, even contributing to inflammation-induced BBB disruption (Thanabalasundaram *et al.*, 2010; Gaceb *et al.*, 2018). In this regard, lipopolysaccharide (LPS), an *in vitro* potent activator of pericytes, was able to induce both the secretion of pro-

and anti-inflammatory molecules, suggesting the complex role of pericytes in the neuroimmune response regulation (Gaceb *et al.*, 2018). Moreover, studies have concluded that in response to inflammatory stimuli, both endothelial cells and pericytes express similar patterns of transcription factor activation, but with distinct secretome profiles (Smyth *et al.*, 2018)(doi:10.1186/s12974-018-1167-8). Pericytes mostly secrete inflammatory chemokines, rather than the chemokines involved in homeostatic leukocyte surveillance. Specific cell-type responses have been identified, with IGFBP2 and IGFBP3 being pericyte-specific (Smyth *et al.*, 2018).

Metabolome assessment

Recently, new insights into the pericyte metabolism were found in an *in vitro* culture system of human primary cells, in the presence and absence of exposure to methamphetamine. In this untargeted metabolomics study, pericytes and astrocytes exhibited the most similar secretion patterns. However, pericytes presented a lactate/pyruvate ratio similar to astrocytes and very low glutamate and glutamine secretion (Herland *et al.*, 2020) (doi:10.1002/adbi.201900230). Metabolic changes observed in pericytes after 24h exposure to methamphetamine were an up-regulation of nucleoside degradation, which could be a cellular compensation mechanism for energy depletion (Herland *et al.*, 2020).

A new study on neural metabolic imbalance induced by MOF (MYST family histone acetyltransferase) dysfunction that triggers pericyte activation and breakdown of vasculature was published last year (Sheikh *et al.*, 2020) (doi:10.1038/s41556-020-0526-8). Their complementary *in vivo* and *in vitro* studies showed that metabolic defects, especially an increase in free long-chain fatty acids (LCFAs) induce a TLR4–NFκB-mediated inflammatory reaction in pericytes that leads to increased vascular permeability and hemorrhaging. Their results suggested that a deficient neural metabolic environment can activate vascular inflammation, pericyte dysfunction and increased vascular permeability. Therefore, changes in the metabolic milieu of a single cell type in a complex organ can impact the functionality of neighboring cells and subsequently disturb overall organ function (Sheikh *et al.*, 2020).

Moreover, administration of the pro-inflammatory TLR4 agonist LPS to mice produces morphological changes in brain pericytes, detachment from the vasculature, remodeling of ECM components and increased vascular permeability (Nishioku *et al.*, 2009) – all of which

are defects that were observed in Mof-nKO brains. In conclusion, this study provides evidence that the metabolic environment can regulate and control the functionality of brain pericytes and thus vascular function (Sheikh *et al.*, 2020).

Functional diversity

Various other studies stressed out the ***functional heterogeneity*** of pericytes***.*** For example, in 2014, Birbrair *et al.* identified a pericyte sub-population, called type-2 pericytes, which only participates in normal angiogenesis (Birbrair, Zhang, Wang, *et al.*, 2014), while another sub-population, called type-1 pericytes, is involved in scar tissue formation, after brain injury (Birbrair, Zhang, Files, *et al.*, 2014). Thus, different sub-populations of pericytes can contribute to different pathological conditions.

Modern functional imaging techniques, such as ***in vivo*** **two-photon microscopy** are frequently used to study pericytes in real time, their dynamic changes of capillary diameter and blood flow in the cortex of anesthetized mice.

To this end, in 2018 Berthiaume *et al.*, performed **long-term *in vivo* two-photon imaging** in the cortex of three inducible transgenic adult mouse models to show that active/dynamic *in vivo* remodeling of pericytes maintains capillary coverage in the adult mouse brain. Following the measurements, they concluded that brain capillary pericytes bargain vascular areas with neighboring pericytes and can extend or retract their processes. Moreover, they showed that selective ablation of a single pericyte induces extension of processes from adjacent pericytes to contact uncovered regions of the endothelium. Furthermore, loss of pericyte contact led to local capillary dilation until interaction was recovered (Berthiaume, Grant, *et al.*, 2018).

Pericyte plasticity

Increasing evidence suggests that brain pericytes alter their traits following stimuli and develop stemness, demonstrating their plasticity.

Cell plasticity is a permissive concept, broadly used to indicate a change in cellular morphology, or a phenotypic change from one cell type to another (an overview on cell plasticity can be found here https://www.sciencedirect.com/topics/neuroscience/cell-plasticity).

Frequently in the literature, plasticity of pericytes refers to their ability to change their phenotype into myofibroblasts, with which they share developmental origin (Armulik, Abramsson and Betsholtz, 2005; Yamaguchi *et al.*, 2020; Kuppe *et al.*, 2021) (for a recent review see (Santos *et al.*, 2019). This is not a feature unique for BBB pericytes, but rather a common feature of pericytes throughout the body, which has been described for liver (Zhang *et al.*, 2014), (Zhang *et al.*, 2014), lung (Shammout and Johnson, 2019), kidney (Shaw *et al.*, 2018), ovary (Kizuka-Shibuya *et al.*, 2014), uterus (Andersson *et al.*, 2015), skin (Noishiki *et al.*, 2019), adipose tissue (Szöke, Beckstrøm and Brinchmann, 2012), and bone marrow pericytes (Herrmann *et al.*, 2016) (for a recent review on various locations and involvement in angiogenesis see (Castro *et al.*, 2018)).

Depending on tissue and environmental cues, pericytes can differentiate into other specialized cells, such as osteoprogenitor cells (Supakul *et al.*, 2019) or adipocytes (Alexander *et al.*, 2016). This versatility, similar to that of stem cells, raised the question whether these cells are *bona fide* mesenchymal stem cells (Crisan *et al.*, 2008), a concept which has been widely embraced, up to the point that pericytes were considered a source for cell transplantation in regenerative therapies (Crisan *et al.*, 2012; Karow, 2013; Vezzani *et al.*, 2018; Supakul *et al.*, 2019), including following CNS pathological states such as stroke (Caporarello *et al.*, 2019; Ogay *et al.*, 2020).

This perspective of using classic PDGFRβ in lineage tracing studies has been challenged a few years ago. Using the transcription factor Tbx-1 for lineage tracing, the authors argued that pericytes are not adipogenic, nor fibrogenic or myogenic progenitors (Alexander *et al.*, 2016). Their argument, soundly supported by *in vivo* models, remains to be further validated by other studies, including those in human models.

In the CNS, pericyte plasticity becomes evident in pathologic conditions, such as ischemia or stroke, conditions which induce dedifferentiation into vascular stem cells with multipotency towards both neural and vascular lineage (Nakagomi *et al.*, 2015; Nakata *et al.*, 2017), but also a microglial phenotype (Özen *et al.*, 2014; Sakuma *et al.*, 2016). The presence of these “induced stem cells” was also highlighted in brain samples of two stroke patients. Their multipotency was further investigated *in vitro* and their neurogenic potential confirmed

(Tatebayashi *et al.*, 2017). The pericyte plasticity potential towards neurogenic progenitors was exploited *in vitro* to directly reprogram them into immature human neurons, capable of generating action potentials (Karow *et al.*, 2012), and of further differentiation into both GABAergic and glutamatergic neurons (Karow *et al.*, 2018). Ontogenically, BBB pericytes may derive from both neural crest and mesoderm (Crisan *et al.*, 2008) [(Paul *et al.*, 2012) (reviewed in (Lange *et al.*, 2013)], which might influence their decision towards one cell type or another.

BBB pericytes are also characterized by "**structural plasticity**" – changes in the distal ramifications of pericyte cell processes (also called terminal processes), which can retract and/or extend over time (Berthiaume, Grant, *et al.*, 2018). Following cell death, neighboring pericyte processes extend during the following days and weeks to cover the denuded epithelium area, with a growth rate of up to 8 µm per day (Berthiaume, Grant, *et al.*, 2018). This topographic plasticity was reported to be enhanced in epileptic seizures in a mouse model (Arango-Lievano *et al.*, 2018).

PERICYTES IN NEUROLOGICAL DISEASES

An essential role of pericytes in the pathophysiology of cerebrovascular diseases is the regulation of blood flow at the NVU level (Cai *et al.*, 2017). Under normal conditions, the variation of systemic blood pressure has no impact on the cerebral circulation flow, due to cerebral vascular autoregulation (Fernández-Klett and Priller, 2015). VSMCs play the most significant part in this process (Poittevin *et al.*, 2014). Pericytes are involved in cerebral autoregulation only in terminal vessels, where VSMCs are not present (Cai *et al.*, 2017). Pericytes regulate the cerebral flow by dilation, thus increasing the capillary flow, in hypoperfusion and hypoxia conditions, and by constriction, thus decreasing the capillary flow, in hyperperfusion conditions (Cai *et al.*, 2017).

Pericytes in ischemic stroke

Paradoxically, in acute ischemic stroke, especially in cases of reperfusion after acute ischemic stroke, pericytes are contracting, thus altering the flow in the microcirculation and

contributing to the no-reflow phenomenon (Yemisci *et al.*, 2009). The mechanism that induces the paradoxical constriction seems to be related to the high sensitivity of pericytes to the oxidative stress created in the hyper-acute stage of ischemic stroke (Yemisci *et al.*, 2009).

Another role of pericytes in the pathophysiology of acute ischemic stroke refers to BBB maintenance during subsequent inflammation (Su *et al.*, 2019). Pericytes, especially the ones from postcapillary venules, are involved in blocking the diapedesis of inflammatory cells in the CNS (Allt and Lawrenson, 2001). In acute ischemic stroke, inflammation enlarges the gaps between pericytes, allowing the inflammatory cells to enter the CNS (Proebstl *et al.*, 2012).

In the subacute and chronic phases of ischemic stroke, pericytes are involved in the recovery stages (Su *et al.*, 2019). They detach from their BBB site, migrate to the injured site and give rise to microglial cells (Özen *et al.*, 2014) participating in the clearance of debris and in the process of angiogenesis and neurogenesis (Gonul *et al.*, 2002; Liu *et al.*, 2012). Although this migration is beneficial to the injured tissue, it can lead to an alteration of the BBB in the previously healthy site by decreasing the number of pericytes (Armulik *et al.*, 2010b; Cai *et al.*, 2017).

Moreover, angiogenesis that occurs inside and around the infarcted area also requires pericyte intervention. ~~(PDGF) B/PDGF receptor (PDGFRβ PDGFR) b~~ system mediates their interactions with endothelial cells (Renner *et al.*, 2003) for blood vessel growth and maturation during stroke vascular remodeling.

Pericytes and CADASIL

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is a hereditary angiopathy caused by a mutation in the Notch-3 gene that favors the aggregation of the Notch-3 protein in vascular cells, promoting a small-vessel disease and numerous subcortical infarcts in young people, that eventually lead to dementia (Chabriat *et al.*, 2009). Although it is well known that VSMCs are altered in CADASIL, pericytes also express the Notch-3 protein and they are severely damaged in this genetic

disease (Dziewulska and Lewandowska, 2012). Moreover, it appears that pericytes are the first cells to be altered in CADASIL (Ghosh *et al.*, 2015). Pericyte damage contributes to BBB leakage and microvascular dysfunction, leading to multiple ischemic strokes and leukoencephalopathy (Ghosh *et al.*, 2015).

Pericytes in aging

Several studies suggest that aging is associated with a loss of pericytes, thus contributing to the alteration of the BBB in the aging brain (Bell *et al.*, 2010). In addition, pericytes in the aging brain have a large number of inclusions and dense bodies in the cytoplasm (Alba *et al.*, 2004). This might suggest a dysfunction of the lysosomes and increased phagocytosis in the aging brain in an attempt to remove the cellular debris that have the potential to become pathological and induce neurodegeneration (Alba *et al.*, 2004). In the aging brain, there is an alteration of the contraction mechanism in the pericytes, through dilation of the microcirculation vessels due to sclerosis and loss of pericytes, or through constriction due to the increased oxidative stress associated with aging, which activates the contractile proteins (Bell *et al.*, 2010; Cai *et al.*, 2017). Whatever the process, the pericytes are no longer able to properly regulate the microcirculation of the aging brain (Bell *et al.*, 2010; Cai *et al.*, 2017).

Pericytes in Alzheimer disease

Regarding the pathophysiology of Alzheimer disease (AD), there are two main theories trying to explain the neurodegeneration process: the amyloid-beta cascade hypothesis and the vascular hypothesis. The vascular hypothesis of AD claims that the main cause is age-related chronic brain hypoperfusion (de la Torre, 2018). The inadequate cerebral blood flow is thought to induce an ischemic-hypoxic state in the neuronal cells and therefore a depletion of glucose and oxygen delivery to brain cells, while the beta-amyloid plaques are a by-product of chronic hypoperfusion (de la Torre, 2018). Pericytes, as part of the NVU, take part in cerebral blood flow maintenance (Lachlan S. Brown *et al.*, 2019). AD is associated with a **decrease in the number of pericytes** (Sagare *et al.*, 2013). This leads to an altered cerebral vascular autoregulation, which may induce the ischemic-hypoxic state that favors

the formation of amyloid-beta plaques. However, Klett and colleagues published an interesting study suggesting that pericyte loss is not a general feature in AD, emphasizing the vasculature remodeling that occurs commonly among different brain regions (Fernandez-Klett *et al.*, 2020)

In healthy people, pericytes contribute to beta-amyloid clearance by expressing the **amyloid-beta clearance receptor LRP-1,** which binds and internalizes different amyloid-beta species for lysosomal degradation (Ma *et al.*, 2018). It has been shown that pericyte loss decreases the clearance of amyloid-beta, thus favoring the aggregation of the protein (Sagare *et al.*, 2013; J. Alcendor, 2020).

Pericytes play a role in the **glymphatic system** by regulating the polarization of aquaporin-4 (AQP4), which consists in the distribution of the receptor on the end-feet of astrocytes, an essential process in the function of the glymphatic system (Gundersen *et al.*, 2014; Zheng, Chopp and Chen, 2020). Thus, the loss of pericytes that appears in AD reduces the polarization of AQP4 and alters the clearance of the amyloid beta, enhancing plaque formation (Gundersen *et al.*, 2014; Zheng, Chopp and Chen, 2020). Moreover, the decrease in pericyte number alters the abluminal structure along the capillary bed, which morphologically affects the glymphatic system's CSF drainage (Zheng, Chopp and Chen, 2020). The whole process becomes a vicious circle: the loss of pericytes affects the beta-amyloid clearance, which accumulates as amyloid beta plaques, leading to the death of more pericytes, which further impedes normal beta-amyloid clearance (Zheng, Chopp and Chen, 2020).

The BBB alteration is an important factor in the pathophysiology of AD. It is worth mentioning that homozygous individuals for the **APOE4 gene** have a strong genetic risk for AD. Even though the mechanisms are not yet clear enough, pericytes seem to play important roles (Winkler, Sagare and Zlokovic, 2014). ApoE4 secreted by the astrocytes fails to inhibit the matrix metalloproteinase-9 (MMP-9) pathway, as apoE2 and apoE3 do, resulting in over-expression of MMP-9 in pericytes. In turn, MMP-9 degrades the proteins of tight and adherens junctions from the endothelial cells, leading to a leaky BBB (Winkler, Sagare and Zlokovic, 2014).

Conclusion

Pericytes exhibit structural plasticity during embryonic cerebral development and as structural elements of the NVU and BBB, they play central roles in various processes, from angiogenesis to vascular remodeling and brain microenvironment regulation. Understanding pericyte behavior in the adult brain is a hot topic in neuroscience, as they may play key roles in regenerative processes and the pathogenesis of neurodegenerative disorders. However, their precise identification and sub-classification is challenging, as fluorescence and electron microscopy are still the most accessible technologies to study these cells. Recent advances combining genetic tools with state-of-the art microscopy for lineage tracing experimental designs opened new perspectives in understanding the complex intervention of pericytes in the homeostasis of the brain, as well as their contribution to different types of pathological states.

As future perspectives, the investigation of BBB pericyte plasticity is yet to be consolidated in human studies, especially in terms of neurogenic potential. If such latent potentiality could be activated *in vivo*, beyond its natural, limited ability, it may prove as a useful tool for neurodegenerative diseases. One major limitation is, however, the specific location of pericytes within the NVU. Although they display migratory properties in pathologic circumstances, their tight relationship to the vascular bed could represent a major impairment in relocation of induced neurons into neuronal networks.

List of Symbols and Abbreviations.

Abca9 − ATP-binding cassette sub-family A member 9

ABCC9 − ATP-binding cassette transporter subfamily C member 9

ACE2 – Angiotensin I Converting Enzyme 2

Agtrl1 – Apelin receptor

APOE4 – Apolipoprotein E4

AQP4 – Aquaporin-4

Atp13a5 – Probable cation-transporting ATPase 13A5

Atp1a2 – ATPase Na+/K+ Transporting Subunit Alpha 2

Atp1b2 – ATPase Na+/K+ Transporting Subunit Beta 2

Atp2a3 – ATPase Sarcoplasmic/Endoplasmic Reticulum Ca2+ Transporting 3

BBB – Blood-brain barrier

C1QTNF1 – Complement C1q Tumor Necrosis Factor-Related Protein 1

CADASIL – Cerebral Autosomal Dominant Arteriopathy with Sub-cortical Infarcts and Leukoencephalopathy

CD13 – Alanyl aminopeptidase N

CFTR/MRP – ATP binding cassette, subfamily C

CNS – Central nervous system

COLEC12 – Collectin Subfamily Member 12

CSF – Cerebrospinal fluid

CSPG4 – Chondroitin sulfate proteoglycan 4

DES – Desmin

DLK1 – Delta-like non-canonical Notch ligand 1

EHD2 EH – Domain Containing 2

FHL5 – Four and a Half LIM Domains 5

FOXL1 – Forkhead Box Protein L1

FRZB – Frizzled Related Protein

GEM – GTP-binding protein

GLAST – Sodium-dependent glutamate/aspartate transporter 1

Gli1 – Glioma-associated oncogene

IGFBP2 – Insulin-like Growth Factor Binding Protein 2

IGFBP3 – Insulin-like growth factor-binding protein 3

KIAA0040 – Uncharacterized Protein KIAA0040

Kir 6.1 – Potassium inwardly-rectifying channel subfamily J member 8

LCFAs – Free long-chain fatty acids

LCM – Laser capture microdissection

LepR – Leptin receptor

LPL – Lipoprotein lipase

LRP-1– Amyloid-beta clearance receptor

LRRC32 – Leucine Rich Repeat Containing 32

MMP-9 – Matrix Metalloproteinase-9

MOF – MYST family histone acetyltransferase

MudPIT – Multidimensional protein identification technology

Myh11 – Myosin heavy chain 11

NDUFA4L2 – Mitochondrial Complex Associated Like 2

Nes – Nestin

NG2 – Proteoglycan neuron-glial antigen 2

Notch-3 – Neurogenic locus notch homolog protein 3

NVU – Neurovascular unit

PDGFB – Platelet Derived Growth Factor Subunit B

PDGFRβ – Platelet-derived growth factor receptor beta

PRELP – Proline and Arginine Rich End Leucine Rich Repeat Protein

PTGDR2 – Prostaglandin D2 Receptor 2

RGS5 – G protein signaling 5

Slc12a2 – Solute Carrier Family 12 Member 2

SLC12A7 – Solute Carrier Family 12 Member 7

Slc1a2 – Solute Carrier Family 1 Member 2

Slc4a4 – Neutral amino acid transporter A

SLC6A12 – Solute Carrier Family 6 Member 12

SMOC2 – SPARC Related Modular Calcium Binding 2

sPDGFRβ – Soluble PDGFRβ

SUR2 – Sulfonylurea receptor 2

SVZ – Subventricular zone

Tbx-1 – T box transcription factor

Tbx18 – T-box transcription factor 18

TEM – Transmission electron microscopy

Tem1 – Endosialin

TLR4-NFκB – Toll-like receptor 4-nuclear factor-kappa B

VIM – Vimentin

VSMC – Vascular smooth muscle cell

VTN – Vitronectin

α-SMA – Alpha-smooth muscle actin

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Competing interests/conflict of interests

No competing interests to declare'

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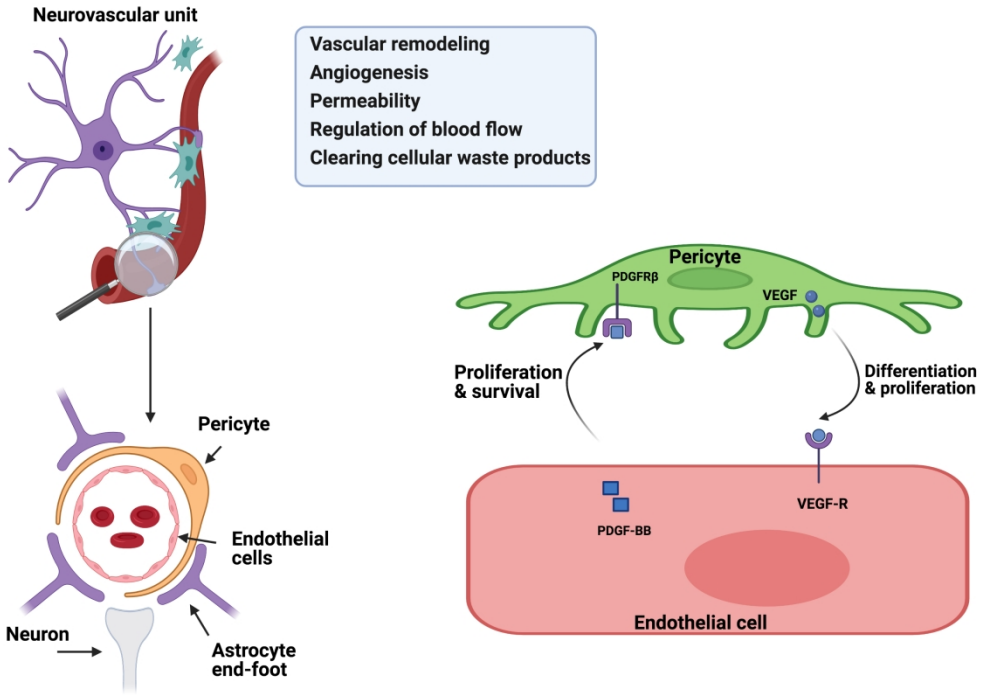
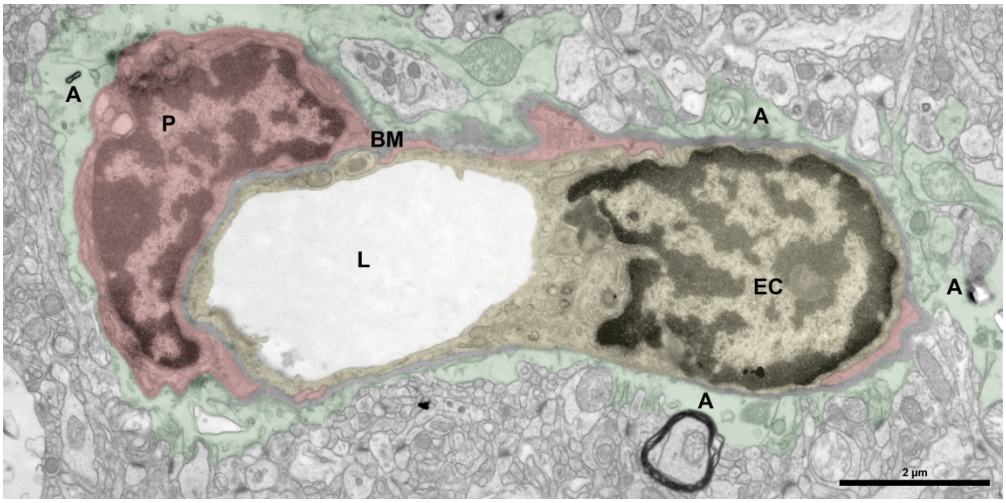


Pericytes and the neurovascular unit. Pericytes regulate the blood-brain barrier and play an important role in vascular remodelling, regulation of blood flow, angiogenesis and vascular permeability



Neurovascular unit in the mouse brain. The pericyte (P) is enclosed by the endothelial cell (EC) basement membrane (BM), which separates it from the surrounding astrocytes (A). The pericyte extends long cell projections embracing the endothelial cell. Digitally colored transmission electron micrograph.