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REVIEW ARTICLE

Astrocytes in the regulation of cerebrovascular functions

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Abstract

Astrocytes are the most numerous type of neuroglia in the brain and have a predominant influence on the cerebrovascular system; they control perivascular homeostasis, the integrity of the blood–brain barrier, the dialogue with the peripheral immune system, the transfer of metabolites from the blood, and blood vessel contractility in response to neuronal activity. These regulatory processes occur in a specialized interface composed of perivascular astrocyte extensions that almost completely cover the cerebral blood vessels. Scientists have only recently started to study how this interface is formed and how it influences cerebrovascular functions. Here, we review the literature on the astrocytes' role in the regulation of the cerebrovascular system. We cover the anatomy and development of the gliovascular interface, the known gliovascular functions, and molecular factors, the latter's implication in certain pathophysiological situations, and recent cutting-edge experimental tools developed to examine the astrocytes' role at the vascular interface. Finally, we highlight some open questions in this field of research.

KEYWORDS

astrocyte, cerebrovascular system, gliovascular interface

1 | GENERAL INTRODUCTION

More than 100 years now, separate Camillo de Golgi and Ramon y Cajal's work on the anatomy of astrocytes and their relationship with cerebral blood vessels from today's studies of these cells' fundamental roles in the regulation of cerebrovascular functions. However, this cellular interface remained unexplored for a very long time. There are several possible explanations for this lack of interest. First, although astrocytes are the most numerous glial cells in the brain, they have long been confined to a secondary, supporting role for neurons. In 2009, Ben Barres entitled his review "Glia—more than just brain glue" and summarized advances in our knowledge of the astrocytes' role in the regulation of synaptic functions (Allen & Barres, 2009). However, his review also illustrated the second reason for a lack of interest in the gliovascular interface: the predominance of studies on the astrocyte's perisynaptic roles (i.e., on the development of synapses and the regulation of synaptic transmission) and the cells' influence on cognitive functions. Finally, the lack of interest in the gliovascular interface also stems from its multicellular composition; in the absence of dedicated experimental tools and models, the functional dialogue between astrocytes and blood vessels was difficult to explore.

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In recent years, there has been a considerable increase in studies of the gliovascular interface. These have revealed the interface's anatomy and emphasized the astrocytes' physiological and pathological roles in the control of perivascular homeostasis and the maintenance of the blood–brain barrier (BBB). These investigations also highlighted the astrocytes' ability to control the entry of blood-borne molecules and immune cells into the brain and to determine their fate. Finally, these studies pinpointed the astrocytes' role in regulating vascular contractility in response to neuronal activity. Here, we provide a critical overview of the field and identify some new topics for future investigation.

2 | THE ANATOMY OF THE GLIOVASCULAR INTERFACE

The vasculature of the central nervous system (CNS) forms a continuous network from pial arteries, penetrating arteries, arterioles, and capillaries to postcapillary venules and veins, the cellular composition and properties of which vary along the vascular bed (Chow et al., 2020; Rungta, Chaigneau, Osmanski, & Charpak, 2018; Saubamea, Cochois-Guegan, Cisternino, & Scherrmann, 2012). Endothelial cells form the core of the vessels, are embedded in a basal lamina and covered by mural cells: pericytes around capillaries, smooth muscle cells around arterioles/arteries and veins. Perivascular fibroblasts are also present around all vessels other than capillaries (Vanlandewijck et al., 2018). Astrocytes are key components of the CNS vasculature. In adults, they completely cover vessels with perivascular astrocytic processes (also referred to as endfeet) (Mathiisen, Lehre, Danbolt, & Ottersen, 2010; McCaslin, Chen, Radosevich, Cauli, & Hillman, 2011) and together with the vascular and perivascular cells, form the gliovascular unit. In the hippocampus, endfeet account for about 4% of the total astrocyte volume (Figure 1). On average, each astrocyte has 3.5 endfeet (range: 1–7) that originate from one or more processes and that wrap around continuous or noncontinuous vessel fragments (Bindocci et al., 2017). This perivascular astrocytic coverage is probably plastic, since laser-ablated endfeet reform in a few days (Kubotera et al., 2019). Along with the endfeet, about a third of astrocyte somata are in direct contact with vessels (Bardehle et al., 2013). This specific subtype of astrocyte is more abundant around arteries and veins than around capillaries (McCaslin et al., 2011).

Astrocytes are functionally and morphologically heterogeneous (Batiuk et al., 2020; Lanjakornsiripan et al., 2018) and this heterogeneity is also observed at the perivascular level. First, astrocyte perivascular coverage is not uniform in the parenchyma but varies according to the position on the vascular tree. At the capillary level, endfeet are in direct contact with the blood vessels, while perivascular spaces filled with cerebrospinal fluid (CSF) separate endfeet from the vessel walls around larger vessels (Abnet, Fawcett, & Dunnett, 1991; Brochner, Holst, & Mollgard, 2015). The length and thickness of endfeet vary. The processes are thicker and contain more glial fibrillary acidic protein (GFAP) around arteries and veins but are thinner and contain less GFAP around capillaries (Cali et al., 2019; McCaslin et al., 2011; Simard, Arcuino, Takano, Liu, & Nedergaard, 2003). Around meningeal vessels and penetrating arteries and veins, endfeet form a dense meshwork of processes called the *glia limitans* covered by a basal lamina. The *glia limitans* forms an additional barrier distinct from the BBB (Balslev, Dziegielewska, Mollgard, & Saunders, 1997). In the context of neuroinflammation, the *glia limitans* can form tight junctions that preclude the passage of blood-borne cells into the parenchyma (Horng et al., 2017). Another specific gliovascular interface is found in the circumventricular organs, which display specific neurosensory and neurosecretory functions. The circumventricular organ's capillaries are fenestrated, which facilitates the exchange of neurohormones and other molecules between the blood and the parenchyma (for a review, see Gabery et al., 2020; Miyata, 2015; Schaeffer et al., 2013). Astrocytes and astrocyte-like cells called tanycytes develop endfeet around some (but not all) of the blood vessels in the CVOs (Morita et al., 2016). In addition, CVO's blood vessel coverage is possibly partial (Benz et al., 2019; Pocsai & Kalman, 2015). Interestingly, these astrocytes/tanycytes display tight junctions at the level of their cell bodies and limit the diffusion of blood-born elements (Langlet, Mullier, Bouret, Prevot, & Dehouck, 2013; Morita et al., 2016; Mullier, Bouret, Prevot, & Dehouck, 2010), as does the BBB in other brain areas. The

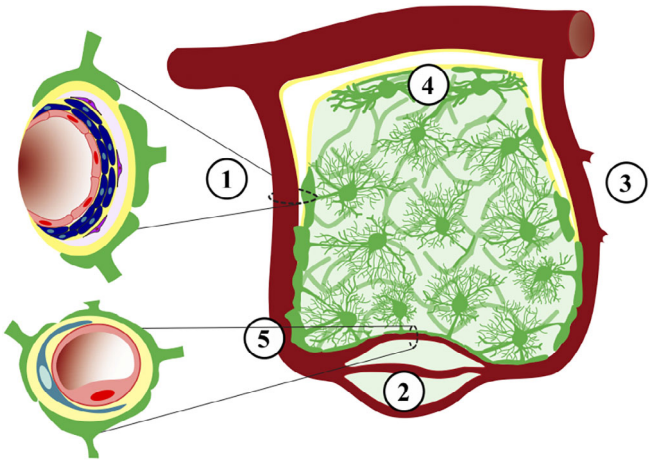

FIGURE 1 The anatomy of the gliovascular interface. Astrocytes cover the whole vasculature of the brain with their endfeet, from penetrating arterioles (1) to capillaries (2) and veins (3). At arterioles/venules, the gliovascular interface is composed of blood–brain barrier (BBB)-forming endothelial cells (ECs), vascular smooth muscle cells (VSMCs) embedded in a first layer of basal lamina (basal lamina, yellow), and fibroblasts. They are surrounded by a perivascular (PV) space filled with cerebrospinal fluid (CSF), a second layer of basal lamina (secreted by astrocytes) and, finally, the endfeet. At capillaries, ECs are surrounded by pericytes. The two basal laminas are fused, and the PV space is absent. Specific astrocytes form the *glia limitans*, where processes form a dense meshwork that separates the parenchyma from the meninges (4). Astrocyte endfeet vary in thickness and size (depending on the type of vessel). An astrocyte can form several endfeet (5). Color code: ECs in red, and VSMCs in dark blue

astrocytes/tanycytes in CVOs are also essential for the integration of signals from the blood or the CSF. For instance, they regulate sympathetic tone as a function of the sodium concentration in the blood (Nomura et al., 2019).

3 | THE DEVELOPMENT OF THE GLIOVASCULAR INTERFACE

The development of the gliovascular interface is a complex, multistep process (Figure 2). In rodents, blood vessels in the brain start to develop around embryonic Day (E) 9. Brain endothelial cells derived from a vascular plexus originating from mesodermal angioblasts first invade the neurectoderm to form intraneural vessels. This angiogenic phase is followed by a differentiation phase at approximately E15, during which mural cells and (in particular) pericytes are recruited to the endothelial surface and induce BBB properties such as tight junction protein expression and the absence of transcytosis (for a review, see Langen, Ayloo, & Gu, 2019; Daneman et al., 2010). A second wave of angiogenesis and BBB maturation occurs postnatally (for a review, see Coelho-Santos & Shih, 2020) (Gilbert, Vidal, Estevez, Cohen-Salmon, & Boulay, 2019; Lunde et al., 2015), suggesting that angiogenesis and barrier genesis are enmeshed processes (Daneman et al., 2009; Daneman et al., 2010; Liebner et al., 2008).

Astrocytes are first generated from radial glial cells in late embryogenesis (around E17.5, in the mouse). Astrocytogenesis continues in neonates—mainly by local division (Ge, Miyawaki, Gage, Jan, & Jan, 2012; Nagao, Ogata, Sawada, & Gotoh, 2016). Recent data have further shown that the developing perinatal cortical astrocytic network is plastic; a dynamic proliferation and spatial dispersion phase (P0–P7) is followed by a maturation phase (P7–P21) characterized by a growing morphological complexity and an increase in the cell volume (Clavreul et al., 2019). At around the time of birth, astrocytes extend polarized processes toward the blood vessels (Ezan et al., 2012; Lunde et al., 2015). It is not known in detail how endfeet develop. A two-step process has been suggested, in which an initial vascular contact is followed by the development of complete coverage (Foo et al., 2011).

4 | THE ASTROCYTES' CONTRIBUTIONS TO CEREBROVASCULAR FUNCTIONS IN THE HEALTHY BRAIN

4.1 | Vascular development and maintenance of the BBB

Angiogenesis and barrier genesis are known to be influenced by radial glial cells and differentiated astrocytes. Conditional deletion of integrin β8 in the neuroepithelium has been shown to alter the differentiation of astrocytes and vascular morphogenesis, resulting in a leaky vasculature (Proctor, Zang, Wang, Wang, & Reichardt, 2005). The link between integrin β8 in radial glial cells and the vasculature might be the activation of extracellular matrix-bound latent transforming growth factor β (TGFβ), which in turn leads to the activation of TGFβ receptors on endothelial cells (Hirota et al., 2015). The TGFβ1 secreted by the radial glial cells might also control vascular morphogenesis (Siqueira, Francis, Gisbert, Gomes, & Stipursky, 2018). The radial glial cells are known to regulate the stability of the vascular network by inhibiting Wingless and Int-1 (Wnt) signaling and the expression of matrix metalloproteinases in endothelial cells (Ma, Kwon, Johng, Zang, & Huang, 2013). Finally, retinoic acid (probably secreted by the radial glial cells) has also been shown to induce BBB differentiation (Mizee et al., 2013).

Astrocyte-secreted Sonic Hedgehog (Shh) has been shown to protect the BBB (Alvarez et al., 2011) but probably only does so under inflammatory conditions, since astrocytes express very low levels of Shh (Vanlandewijck et al., 2018; Zhang et al., 2014). Similarly, astrocytic src-suppressed C-kinase substrate (SSeCKS) was found to regulate BBB integrity after stroke and during BBB maturation (Lee et al., 2003). However, recent transcriptomic data show that the

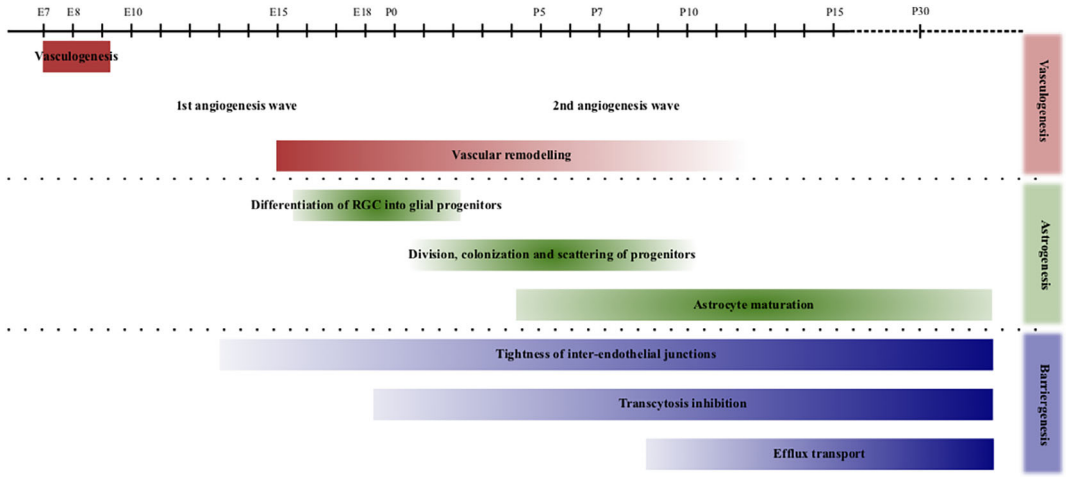

FIGURE 2 Timeline of key developmental processes of the gliovascular interface in the mouse brain

level of SSeCKS expression in astrocytes is much lower than in vascular cells (Vanlandewijck et al., 2018). The role of proteins secreted by mature astrocytes in the development of the brain vasculature has been mainly explored in vitro but has yet to be confirmed in vivo. Nevertheless, astrocytes appear to have an important role in the maintenance of BBB functions in endothelial cells, for instance by maintaining contact with the vessels through the laminins that contribute to the basement membrane (Yao, Chen, Norris, & Strickland, 2014).

Endothelial cells secrete factors such as leukemia inhibitory factor and bone morphogenetic proteins (Imura, Tane, Toyoda, & Fushiki, 2008; Levy, Zayats, Guerrero-Cazares, Quinones-Hinojosa, & Searson, 2014; Mi, Haeberle, & Barres, 2001), which might also induce astrocytes to differentiate—at least in vitro. Nitric oxide production by endothelial cells has been shown to enhance glycolysis in astrocytes in vitro, which suggests that endothelial cells might be involved in the induction of astrocyte-specific metabolic functions (Brix, Mesters, Pellerin, & Johren, 2012). Endothelial cells also induce the polarized expression of the water channel protein aquaporin 4 (Aqp4) in astrocyte endfeet (see chap. 5.1) through both the secretion of factors and mechanical contact with astrocytes (Camassa et al., 2015). Astrocyte-endothelial contact also seems to be necessary for expression of the glutamate transporter GLT-1, which contributes to glutamate uptake—an essential function of mature astrocytes (Lee, Martinez-Lozada, Krizman, & Robinson, 2017). Taken as a whole, these data strongly suggest that the maturation of astrocytes and endothelial cells are co-dependent—especially at postnatal stages. Segarra et al. recently proposed a model in which the endothelial expression of Disabled-1 (Dab1) regulates the astrocytes' expression of integrin-β1, which in turn influences the establishment of mature BBB functions (Segarra et al., 2018).

4.2 | Regulation of fluid and solute movements in the brain

The distribution of ions and metabolites throughout the brain and the removal of waste products requires efficient circulation of CSF and interstitial fluid (ISF) (Figure 3). The ISF is partly secreted by brain cells and the BBB (for a review, see Hladky & Barrand, 2016). The CSF is produced by the choroid plexuses, secreted into the ventricular system, and eliminated by extracranial veins and lymphatic vessels. Following on from pioneering work (Cserr, Cooper, Suri, & Patlak, 1981; Rennels, Blaumanis, & Grady, 1990; Rennels, Gregory, Blaumanis, Fujimoto, & Grady, 1985; for a review, see Abbott, 2004), recent research has evidenced the circulation of CSF and ISF within the perivascular spaces (Hannocks et al., 2018; Iliff et al., 2012). All these studies have found that the *glia limitans* and the endfeet are likely to have roles in the influx/efflux of CSF and ISF—especially with regard to water exchanges.

In the glymphatic model, Aqp4 has been proposed to control perivascular CSF and ISF water exchange (Iliff et al., 2013; Mestre et al., 2018) using several AQP4KO mice (Iliff et al., 2013; Mestre et al., 2018). High polarization of Aqp4 at the perivascular interface

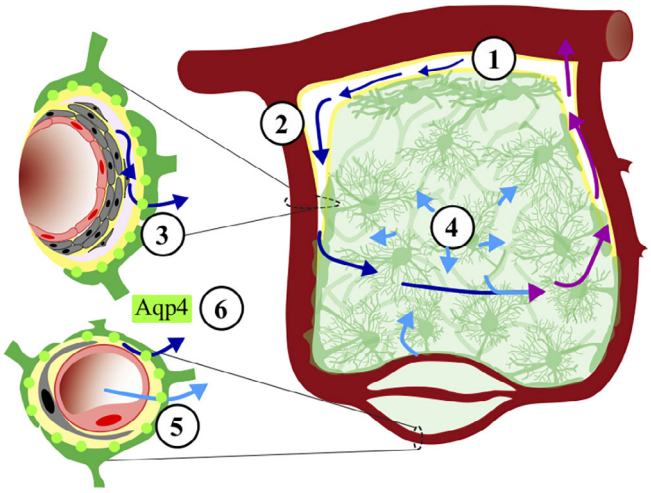

FIGURE 3 The role of astrocyte endfeet in CSF-ISF circulation. Cerebrospinal fluid (CSF, in dark blue) secreted in the ventricles is present in the meninges (1) and recirculates into the parenchyma via the perivascular space (2) and entry across endfeet (3). Interstitial fluid (ISF, in light blue) secreted by brain cells (4) and present at the blood–brain barrier (5) mixes with the recirculating CSF and is drained perivascularly (6). The glymphatic model features unidirectional CSF movement, penetrating the parenchyma via the periarterial space and drained via the venous compartment. In this model, aquaporin 4 (Aqp4) channels in endfeet are important mediating factors for water flux (7)

would favor CSF flux in the parenchyma. Lower amount of Aqp4 in nonperivascular astrocytic membranes would participate to CSF/ISF exchanges. Aqp4 might also be involved in the circulation of various compounds such as amyloid β (Iliff et al., 2012; Ren et al., 2017), lactate (Lundgaard et al., 2017), apolipoprotein E (ApoE) (Achariyar et al., 2016), and adeno-associated virus vectors (Murlidharan, Crowther, Reardon, Song, & Asokan, 2016) mainly in the brain but also in the spinal cord (Wei et al., 2017). Deletion of Aqp4 was shown to alter the CSF flux (Mestre et al., 2020) and the clearance of amyloid β (Iliff et al., 2012) upon ischemia. A correlation between Aqp4 perivascular polarization in endfeet and the parenchymal circulation of CSF has been found during development and aging (Kress et al., 2014; Munk et al., 2019). However, contradicting results regarding Aqp4 contribution have been found (Smith, Yao, Dix, Jin, & Verkman, 2017). It remains to determine whether Aqp4-mediated water flux is uni- or bidirectional. The precise driving mechanisms and directionality of perivascular fluxes are also still subject to debate. In the glymphatic model, influx is periarterial and the efflux is perivenous (Iliff et al., 2012). However, others suggested that the flow is bidirectional (Hannocks et al., 2018). Our knowledge of the astrocytes' and PvAPs' contributions to the fluid's circulation is also incomplete. In particular, it is not clear how solutes pass through the endfeet layer because only passive diffusion is not enough (Korogod, Petersen, & Knott, 2015). These questions are essential to improve the actual models of the fluid circulation, which cannot yet explain all physiological observations (for a review, see Hladky & Barrand, 2018).

4.3 | Immune quiescence and surveillance

The CNS is often considered to be an immune-quiescent organ, with low expression of major histocompatibility complex (MHC) proteins, low numbers of resident immune cells and an anti-inflammatory environment. However, the CSF-filled compartments (i.e., ventricles and meninges) connected to meningeal lymph vessels (Aspelund et al., 2015; Louveau et al., 2015) contain large numbers of peripheral immune cells, including lymphocytes and professional antigen-presenting cells involved not only in classical immune processes but also in cognitive functions (for a review, see Norris & Kipnis, 2019). These compartments are separated from the parenchyma by the *glia limitans*, which impedes the penetration of leukocytes into the parenchyma. The basal lamina secreted by astrocytes is also crucial for restricting lymphocyte access to the parenchyma (Agrawal et al., 2006). Finally, the almost complete absence of peripheral immune cells in the parenchyma is also due to the low degree of endothelial reactivity, that is, low expression levels of leukocyte adhesion molecules. Even though astrocytes are known to have a role in the maintenance of the brain immune quiescence, few studies have highlighted the cells' involvement under physiological conditions. We found that in the absence of overall inflammation and BBB rupture, connexin 43 (Cx43), an astrocyte-specific protein of which high levels are found in endfeet (see Section 5.4.) controls leukocyte recruitment (Boulay, Mazeraud, et al., 2015).

When inflammation occurs, astrocytes actively participate in the innate and adaptive immune responses. By facilitating the circulation of brain fluids (see the previous section), astrocytes drain antigens from the parenchyma into the lymph circuits. The astrocytes also express MHC proteins (to activate infiltrated leukocytes), programmed death-ligand 1 (PD-L1) (to activate T-cell differentiation) (Pavelko, Bell, Harrington, & Dong, 2017:Shwetank, 2019 #241; Prasad et al., 2017), B-cell activating factor and a proliferation-inducing ligand (BAFF and APRIL) (to maintain intracerebral B-cells) (Metcalf, Baxter, Nilaratanakul, & Griffin, 2013; Thangarajh, Masterman, Hillert, Moerk, & Jonsson, 2007) (for a review, see Sofroniew, 2015). Astrocytes can also induce lymphocyte death via the PD-L1 (Schachtele, Hu, Sheng, Mutnal, & Lokensgard, 2014) and Fas/Fas ligand pathways (Bechmann et al., 2002) and by secreting galectin 9 (Steelman, Smith, Welsh, & Li, 2013). At the *glia limitans*, astrocytes express tight junctions and thus block leukocyte penetration (Horng et al., 2017). Astrocytes also induce BBB repair through several mechanisms, such as the release of Shh (Alvarez et al., 2011) and ApoE (Bell et al., 2012). Finally, the formation of a glial scar by astrocytes is also crucial for the repair and maintenance of the BBB because it further restricts the spread of leukocytes and microbial pathogens (for a review, see Sofroniew, 2015).

4.4 | Metabolism and blood flow

Astrocyte interfaces are often represented as distinct, distant entities, with the neuroglial interface on one side and the gliovascular interface on the other. Although most perisynaptic astrocytic processes do not contact blood vessels, endfeet are in contact with both vessels and synapses (Boulay et al., 2017). The proximity between endfeet, vessels and synapses might facilitate the integration of vascular, neuronal and astrocytic signals. Thus, the concept of the gliovascular interface extends to that of the neuro-glio-vascular interface, or more generally, the neurovascular unit.

The astrocytic interface is situated between the blood and the neurons and thus enables metabolites to reach the latter. The brain is the body's organ that consumes the most energy, due to the need to constantly modulate ion concentrations. Glucose (shuttled from the blood to the astrocytes by the transporter GLUT1, where it is converted into lactate) is probably the main energy metabolite for neurons (Pellerin & Magistretti, 1994). This concept was termed the astrocyte-to-neuron lactate shuttle but is still debated. Indeed, in vitro experiments have shown that neurons uptake glucose directly from the extracellular medium (Bak, Schousboe, Sonnewald, & Waagepetersen, 2006). Furthermore, using a fluorescent analog of glucose, it was shown that direct uptake in neurons was more significant than uptake by astrocytes in vivo (Patel et al., 2014). As part of the astrocyte-to-neuron lactate shuttle, astroglial Cx30 and Cx43 (present in large amounts in endfeet; see Section 5.4) might funnel the glucose through the astrocyte gap junction network to sustain neuronal activity (Rouach, Koulakoff, Abudara, Willecke, & Giaume, 2008).

To ensure that neurons receive enough energy to match their high demand, the coupling between blood vessels and neurons increases blood flow to regions of higher neuronal activity during a specific spatiotemporal window—a process referred to as functional hyperemia. This process might be regulated (at least in part) by the endfeet. Interestingly, variation of the cerebral blood flow may also control the intracellular calcium in astrocyte perivascular processes and regulate neuronal activity, suggesting a retrograde vascular to neuronal information processing (Kim, Ramiro Diaz, Iddings, & Filosa, 2016; Moore & Cao, 2008). In 2003, Zonta et al.'s study of juvenile brain slices (Zonta et al., 2003) demonstrated that the glutamate acting on the metabotropic glutamate receptor mGluR5 in astrocytes triggered an increase in intracellular Ca^{2+} levels, cyclooxygenase activation, the release of prostaglandin E2, and arteriolar dilation. In endfeet, large-conductance Ca^{2+}-activated K^+ channels (also known as BK channels) were also shown to increase extracellular K^+ levels by acting on inward-rectifier K^+ (Kir) channels on vascular smooth muscle cells (VSMCs) to dilate arterioles (Girouard et al., 2010). in vivo investigations with the photolysis of caged Ca^{2+} in endfeet confirmed these mechanisms, although the process was slow (from 0.5 to 5 s) (Takano et al., 2006). More recently, fast intracellular Ca^{2+} elevation was detected in vivo in endfeet following artificial whisking (Lind et al., 2018; Lind, Brazhe, Jessen, Tan, & Lauritzen, 2013) or in active mice (i.e., natural whisking) (Tran, Peringod, & Gordon, 2018)—suggesting that the Ca^{2+} response in endfeet might correlate with cerebral blood flow responses. However, it is not clear if and how astrocyte Ca^{2+} dynamics in endfeet shape functional hyperemia (for a review, see Cauli & Hamel, 2018). First, neurovascular coupling

remains intact in mice lacking astrocytic IP3R-dependent Ca^{2+} signaling (Bonder & McCarthy, 2014; Nizar et al., 2013). Second, results vary with the vascular level (i.e., arterioles containing VSMCs vs. capillaries containing pericytes). Mishra et al. (2016) reported that the Ca^{2+} rise in astrocytes was required for the neuron-activity-evoked dilation of capillaries but not arterioles. Third, results vary with the age. In particular, the metabotropic glutamate receptor mGluR5 is not expressed in mature astrocytes (Sun et al., 2013).

5 | KEY COMPONENTS OF THE MOLECULAR REPERTOIRE IN PERIVASCULAR ASTROCYTIC PROCESSES

Astrocytic functions at the gliovascular interface are sustained by a specific but only partially characterized molecular repertoire. From a morphological and functional point of view, the astrocytes are heterogeneous. It is therefore likely that this diversity is also reflected in the astrocytes' perivascular repertoire. Here, we focus on a number of "flagship" markers of endfeet (Figure 4).

5.1 | Aquaporin 4

Is a bidirectional water channel specifically expressed by astrocytes and highly polarized in astrocytic membranes facing the vessels (Nagelhus et al., 1998; Nielsen et al., 1997). Perivascular polarization in the human cortex is a third less pronounced than in the mouse cortex (Eidsvaag, Enger, Hansson, Eide, & Nagelhus, 2017). In mice, immunostaining experiments show that Aqp4 is present around all the vessels and so—probably—in all endfeet (Ezan et al., 2012). However, its density varies along the vasculature, with higher expression levels in endfeet facing pericytes (Gundersen, Vindedal, Skare, & Nagelhus, 2014). Aqp4 is responsible for water movement in the brain and represents the main water influx route in brain edema (for a review, see Amiry-Moghaddam & Ottersen, 2003). The channel may

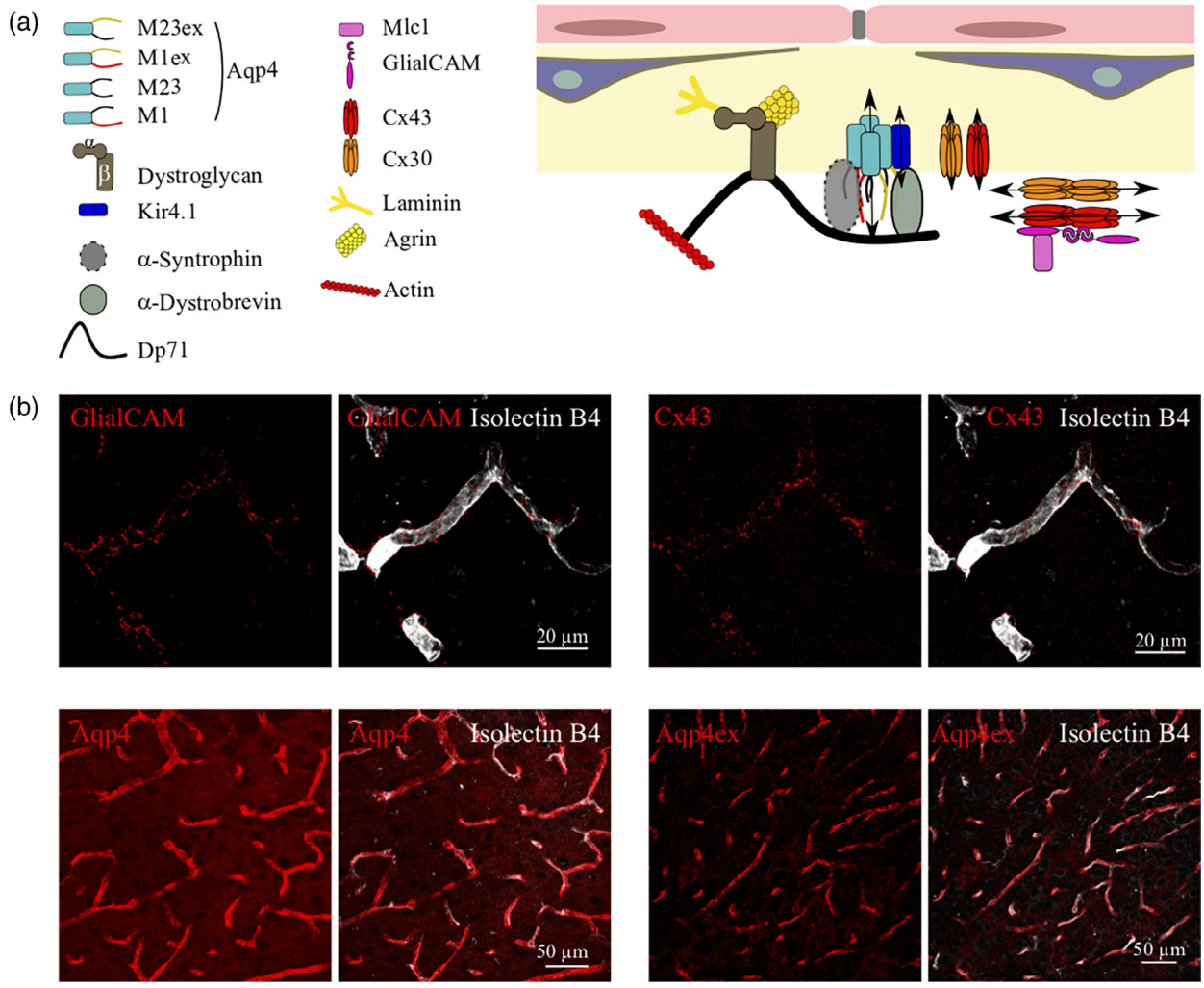

FIGURE 4 The molecular repertoire of endfeet. (a) Schematic drawing of gliovascular interface with "flagship" markers of endfeet. (b) Representative confocal projection images of mouse adult brain slices immunolabeled for GlialCAM, Cx43, Aqp4, and Aqp4ex (all in green). Blood vessel wall are labeled with isolectin B4 (IB4) (gray) on merged images

also contribute to the clearance of ISF (see Section 4.2). At the *glia limitans*, Aqp4 also mediates CSF movement from subarachnoid spaces into the spinal cord parenchyma (Wei et al., 2017). In vitro, Aqp4 can enhance astrocyte process formation and branching (Sato et al., 2018). The latter observation is reminiscent of Nicchia's report that Aqp4 knockdown causes F-actin depolarization and drastic morphological changes in vitro in human and rat astrocytes (but not in mice) (Nicchia et al., 2005).

Aqp4 expression starts around the time of birth in the mouse and between gestational weeks 19–22 in humans (El-Khoury et al., 2006). According to Ezan et al, the mouse Aqp4 anchorage in endfeet develops progressively until P5, when it is detected in all endfeet (Ezan et al., 2012). In contrast, Lunde et al. reported that Aqp4 appeared in endfeet around P7 only (Lunde et al., 2015). Aqp4 continued to accumulate until P21 in endfeet and until P13 in the *glia limitans*—reinforcing the concept of postnatal endfeet maturation (Lunde et al., 2015).

Perivascular astrocytic Aqp4 polarization relies on its anchoring by the dystrophin-associated protein complex (dystrophin-associated protein complex, see below) (Amiry-Moghaddam, Frydenlund, & Ottersen, 2004; Enger et al., 2012; Neely et al., 2001; Noell et al., 2011) and the link between dystrophin-associated protein complex and basal lamina (Noell et al., 2007; Tham, Joshi, & Moukhles, 2016). However, dystrophin/dystrophin-associated protein complex-independent pools of Aqp4 are found in the cerebellum (Nicchia et al., 2008). The organization of the Aqp4 channel in endfeet is very specific. Functional tetramers of Aqp4 are arranged into orthogonal array particles (OAPs) at the membrane (Dermietzel, 1973; Rash, Davidson, Yasumura, & Furman, 2004; Wolburg, 1995). Most of the OAPs are composed of two Aqp4 isoforms transcribed from different initiating methionine (M) start sites and driven by distinct promoters: AQP4a (M1: 32 kDa) and AQP4c (M23: 30 kDa) (Furman et al., 2003). Other cytoplasmic isoforms have been described in vitro (Lisjak, Potokar, Rituper, Jorgacevski, & Zorec, 2017; Lisjak, Potokar, Zorec, & Jorgacevski, 2020). Furthermore, recent studies revealed the existence of Aqp4ex (M1ex, 38 kDa; and M23ex, 35 kDa), a novel Aqp4 isoform accounting for about 10% of all Aqp4 and in which a 29-amino-acid C-terminal extension is generated by translational readthrough (de Bellis et al., 2017; Palazzo et al., 2019). Surprisingly, Aqp4ex is expressed several days later than Aqp4 in the course of development because it is not detected in P9 brain extracts (Sapkota et al., 2019). Aqp4ex is strongly confined to endfeet and, to a lesser extent, the *glia limitans* (Palazzo et al., 2019). In vitro, Aqp4ex modulates the size of OAPs, and the phosphorylation of Aqp4ex's additional C-terminal tail regulates the water channel gating as well as the vesicular trafficking of Aqp4 to the plasma membrane (de Bellis et al., 2017). In a very elegant study, Palazzo et al. generated an Aqp4ex knock-out mice in which the weak stop codon was replaced by a strong one. It was found that the presence of Aqp4ex in the CNS is necessary for anchoring Aqp4 in endfeet. In the Aqp4ex knock-out mice, Aqp4 diffused in astrocyte membranes away from endfeet. Although these results are convincing, the absence of Aqp4ex expression at stages when only Aqp4 is present in endfeet suggests that OAP assembly depends on other mechanisms during endfeet maturation. It is noteworthy that the conformational changes operated by the presence of Aqp4ex in OAPs are targeted by neuromyelitis optica (NMO) autoantibodies (Palazzo et al., 2019).

5.2 | Kir4.1

Is an inward rectifying K^+ channel expressed by astrocytes and oligodendrocytes. In astrocytes, its expression is highly polarized in endfeet and perisynaptic astrocytic processes (Higashi et al., 2001). In perisynaptic astrocytic processes, Kir4.1 allows the rapid uptake of K^+ released into the extracellular space during neuronal activity. This uptake is believed to be coupled to K^+ redistribution via the gap junction-coupled astrocyte network—a mechanism known as spatial buffering (for a review, see Butt & Kalsi, 2006). Kir4.1 channels are responsible for the astroglial membranes' major potassium conductance and are essential for the maintenance of hyperpolarization in mature astrocytes (for a review, see Dallerac, Chever, & Rouach, 2013; Nwaobi, Cuddapah, Patterson, Randolph, & Olsen, 2016). The role of Kir4.1 in endfeet has not been extensively explored. As a weak inward rectifier, the Kir4.1 channel allows K^+ inflow and moderate K^+ outflow. Kir4.1's association with Aqp4 is mediated by the dystrophin-associated protein complex (see below) and is believed to be functionally coupled to Aqp4 for coordinated water and K^+ flux in the water/volume regulation of astrocytes (Amiry-Moghaddam et al., 2004; Holthoff & Witte, 2000). Although pioneering work on neurovascular coupling in the retina failed to identify the prominent role of this channel in neuronal activity-mediated arteriolar vasomotricity (Metea, Kofuji, & Newman, 2007), this channel is still viewed as a putative component in the mechanism of neurovascular coupling (Nippert, Biesecker, & Newman, 2018). Kir4.1 is weakly detected at P7 and its level progressively rises until P60 (Lunde et al., 2015). In the mouse cortex, Kir4.1's localization in endfeet starts at around P7-P14 (Moroni, Inverardi, Regondi, Pennacchio, & Frassoni, 2015). The mechanisms involved in K^+ homeostasis in the perinatal period have not been characterized.

5.3 | The dystrophin-associated protein complex

Is an assembly of proteins that physically links the endfeet's intracellular actin cytoskeleton to the perivascular membrane and the extracellular matrix. The core functional unit of the dystrophin-associated protein complex is constituted by α-dystroglycan, β-dystroglycan, and dystrophin. Dystrophin is associated with the intracytoplasmic proteins α-syntrophin and α-dystrobrevin, which bind to Aqp4 and Kir4.1. The interconnection of these proteins implies that the absence or mislocation of one member of the dystrophin-associated protein complex will alter the perivascular location of Aqp4 and Kir4.1—thus compromising perivascular water and potassium homeostasis. Interestingly, and as also suggested for Aqp4, the dystrophin-associated protein complex might also influence astrocyte process formation

(Sato et al., 2018). The developmental data concerning the dystrophin-associated protein complex and the way it progressively assembles in endfeet are difficult to reconcile because of the diversity of models and tools used. However, most studies indicate a progression from the early postnatal stages onward—indicating that the postnatal expression of dystrophin-associated protein complex proteins is involved in endfeet maturation.

The dystroglycan complex is composed of two protein subunits translated from a single mRNA transcript of the *Dag1* gene (Ibraghimov-Beskrovnaya et al., 1992). The α-subunit (α-dystroglycan) resides at the outer surface of the plasma membrane and interacts with the membrane-spanning β-subunit (β-dystroglycan) (Akhavan, Crivelli, Singh, Lingappa, & Muschler, 2008; Holt, Crosbie, Venzke, & Campbell, 2000). In astrocytes, the dystroglycan complex links the laminin of the basal lamina to specialized membrane domains within endfeet. This interaction has a key role in tethering Aqp4 in the endfeet (Noell et al., 2011). A recent study found that the endfeet of Nestin-positive differentiating astrocytes of the forebrain contained β-dystroglycan as early as E20, which might designate β-dystroglycan as the earliest known marker of endfeet (Kalman, Oszwald, & Adorjan, 2018). However, these results contradict a previous study in which β-dystroglycan in endfeet was only detectable from P7 onward (Lunde et al., 2015).

Dystrophin (Dp) is encoded by *Dmd*, one of the longest known genes; it comprises 79 exons and has at least 7 different promoters. In the dystrophin-associated protein complex, dystrophin mainly consists of Dp71d isoforms (promoter between Exons 62 and 63), which correspond to the major isoform type (around 80%) in the adult brain (Aragon et al., 2018). The expression profile of Dp71 has been studied in the mouse hypothalamic supraoptic nucleus, where it first appears at birth and progressively increases until P60. However, the precise moment of its anchorage in endfeet membranes in this brain structure has not been determined (Souttou et al., 2019). Dystrophin's role in the brain has been studied in vivo using the X linked muscular dystrophy 3, Verne Chapman (mdx3cv) mouse model, in which all dystrophin isoforms are inactivated (Chapman, Miller, Armstrong, & Caskey, 1989). In hypothalamic nuclei, this inactivation alters the perivascular localization of α-syntrophin (Benabdesselam et al., 2012). In Müller cells of the retina, the specific deletion of Dp71 affects astrocyte morphology and Aqp4 and Kir4.1 clustering in perivascular astrocytic membranes; in turn, this results in defective osmoregulation, increased permeability of the blood-retina barrier, and sensitivity to edema (Dalloz et al., 2003; Giocanti-Auregan et al., 2016; Sene et al., 2009). Interestingly, inactivation of Dp71 (normally expressed from P6 onward in Müller cells) also alters postnatal angiogenesis (Giocanti-Auregan et al., 2016). Although these observations were performed in the retina, they suggest that in addition to endothelial cells, the astrocytes' perivascular properties (such as the assembly of the dystrophin-associated protein complex) influence the postnatal maturation of the perivascular network.

The syntrophins constitute a family of five adaptor proteins (α, β1, β2, γ1, and γ2) that link signaling proteins to the dystrophin-associated protein complex (Albrecht & Froehner, 2002). α-syntrophin is the prevalent endfeet isoform; it is present at higher densities in membrane domains facing pericytes (Gundersen et al., 2014). Its depletion does not affect expression patterns of laminin, agrin, and β-dystroglycan but does cause the loss of, respectively, 79 and 94% of Aqp4 in the endfeet of the spinal cord and cortex (Hoddevik et al., 2017; Neely et al., 2001). α-Syntrophin is therefore probably the most important factor known to determine the size of the endfeet's Aqp4 pools (Hoddevik et al., 2020). Nevertheless, in terms of development, α-syntrophin is probably not involved in the development of the dystrophin-associated protein complex and Aqp4 endfeet anchorage, as it appears only at around P13 in mouse cortical endfeet—after the dystroglycan complex and Aqp4 (Ezan et al., 2012; Lunde et al., 2015).

The dystrobrevins (DBs) are a family of proteins that interact directly with dystrophin and the syntrophins. Two genes (*DntA* and *DntB*) encode α- and β-dystrobrevin, respectively. α-Dystrobrevin is present in endfeet and endothelial cells in the cerebellum (Bragg, Amiry-Moghaddam, Ottersen, Adams, & Froehner, 2006). Although the development of α-dystrobrevin expression in endfeet has not been characterized, immunofluorescence labelings in cortical regions suggest that α-dystrobrevin is absent at the vascular level at P2 but present at P9 (Lien, Vlachouli, Blake, Simons, & Gorecki, 2004). In the latter report, however, the cellular location of the immunolabeling was not specified. The absence of α-dystrobrevin disrupts the perivascular localization of Aqp4 and Kir4.1 in the hippocampus and cerebellum (Bragg, Das, & Froehner, 2010; Lien et al., 2012).

5.4 | Astroglial connexins Cx30 and Cx43

Are the main astroglial Cxs and are strongly concentrated in endfeet. They assemble in sixes into hemichannels to form gap junction channels between adjacent endfeet; hence, they mediate direct cell-to-cell diffusion of ions and small signaling molecules (<1.2 kDa) and provide electrical and metabolic coupling (Giaume, Koulakoff, Roux, Holcman, & Rouach, 2010). Cx30 and Cx43 can be mixed within heterotypic or heterodimeric channels (Koval, Molina, & Burt, 2014). The exact combination of Cxs in endfeet is unknown. However, inactivation of Cx30 or Cx43 abolishes only half the astrocytic coupling—indicating the existence of Cx30- and Cx43-homomeric channels (Rouach et al., 2008). The Cx hemichannels are not only involved in gap junctions but can also persist in an undocked conformation to mediate the direct exchange of molecules between the intracellular and extracellular milieus (Saez & Leybaert, 2014). This hemichannel activity has mainly been described in the context of disease. However, recent studies suggest that hemichannel activity also regulates the normal brain (Chever, Lee, & Rouach, 2014; Ghezali et al., 2020). Finally, Cxs have channel-independent functions, involving protein interactions and intracellular signaling (Elias, Wang, & Kriegstein, 2007; Giepmans, 2004; Pannasch et al., 2014). Through its intracellular carboxy-terminal tail, Cx43 interacts with multiprotein complexes linked to the cytoskeleton, and contributes in vitro to cell polarity, morphology, migratory activity, and adhesion

(Giepmans, 2004; Herve, Derangeon, Sarrouilhe, Giepmans, & Bourmeyster, 2012; Olk, Zoidl, & Dermietzel, 2009; Xu, Francis, Wei, Linask, & Lo, 2006). In vivo, nonchannel Cx30 controls the activity-dependent remodeling of distal astroglial processes (Ghezali et al., 2020; Pannasch et al., 2014). Although Cx30 and Cx43 are mainly concentrated in endfeet and in the *glia limitans*, their role at the vascular interface has been less well investigated than their perisynaptic function. In mice, level of Cx30 rises in astrocytes from P10 onward and the protein concentrates at the perivascular level after P12 (Ezan et al., 2012; Simard et al., 2003). In contrast, the accumulation of Cx43 in endfeet starts at P2 (Ezan et al., 2012), increases until P15, and remains stable thereafter (Gilbert et al., 2019). The Cx30- and Cx43-containing perivascular gap junction channels have been shown to distribute glucose throughout the perivascular astroglial network, thereby sustaining neuronal activity (Rouach et al., 2008). Dual knock-out of Cx30 and Cx43 uncouples astrocytes in the hippocampus, reduces water transport across astrocytic plasma membranes, and increases the leakiness of the BBB upon shear stress (Ezan et al., 2012; Lutz et al., 2009). This dual knock-out is accompanied with the partial loss of β-dystroglycan and Aqp4M23 (but not Aqp4M1 and Aqp4ex) (Ezan et al., 2012; Katoozi et al., 2020). Taken individually, each astroglial connexin has very distinct functions at the gliovascular interface. The absence of Cx30 regulates the transcription level of γ-sarcoglycan (probably in VSMCs) but does not affect BBB integrity (Boulay, Mazeraud, et al., 2015; Vanlandewijck et al., 2018). In contrast, Cx43 regulates BBB immune quiescence and resistance to shear stress. Upon Cx43 inactivation, astrocytes adopt an atypical reactive status with no change in most canonical astrogliosis markers but upregulation of molecules promoting immune recruitment, such as the chemokine (C-C motif) ligand 5 (Ccl5), the C-X-C motif chemokine ligand 10 (Cxcl10) and the programmed cell death1 ligand 1 (CD274) (Boulay et al., 2018). Consistently, the continuous recruitment of peripheral immune cells into the brain parenchyma is observed in astroglial Cx43-knock-out brains (Boulay, Mazeraud, et al., 2015), followed by an autoimmune response against the brain extracellular matrix protein Von Willebrand Factor A Domain Containing 5A (Vwa5a) (Naba et al., 2012) (Boulay, Mazeraud, et al., 2015; Boulay, Saubamea, Decleves, & Cohen-Salmon, 2015). In parallel with this inflammation, anti-inflammatory mechanisms (such as the astrocytes' upregulation of the complement decay factor CD55 or the expression of IL10 by recruited immune cells) may explain why the continual immune recruitment is not associated with brain lesions. These results indicate that astroglial Cx43 is an important factor in the control of astrocyte immunoregulation at the vascular interface.

Recent research indicates that in addition to Cx43 and Cx30, Cx26 is also expressed in endfeet (Boulay et al., 2017) and the *glia limitans* (Lynn, Tress, May, Willecke, & Nagy, 2011). It is noteworthy that Gjb2 and Gjb6 (coding, respectively, for Cx26 and Cx30) are contiguous genes. The original deletion model for Cx30 included common regulatory elements leading to the co-inactivation of Cx30 and Cx26 (Boulay et al., 2013; Teubner et al., 2003). One should therefore be extremely cautious when analyzing data generated in this model.

5.5 | The MLC1/GlialCAM complex

Megalencephalic leukoencephalopathy with subcortical cysts-1 (MLC1) and glial cell adhesion molecule (GlialCAM) are both membrane proteins forming a junctional complex (Lopez-Hernandez, Sirisi, et al., 2011) between endfeet (Hoegg-Beiler et al., 2014; Teijido et al., 2007). Mutations in the associated genes are associated with a rare type of leukodystrophy: megalencephalic leukoencephalopathy with subcortical cysts, characterized by progressive white matter vacuolation, slowly progressive ataxia, spasticity, and cognitive decline (van der Knaap, Boor, & Estevez, 2012). MLC1 is specifically expressed in astrocytes, including the Bergmann glia of the cerebellum. High expression levels are found in perivascular astrocytes (Toutounchian & McCarty, 2017). GlialCAM is an adhesion molecule from the immunoglobulin superfamily expressed predominantly in astrocytes and oligodendrocytes (Chung Moh, Hoon Lee, & Shen, 2005; Favre-Kontula et al., 2008) and forms a complex with MLC1; both proteins are necessary for localization of the complex at endfeet junctions (Lopez-Hernandez, Ridder, et al., 2011; Hoegg-Beiler et al., 2014). The MLC1/GlialCAM complex's exact function at the gliovascular interface is still unknown, although several datasets point toward a role in perivascular ion and water homeostasis. Mice lacking MLC1 or GlialCAM display disturbed brain potassium dynamics (Dubey et al., 2018) and endfeet swelling (Bugiani et al., 2017; Dubey et al., 2015). The mechanisms behind these defects have not been defined but may be linked to the fact that GlialCAM is an auxiliary subunit of the inward rectifier chloride channel ClC-2 and so might be necessary for the channel's membrane targeting and function at the gliovascular interface (Bugiani et al., 2017; Hoegg-Beiler et al., 2014; Jeworutzki et al., 2012; Lopez-Hernandez, Ridder, et al., 2011; Sirisi et al., 2017). Additional studies have shown that the MLC1/GlialCAM complex indirectly regulates the TRPV4 and LRRC8 ion channels (Elorza-Vidal et al., 2018; Estevez et al., 2018). These data were mostly obtained in vitro and therefore require in vivo confirmation. Indeed, the channels' expression profiles in endfeet have not been well characterized. LRRC8a (the main astroglial LRRC8 isoform) might be predominantly expressed in endfeet surrounding cortical arteries (Formaggio et al., 2019), and ClC-2 might only be expressed by a subtype of astrocytes (Benesova et al., 2012). Finally, TRPV4 is not expressed in mouse astrocytes in vivo (Pivonkova et al., 2018; Valiente et al., 2014; Vanlandewijck et al., 2018). It has been suggested (but not confirmed) that the MLC1/GlialCAM complex interacts with the dystrophin-associated protein complex, Aqp4 and Kir4.1; no effect on the localization of these proteins was detected in the mouse knock-out models (Bugiani et al., 2017; Hoegg-Beiler et al., 2014). Apart from ion homeostasis, MLC1 regulates in vitro membrane actin dynamics (Hwang, Vu, Kim, & Lim, 2019) and signaling cascades (Lanciotti et al., 2016). In vitro, both GlialCAM and MLC1 influence the turnover of Cx43 at the membrane (Helms et al., 2016; Lanciotti et al., 2020). Again, all these effects require in vivo confirmation. The expression of MLC1 and GlialCAM in the brain starts a few days after birth and increases until P21 (Bugiani et al., 2017; Dubey et al., 2015). A recent study of endfeet showed

that GlialCAM expression precedes MLC1 expression and that levels of both increase in formed, Aqp4-expressing endfeet between P10 and P15 (Gilbert et al., 2019). These results suggest that the MLC1/GlialCAM complex is a marker of astrocyte endfeet maturation.

5.6 | Laminins

Are major components of the basal lamina and serve as ligands for receptors such as integrins (del Zoppo & Milner, 2006; Sixt et al., 2001) and the dystroglycan complex (Moore et al., 2002). They are heterotrimeric molecules composed of α, β, and γ chains. Laminins are produced by all the cells of the gliovascular interface and together form a core basal lamina component. The only brain areas where astroglial and vascular laminins do not interact and separate basal laminas can be observed are the Virchow-Robin spaces, where endfeet are morphologically separated from the vessel surface (Figure 1). This partly explains why astroglial laminins have not yet been well defined. Laminin α2 is expressed in astrocytes in general (Vanlandewijck et al., 2018; Zhang et al., 2014) and in endfeet in particular (Sixt et al., 2001). In contrast, laminin β1 (typically detected in "parenchymal basement membranes") (Sixt et al., 2001) is not expressed by astrocytes (Vanlandewijck et al., 2018; Zhang et al., 2014). Laminin γ1 (encoded by *Lamc1*) is mainly expressed by pericytes (Vanlandewijck et al., 2018). However, its function was investigated in astrocytes by using Nestin-promoter-driven deletion, which therefore also impacted neural precursor cells (Chen et al., 2013). The absence of laminin γ1 in astrocytes was found to affect several properties of the gliovascular interface (BBB integrity, VSMC/endfeet contact, and expression of VSMC and pericyte contractile proteins) but only in the striatum and thalamus (Chen et al., 2013). It is noteworthy that inactivation of *Lamc1* also leads to lower levels of laminin α1 and α2 in the gliovascular interface, indicating that impairment of the gliovascular interface might not be solely due to the specific absence of laminin γ1. The same laboratory produced a twin article in the same year and described other molecular alterations related to the absence of laminin γ1 in astrocytes. First, levels of Aqp4 were abnormally low in endfeet. On the same lines, treatment of primary astrocytes with laminin γ1 antibodies has been shown to inhibit the clustering of Aqp4 and β-dystroglycan (Noel, Tham, MacVicar, & Moukhles, 2020). Second, a higher level of Pdgfrβ expression in pericytes suggested the presence of differentiation defects in these cells; this has not, however, been observed in all laminin γ1 inactivation models (Yao et al., 2014). Although further work is needed to fully understand these results, they suggest that astroglial laminins are involved in pericyte and VSMC differentiation. These results also emphasize the need to better characterize the astroglial laminins' developmental expression profile and their localization in the brain.

5.7 | Agrin

Is an heparan sulfate proteoglycan and another important component of the basal lamina. It is ubiquitously expressed by the cells of the gliovascular interface. Agrin's role in astrocytes has mainly been investigated in vitro. This proteoglycan exerts a strong effect on the clustering of β-dystroglycan and (possibly) Aqp4 (Noel et al., 2020). However, these findings contradict a previous analysis of primary astrocytes derived from agrin knock-out mice in which no effect on OAP densities was found in a freeze-fracture analysis (Noell, Fallier-Becker, Deutsch, Mack, & Wolburg, 2009). In contrast, an analysis of astrocyte membranes in vivo at E19.5 showed a drastic reduction in OAPs (Noell et al., 2009). These observations indicate that the polarized distribution of OAPs requires the presence of agrin (probably provided by the vascular compartment) in the basal lamina. As is the case for laminins, further work is needed to characterize the agrin isoforms provided by astrocytes.

5.8 | Local translation and the identification of new markers for perivascular astrocytic processes

Our knowledge of the molecular repertoire of endfeet is still partial. Most studies to date have focused on the most prevalent molecular markers mentioned above. A novel way of further exploring this repertoire was recently highlighted by the discovery of local translation in endfeet. As is the case for most morphologically complex cells, mRNAs are present in astrocyte processes and are locally translated. Some of these RNAs (such as those coding for Aqp4 or Kir4.1) are more extensively translated in the endfeet than in the soma (Boulay et al., 2017). This molecular repertoire—referred to as the "endfeetome"—probably constitutes a reservoir of novel markers of endfeet and warrants further investigation.

6 | THE GLIOVASCULAR INTERFACE IN BRAIN DISEASES

Since astrocytes represent an essential functional crossroads in the brain, their vascular interfaces are involved in many brain diseases. Below, we summarize the literature data on the most extensively studied diseases in this field.

6.1 | Alzheimer's disease and Parkinson disease

In Alzheimer's disease (AD), astrocytes become reactive, undergo drastic morphological and molecular changes, release inflammatory factors, and thus contribute to cellular damage at the gliovascular interface (for a review, see Arranz & de Strooper, 2019; Ben Haim, Carrillo-de Sauvage, Ceyzeriat, & Escartin, 2015). Several studies have demonstrated that the anatomy of the gliovascular interface is altered during AD, with endothelial cell and pericyte degeneration, reduction of endfeet coverage, capillary distortions, BBB abnormalities, and thickening of the basal lamina (Montagne et al., 2015; Montagne et al., 2016; Montagne, Zhao, & Zlokovic, 2017; van de Haar et al., 2016). In endfeet, Aqp4 loses its polarization at amyloid

deposition sites (Yang et al., 2011). In capillary cerebral amyloid angiopathy (CAA), the amyloid deposits can form a ring around the vessel; this separates the endfeet from the endothelial vessel wall and alters the astrocytic Ca^{2+}-mediated hyperemic responses (Kimbrough, Robel, Roberson, & Sontheimer, 2015). CAA also causes the loss of Kir4.1 and dystrophin expression, which in turn perturbs potassium homeostasis and the mechanical and functional links between astrocytes and other cellular components of the gliovascular interface (Wilcock, Vitek, & Colton, 2009). A recent human transcriptomic study demonstrated that AD is also associated with changes in the mRNAs encoding prominent endfeet proteins, such as dystroglycans, dystrobrevin, α-syntrophin, and MLC1 (Simon et al., 2018). Other astrocyte-driven changes in vascular functions in AD have been reported. In CAA, a high level of angiopoietin-like 4 (ANGPTL4) has been found in reactive astrocytes close to vessels (Chakraborty et al., 2018). In vitro, ANGPTL4 stimulates endothelial cell migration and sprouting, and may thus act as a potent angiogenic mediator contributing to a pathological vascular remodeling. Since ANGPTL4 levels are significantly higher in plasma from patients with vascular dementia, this marker might be of value for diagnosing CAA (Chakraborty et al., 2018). ApoE is an astrocyte protein that acts as a cholesterol and phospholipid transporter and is required for integrity of the BBB (Hafezi-Moghadam, Thomas, & Wagner, 2007). Three main isoforms (E2, E3, and E4) are found in humans. In vitro, APOE4 regulates TJ integrity (Nishitsuji, Hosono, Nakamura, Bu, & Michikawa, 2011). However, in mutant mice in which Apoe is replaced by the human APOE4, the latter promotes BBB disruption by activating a pro-inflammatory CypA—nuclear factor—matrix-metalloproteinase-9 (MMP-9) pathway in pericytes (Bell et al., 2012; Halliday et al., 2016). These results are consistent with the 10-fold greater risk of developing late-onset AD in homozygous E4 individuals. In addition to affecting BBB integrity (Montagne et al., 2020), ApoE4 also influences the astrocyte's glucose metabolism in general and lactate synthesis in particular, which may cause an imbalance in the astrocyte–neuron lactate shuttle (Williams et al., 2020). Low expression of the astrocytic glucose transporter GLUT1 at the gliovascular interface has been found in postmortem AD brain samples (Simpson & Davies, 1994), and low expression of GLUT1 and lactate transporters has also been reported in transgenic arctic β-amyloid mice expressing human amyloid precursor protein (Merlini, Meyer, Ulmann-Schuler, & Nitsch, 2011). These changes in the astrocytes undoubtedly compromise the brain's metabolism.

As in AD, Parkinson's disease (PD) is associated with cerebrovascular alterations; indeed, the term "vascular parkinsonism" has been suggested (Jellinger, 2003; Kummer et al., 2019). Interestingly, the blockade of astrocyte reactivity might be protective in mouse models of PD (Yun et al., 2018). With regard to endfeet markers, it was recently shown that injection of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine into the mouse striatum (which reproduces PD neurodegeneration) upregulates Cx30 and Cx43 expression, whereas Cx30 deficiency attenuates A2 astrocyte responses and gives a protective phenotype; these findings suggest that Cx30-mediated functions are neuroprotective in this context (Fujita et al., 2018).

6.2 | Stroke

The astrocytes' perivascular functions (such as BBB maintenance and immunoregulation) are strongly modified in stroke, leading to ischemia, neuroinflammation, edema, and intracerebral bleeding. After the onset of stroke, astrocytes become reactive following the release of damage-associated molecular patterns released by neurons and other glial cells. The reactive astrocytes secrete pro-inflammatory cytokines, chemokines, and MMPs, such as MMP-9, which subsequently disrupt the BBB and recruit leukocytes from the peripheral blood (Pekny, Wilhelmsson, Tatlisumak, & Pekna, 2019). Reactive astrocytes also secrete inflammatory cytokines (e.g., IL-15), which recruit $CD8^+$ T cells and natural killer cells and thereby aggravate the brain lesions (Li et al., 2017; Shi et al., 2020). A recent study using the middle cerebral artery occlusion (MCAO) model suggested that CD147 (EMMPRIN/basigin), an extracellular MMP-inducer that induces MMP activity and promotes leukocyte extravasation and inflammation (Agrawal & Yong, 2011) is produced by astrocytes during stroke (Patrizz et al., 2020). With regard to the astrocyte endfeet repertoire, Aqp4 expression increases during edema and ischemia. Hence, inhibition of this increase might prevent and/or mitigate edema (Tang & Yang, 2016; Zheng et al., 2017). Aqp4 polarization is lost after MCAO in mice (Frydenlund et al., 2006). In contrast, levels of Cx43 rise in the peri-infarct area several days after MCAO in mice. Interestingly, the inhibition of Cx43 hemichannel activity decreases infarct volume and improves behavioral performance (Chen et al., 2019; Freitas-Andrade et al., 2019; Kozoriz et al., 2010). Together, these studies indicate that Aqp4 and Cx43 are potential therapeutic targets. Astrocytes exert also compensatory functions in the event of stroke. Following MCAO, they secrete pentraxin 3 (Shindo et al., 2016), which (in vitro, at least) increases the expression of endothelial TJ proteins. Pentraxin 3's effects might be linked to its ability to bind and inhibit vascular endothelial growth factor (VEGF), a key regulator of endothelial permeability during stroke and an inhibitor of FGF2- and FGF8-mediated angiogenesis (Rusnati et al., 2004). The in vivo role of astrocytic pentraxin 3 needs to be determined. Another recently described post-stroke astrocytic mechanism contributing to the brain repair is the expression of SorCS2, a member of the VPS10P domain receptor family that is usually expressed solely by neurons. SorCS2 expression was found to be upregulated in astrocytes following ischemia; the protein might control the secretion of endostatin and thus promote angiogenesis (Malik et al., 2020).

6.3 | Multiple sclerosis

The loss of astrocyte functions at the vascular interface is a component of the disease mechanism in multiple sclerosis (MS). The endfeet's vascular coverage is altered in patients and in animal models of MS (Eilam et al., 2018; Prineas & Lee, 2019). Notably, the endfeet's Aqp4 and Cx43 polarity is lost (Fournier et al., 2018; Masaki, 2015; Masaki et al., 2013; Prineas & Lee, 2019; Sharma et al., 2010). Importantly, this change in Aqp4 polarization occurs at sites of leukocyte

infiltration and is associated with perturbation of the glymphatic circulation in the spinal cord (Fournier et al., 2018). Given that Aqp4 knock-out impairs the systemic and intracerebral synthesis of transforming growth factor-beta 1, loss of Aqp4 might also contribute to neuroinflammation (Xue et al., 2019). During MS, astrocytes secrete various factors that damage or protect the BBB by directly influencing the level of endothelial TJ expression and thus BBB integrity. Higher levels of VEGF and lower levels of Shh impair BBB integrity, activate the endothelium, and prompt the recruitment of cytotoxic immune cells in the brain parenchyma (Alvarez et al., 2011; Argaw et al., 2012). The expression and secretion of cytokines and chemokines by astrocytes influence immune recruitment and immune cell fate (Rothhammer & Quintana, 2015). In contrast, the formation of tight junctions between endfeet restricts immune recruitment (Horng et al., 2017); interestingly, certain antigenic targets of autoimmunity in MS and in NMO (also known as Devic's disease) are strongly expressed in endfeet. In NMO, the binding of anti-Aqp4 IgG activates the complement cascade and leads to astrocyte lysis (Hinson et al., 2012). The resulting displacement of Aqp4 from the endfeet might initiate an inflammatory cascade leading to BBB weakening, leukocyte recruitment, oligodendrocyte apoptosis, myelin loss, and neuronal loss (Pittock & Lucchinetti, 2015). Interestingly, Aqp4ex (the extended Aqp4 isoform produced by translational readthrough (Palazzo et al., 2019), which drives the assembly of OAPs in endfeet) is crucial for the binding of NMO-IgG to OAPs (Palazzo et al., 2019). Finally, about half of all MS patients have serum autoantibodies against the extracellular loop of Kir4.1 (Srivastava et al., 2012). Although these antibodies' immunopathogenic role remains to be established, they may well impair oligodendrocytes and myelination, and prompt neuronal degeneration (as observed in Kir4.1 knock-out mice).

6.4 | Cancer

The vasogenic edema and high ISF pressure related to prominent alterations of the BBB can be a major cause of morbidity in patients with brain tumors and are especially prevalent in patients with glioblastoma (the most aggressive malignant primary tumor in adults). Glioblastomas are characterized by hypoxic and necrotic features and angiogenesis, which make them particularly vascularized tumors. The neovessels have been described as swollen, highly branched, and permeable (Jain et al., 2007); (Ahir, Engelhard, & Lakka, 2020). Most of the neovessels collapse, leaving expanded perivascular spaces surrounded by a basal lamina that directly faces glioblastoma cells (Noell et al., 2012). The gliovascular interface is altered, with a loss of endfeet and changes in the basal lamina (Lee et al., 2009). Consequently, the BBB is physically replaced by a permeable “blood-tumor barrier” in the tumor core. The degree of BBB damage varies, however; in many regions, the BBB is preserved and thus limits the delivery of therapeutic drugs to all tumor cells (for a review, see Stathias et al., 2018).

Glioma cells alter the gliovascular interface, with a loss of endfeet and changes in the basal lamina (Lee et al., 2009). Disruption of the gliovascular interface is also observed in peritumoral tissues (Engelhorn et al., 2009). Peritumoral glioma cells migrate along vessels and thus colonize the brain (for a review, see Cuddapah, Robel, Watkins, & Sontheimer, 2014; Gritsenko, Leenders, & Friedl, 2017). In doing so, they leave the tumor mass and thus accentuate the likelihood of secondary tumors or recurrence after surgery. This perivascular migration is also be associated with greater vascular permeability and major alterations of the gliovascular interface. Indeed, glioma cells intercalate between endfeet and blood vessels and thus disrupt the vascular cells' ability to respond to vasoactive molecules released by endfeet. Neurovascular coupling is impaired (Watkins et al., 2014). The BBB becomes leaky which may favor the entry of seizure-inducing serum components into the peritumoral region (for a review, see Buckingham & Robel, 2013) and in turn lead directly to neuronal hyperexcitability and epileptogenesis. Our knowledge of the cellular and molecular determinants of this endfeet withdrawal is incomplete but the latter might be novel therapeutic targets. First, reactive, proliferating, STAT3-positive astrocytes have been seen to form a scar close to the tumor borders (Campbell et al., 2020). Peritumoral astrocytes display changes in endfeet protein expression. A general decrease in levels of Kcnj10 mRNA (encoding Kir4.1) is detected, and Kir4.1 expression becomes heterogenous with zones of low and high protein expression (Campbell et al., 2020). These changes are associated with electrophysiological defects, such as the loss of barium sensitive- membrane K^+ permeability and (moderate) depolarization of resting astroglial membrane potentials (Campbell et al., 2020). Enhanced Aqp4 expression, the loss of Aqp4 endfeet polarity, and peritumoral edema have been observed in infiltrative zones surrounding the tumor mass—especially in high-grade glioma. Cx43 is overexpressed in reactive astrocytes in peritumoral tissues (Aronica et al., 2001; Darmanis et al., 2017; Kolar et al., 2015; Li, Liu, Liu, & Su, 2015; Sin et al., 2016), and astroglial Cx43-mediated hemichannel functions are enhanced (Sin et al., 2016). Moreover, Cx43 is expressed by glioma cells and forms gap junctions with astrocytes (Venkatesh et al., 2019; Zhang et al., 1999), which constitute a major pathway for cell-to-cell communications.

6.5 | Seizures and epilepsy

Epilepsy defines a large panel of chronic brain disorders characterized by a periodic and unpredictable occurrence of seizures. Major histopathological features in epilepsy include neuronal loss, aberrant synaptic sprouting and neuronal network remodeling, but also microvascular proliferation and BBB disruption as well as gliosis. Astrocytes dysfunctions linked to epilepsy have been extensively studied (for a review, see Clasadonte & Haydon, 2012; Coulter & Steinhäuser, 2015; de Lanerolle, Lee, & Spencer, 2010; Patel, Tewari, Chaunsali, & Sontheimer, 2019; Seifert, Carmignoto, & Steinhäuser, 2010; Steinhäuser & Seifert, 2012; Wetherington, Serrano, & Dingledine, 2008). Regarding astrocytic perivascular functions, astrogliosis and altered expression of Aqp4, K_{ir}4.1, and proteins of the dystrophin-associated protein complex have been described in animal

models of epilepsy and in humans (Eid et al., 2005; Heuser et al., 2012; Lee, Hsu, Seldin, Arellano, & Binder, 2012; Medici, Frassoni, Tassi, Spreafico, & Garbelli, 2011). Due to the role of these proteins in the regulation of perivascular ion concentration, water homeostasis and extracellular space volume, their alteration might contribute to local hyperexcitability during both epileptogenesis and ictogenesis. Naïve transgenic animals lacking α-syntrophin display major loss of perivascular Aqp4 and Kir4.1 and present also more severe hyperthermia-induced seizures than control animals (Amiry-Moghaddam et al., 2003). Early defect in perivascular Aqp4 expression have been detected during the latent phase preceding the chronic epileptic seizures in an animal model of mesial temporal lobe epilepsy (Alvestad et al., 2013), suggesting the early involvement of Aqp4 dysfunction in the pathogenesis of the disease. Interestingly, perivascular Aqp4 loss can be associated with local BBB disruption, neurovascular uncoupling, edema, and serum-albumin extravasation (Bankstahl et al., 2018; Kim et al., 2010; Prager et al., 2019).

BBB disruption is a hallmark of epilepsy linked to alteration of endothelial transporters and tight junction proteins expression, modulation of molecular transport across BBB as well as pathological neo-angiogenesis (for a review, see Gorter, Aronica, & van Vliet, 2019; Löscher & Friedman, 2020; Marchi & Lerner-Natoli, 2013). Transient BBB disruption has been described after a single ictal episode (Alvarez, Maeder, & Rossetti, 2010; Bankstahl et al., 2018; Rüber et al., 2018; van Vliet et al., 2007; van Vliet, Aronica, & Gorter, 2015) and is known to contribute to the development of seizure disorders (Marchi et al., 2007; van Vliet, Aronica, Tolner, Lopes da Silva, & Gorter, 2004). The causal links between seizures, BBB damages, and alterations of the gliovascular interface are not yet understood. One mechanism might be the extravasation of serum albumin following seizures. In astrocytes, it would stimulate reactive astrogliosis and alter astrocytic perivascular properties such as the expression of Kir4.1 and Aqp4 (Ivens et al., 2007; Seiffert et al., 2004).

7 | NEW TOOLS FOR STUDYING ASTROCYTIC FUNCTIONS AT THE VASCULAR INTERFACE

With the emergence of research on brain interfaces, dedicated experimental tools have been developed to characterize the molecular and cellular regulatory interactions between neurons, astrocytes, and vascular cells. Below, we review some of the most relevant tools for specifically studying the gliovascular interface.

7.1 | In vitro coculture systems

In vitro gliovascular cultures consist of cocultures of endothelial cells, astrocytes, and (in some cases) pericytes placed together (either in direct contact or in separate inserts) to mimic intercellular signaling. These cultures have been instrumental in demonstrating the astrocytes' ability to induce BBB differentiation (for a review, see Helms et al., 2016). However, a major limitation of these approaches is that the gliovascular cells' high degree of polarization in vivo is lost in vitro. A number of recent attempts to build polarized in vitro gliovascular interface have been made. Cocultures of primary cortical astrocytes from newborn rats and immortalized bEND3 mouse brain endothelial cells were grown on each side of a membrane with 1 μm pores (Omidi et al., 2003). The GFAP-positive astrocytic processes extended to the top of the filter, and endfeet-like structures showed a distinct peak in Aqp4 immunoreactivity (relative to the cytoplasm) that was absent in α-syntrophin knock-out astrocytes. Endfeet formation was maintained if endothelial cells were replaced by endothelial-conditioned medium or a Matrigel or Agrin deposit but not if laminin alone was used. These results underlined the inductive role of endothelial cells and dystrophin-associated protein complex components and preponderance of agrin over laminin in the polarization of endfeet Aqp4. Interestingly, the addition of beads also influenced astrocytic process development and polarity—indicating that mechanical interaction per se has an inductive effect (Camassa et al., 2015). In a more recent study, astrocytes differentiated from neurospheres were cocultured with mechanically purified brain vessels from newborns (Kawauchi, Horibe, Sasaki, Hirata, & Rikitake, 2019). Under these conditions, well-ramified astrocytes developed endfeet terminated by Aqp4-positive perivascular contacts. Although this model requires more rigorous characterization, this type of approach might be useful for studying the molecular regulation governing endfeet formation and polarity. Finally, microfluidics constitute one of the most dynamic fields for reconstituting cellular interactions. Several attempts have been made to develop microfluidic models of the gliovascular interface. Although most studies to date have focused on the BBB's properties, they will probably also be useful for better characterizing the properties of perivascular astrocytes—particularly in systems in which astrocytes develop perivascular ramifications (Bang et al., 2017; Maoz et al., 2018).

7.2 | Ex vivo and in vivo imaging of calcium in endfeet

Endfeet display prominent Ca^{2+} signals. A wide range of genetic tools have been developed to study Ca^{2+} signals in astrocytes (for a review, see Yu, Nagai, & Khakh, 2020). Coupled to in vivo two-photon microscopy, these tools make it possible to study the profile and role of Ca^{2+} signals in endfeet during neuronal or vascular events in both anesthetized and awake animals. These cutting-edge approaches are decisive for defining the contribution of perivascular astrocytic Ca^{2+} signals to functional hyperemia, and yielded the first comprehensive three-dimensional map of Ca^{2+} activity in individual astrocytes (Bindocci et al., 2017). The approaches also enabled researchers to quantify astrocytic Ca^{2+} activity in endfeet, which appears to correspond to about 10% of the total activity. Interestingly, perivascular signals were mostly restricted to individual endfeet, and endfeet wrapped round the same vessel were asynchronous.

7.3 | Purification of the gliovascular interface

The mechanical purification of brain vessels was initially developed with a view to analysis the vascular compartment (Brendel, Meezan, & Carlson, 1974; Yousif, Marie-Claire, Roux, Scherrmann, & Decleves, 2007). However, we recently demonstrated that astrocyte endfeet remained attached to the vessel surface and became separated from their soma in these preparations (Boulay, Saubamea, et al., 2015). Thus, this "old but refreshed" experimental protocol provided an opportunity for the biochemical and molecular characterization of endfeet (Boulay, Mazare, Saubamea, & Cohen-Salmon,- 2019). Furthermore, we showed that mild digestion of the basal lamina led to partial detachment of endfeet from the vessel surface (Boulay et al., 2017). A comparison of digested and nondigested samples allowed us to identify endfeet components, such as mRNAs transported into the endfeet (Boulay et al., 2017). When combined with an astrocyte-specific ribotagging approach (Doyle et al., 2008), this method was also used to identify polysomal mRNAs in endfeet (Boulay et al., 2017).

7.4 | The morphology and ultrastructure of perivascular astrocytic processes

Dye injection into astrocytes using patch-clamp pipettes (Bushong, Martone, Jones, & Ellisman, 2002) and transgenic approaches in which astrocytes express cytosolic fluorescent proteins have been instrumental in revealing the astrocyte's morphological complexity (for a review, see Yu et al., 2020). Relative to basic immunofluorescent approaches, these techniques might enable researchers to determine the endfeet' precise morphology and describe their diversity and complexity more comprehensively. Indeed, GFAP immunofluorescence only accounts for about 15% of the total astrocyte volume (Bushong et al., 2002). A recent confocal microscopy of enhanced green fluorescent protein expressed under the control of the human GFAP promoter, in mouse hippocampal slices, suggested that the mean volume of astrocytes in the dentate gyrus is about 14,000 μm^3 (range: 3,000–35,000 μm^3) (Bindocci et al., 2017). Transmission electron microscopy (TEM) has also been widely used to study the architecture of endfeet. We recently demonstrated that endfeet in the cortex and hippocampus are all equipped with a continuous network of rough and smooth endoplasmic reticulum (and, for 7% of these, a full Golgi apparatus)—suggesting that secreted proteins and membrane proteins can mature locally (Boulay et al., 2017). TEM studies have also revealed that endfeet contain a dense, extremely plastic mitochondrial network close to the endfeet membrane (Göbel et al., 2020; Mathiisen et al., 2010). Along with confocal microscopy and in vivo Ca^{2+} imaging, recent developments have enabled faster 3D reconstruction of TEM images (Cali et al., 2019) and a more complete definition of the ultrastructure of the neuro-glio-vascular interface (Coggan et al., 2018).

8 | OPEN QUESTIONS FOR FUTURE RESEARCH

Although research on the gliovascular interface is expanding rapidly, many questions have yet to be answered. First, our current picture of perivascular astrocyte coverage is rather general and lacks details. How are endfeet organized? A recent study showed that on average, each astrocyte in the hippocampus has 3.5 endfeet (Bindocci et al., 2017). Is this a general feature, or it is a particularity of the hippocampus? Is there heterogeneity within the brain? Are there astrocytes that lack endfeet? Are all endfeet identical? Do all endfeet cover the same vessel surface? Are there correlations between astrocyte functions and the perivascular architecture? Are the endfeet organized in a particular way, depending on the type of vessel? Answers to these questions would undoubtedly enrich our current understanding of the astrocytes' functional diversity.

Most studies of endfeet development have focused on the expression profiles of Aqp4 and other members of the dystrophin-associated protein complex. However, TEM studies of samples from mouse pups at birth show that some endfeet lack Aqp4 (Lunde et al., 2015). This finding suggests that Aqp4 is probably not the sole marker of early endfeet development. Furthermore, we know very little about the factors regulating endfeet differentiation. The few available data come from studies of in vitro or nonpolarized systems.

The studies of gliovascular functions are based on an extremely limited molecular repertoire. As discussed above, techniques for isolating endfeet should help to bridge this knowledge gap. This approach could be performed for specific brain regions and different types of vessel, and would probably highlight specific molecular mechanisms that regulate the dialogue between astrocytes and vascular cells.

The recent discovery of local translation and a repertoire of polysomal RNAs in endfeet raises the question of how astrocyte polarity is controlled. The putative role of local translation in the establishment and maintenance of astrocyte polarity remains to be investigated. Given that astrocytes are the last elements to join the vessel surface in the course of development, we can assume that signals from vascular cells induce the formation and polarization of endfeet. Addressing these questions will require the development of tools that maintain the gliovascular interface's polarity.

Finally, perisynaptic astrocyte processes are known to be extremely mobile, which contrasts with our current view of a relatively static gliovascular interface. Conceivably, this mobility might also be present at the vessel and might have crucial regulatory functions.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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