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Review

Interplay between metalloproteinases and cell signalling in blood brain barrier integrity

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Summary. The Blood-Brain Barrier (BBB) is a highly specialised interface separating the Central Nervous System (CNS) from circulating blood. Dysregulation of the BBB is a key early event in pathological conditions such as inflammation, in which the entry of activated leukocytes into the CNS is facilitated by BBB breakdown. The metzincin family of metalloproteinases (MPs) is one of the major contributors to BBB permeability as they cleave endothelial cell-cell contacts and underlying basal lamina components. However, the mechanisms by which MPs regulate BBB integrity has not yet been fully elucidated. The aim of this review is to provide an overview of pathways by which MPs could regulate the BBB in the context of neuroinflammation.

Key words: Metalloproteinases, Metzincins, Blood-brain barrier, Signalling mechanisms, Neuro-inflammation

Development of the BBB

The Blood-Brain Barrier (BBB) is a tightly regulated interface between the Central Nervous System (CNS) and the circulating blood that ensures a highly selective paracellular and transcellular exchange of molecules between the two compartments. Structurally, the BBB is formed by highly specialised capillary endothelial cells (ECs) discontinuously wrapped on their luminal side by a cellular layer of pericytes. Together, ECs and pericytes synthesise a basement membrane (comprising extracellular matrix components including collagen IV, laminin and heparan sulphate proteoglycan) that provide structural support and contribute to barrier formation. Astrocyte foot processes externally cover this basement membrane acting as intermediates between the blood vessels and the CNS and promoting BBB stability through the secretion of trophic factors (see Fig. 1 for a depiction of the basic features of the neurovascular unit NVU) (Park et al., 2003; Seo et al., 2012). The perivascular space, previously thought to be absent from capillaries, may well be found throughout the vasculature (for review and discussion see Abbott et al., 2018 and papers therein).

Accumulation of immune cells within the perivascular space does not necessarily trigger full neuroinflammation, as the glia limitans (formed by a parenchymal basement membrane and the astrocyte foot process) forms a second barrier that keeps the immune cells from entering the CNS (reviewed in Engelhardt and Ransohoff, 2012; Engelhardt et al., 2016; Iadecola 2017).

Cell-cell interactions between neighbouring ECs result in a polarized phenotype and very limited transcellular diffusion, resulting in the BBB's limited permeability. Tight (TJ) and adherens (AJ) junctions link adjacent ECs together (Kniesel and Wolburg, 2000). TJs comprise transmembrane proteins such as occludins, junctional adhesion molecules (JAMs) and claudins which are anchored to actin filaments via adaptor proteins including cingulin, zona occludens proteins

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(ZO-1, -2 and -3) and Ca^{2+}-dependent serine protein kinase (CASK). Endothelial AJs link to the cytoskeleton through transmembrane proteins including vascular-endothelial (VE)-cadherin and catenins (α, β and p120) (Fig. 1) (Dejana et al., 2000; Kniesel and Wolburg, 2000; Alvarez et al., 2011a). For a detailed review of endothelial tight junctions, see Stamatovic et al. (2016).

The development of the BBB is a tightly regulated process. In the developing brain, neural progenitor cells secrete Vascular Endothelial Growth Factor (VEGF), which guides embryonic EC migration into the developing brain (Raab et al., 2004). Sprouting, angiogenesis and BBB maturation are promoted by Wnt secreted by neural progenitor cells, which induces transcription of genes implicated in BBB maintenance, such as TJ molecules (Liebner et al., 2008). ECs from emerging vessels release Platelet Derived Growth Factor-b (PDGF-b), promoting pericyte recruitment to the vessel surface (Hellström et al, 1999). Transforming Growth Factor-β (TGF-β)-mediated cross-talk between ECs and surrounding pericytes promotes VE-cadherin upregulation in ECs (increasing pericyte adhesion) and pericyte deposition of ECM components (Hill et al., 2014). Once vessels are formed, neighbouring astrocytes

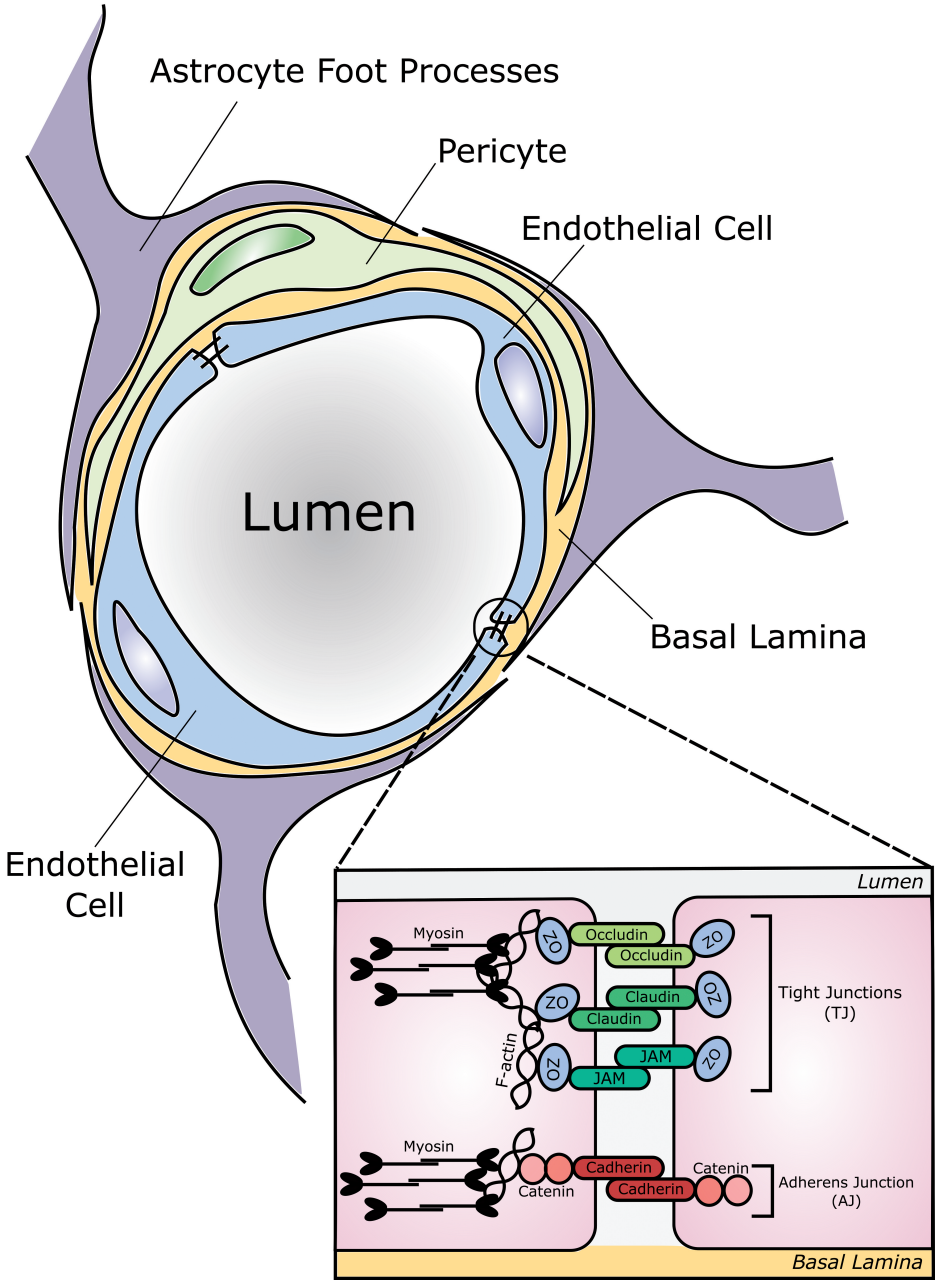

Fig. 1. Schematic representation of the architecture of the blood-brain barrier (BBB). Endothelial cells (ECs) forming the capillaries establish intercellular contacts known as tight junctions. ECs and pericytes are surrounded by a basement membrane, which in turn is also wrapped by astrocyte foot processes. Astrocytes play a major role in BBB maintenance through the secretion of several factors including Shh (not shown).

support BBB maturation through the secretion of Sonic Hedgehog (Shh), a trophic factor responsible for increased TJ protein expression in ECs (Alvarez et al., 2011b).

BBB disruption is a pathological event

BBB dysfunction is widely implicated in the context of brain injury and disease. Cytotoxic edema through BBB disruption is a key pathological event in a wide variety of syndromes such as ischemic stroke (Page et al., 2016), meningitis (Sellner and Leib, 2006) or ketoacidosis (Hoffman et al., 2009). BBB disruption also facilitates the entry of activated immune cells into the CNS and is likely to be an early event in the development of Multiple Sclerosis (MS) lesions (Abbott et al., 2010; Larochelle et al., 2011). In meningitis, BBB disruption, is probably required for the pathogenic invasion of the CNS (Wang et al., 2016).

Molecular and/or environmental factors can enhance the ability of circulating activated T cells to cross the BBB and enter the CNS. Adhesion molecules of the selectin family (such as P-selectin glycoprotein-1) expressed by the circulating activated leukocytes interact with their respective ligands up-regulated on ECs (Man et al., 2007; Engelhardt et al., 2016; Wang et al., 2016). Close interaction with ECs allows circulating leukocytes to detect chemokines and cytokines (released by the damaged tissue) and leukocyte tethering via the activation of integrins. The increased affinity between the immune cell and the endothelium strengthens leukocyte adhesion to the vascular wall, leading to a crawling process mainly mediated by alpha L/M beta 2 and alpha 4 beta 1 integrins and their respective endothelial partners Intercellular Adhesion Molecules (ICAMs) and Vascular Cell Adhesion Molecules (VCAMs) (Man et al., 2007; Engelhard et al., 2016; Wang et al., 2016). During this stage, crawling immune cells scan the endothelial surface looking for a permissive site for extravasation (or diapedesis), where protrusions from the endothelial plasma membrane will surround the adherent leukocyte helping it to migrate across the BBB. This extravasation can take place through two different routes: paracellular diapedesis and transcellular diapedesis. In paracellular diapedesis the activated leukocyte migrates into the CNS by disrupting the endothelial junctions between adjacent ECs, enabling transmigration (Man et al., 2007; Engelhardt and Ransohoff, 2012; Wang et al., 2016). Transcellular diapedesis occurs less frequently and is characterised by clustering of ICAM1 (Millan et al., 2006; reviewed in Engelhardt et al., 2016). In conditions such as MS, once circulating autoreactive T cells have entered the CNS, they secrete proinflammatory cytokines such as interferon-γ (INF-γ) and tumour necrosis factor-α (TNF-α) (Engelhardt, 2006; Man et al., 2007; Engelhardt and Ransohoff, 2012). These cytokines can then activate antigen-presenting cells (APCs) and further promote the migration of T cells across the BBB by increasing the expression of adhesion molecules in both circulating leukocytes and ECs. For further details of extravasation mechanisms see reviews by Vestweber and Engelhardt and colleagues (Vestweber, 2015; Engelhardt et al., 2016) and for review of immunopathology of MS development and T cell subsets involved see Garg and Smith (2015). Thus, cytokines play a crucial role initiating and promoting the entry of pro-inflammatory cells into the CNS.

Many studies have reported the role of cytokines in BBB disruption in neuroinflammatory disorders. Release of pro-inflammatory mediators is an early event in MS leading to BBB disruption (Minagar and Alexander, 2003; Abbott et al., 2010; Larochelle et al., 2011). Elevated levels of circulating TNFα, IL-17A and decreased levels of circulating IL-10 were reported in MS patients (Trenova et al., 2018). Although the exact mechanisms by which cytokines trigger BBB disruption are incompletely understood, reduced levels of TJ proteins (such as ZO-1, claudin-5 and occludin) have been reported in several model systems (Forster et al., 2008; Aslam et al., 2012; Cohen et al., 2013; Labus et al., 2014). Anti-inflammatory cytokines such as IL-4 and IL-10 can ameliorate brain lesions and cellular infiltration in a murine model of MS (Hosseini et al., 2017), and termination of inflammation together with repair mechanisms can be promoted by microglial secretion of IL-10, transforming growth factor β (TGFβ) and insulin-like growth factor 1 (IGF1) (Amantea et al., 2015). For an overview of factors controlling BBB permeability, see Almutairi et al. (2016).

Metzincins play a key role during BBB disruption

Metzincins, comprising Matrix Metalloproteinases (MMPs), A Disintegrin And Metalloproteinases (ADAMs) and A Disintegrin And Metalloproteinase with Thrombospondin motifs (ADAMTS), are a family of zinc containing proteinases widely implicated in the biology of the nervous system (reviewed in (Rivera et al., 2010)).

MMPs

MMPs are synthesized as inactive enzyme precursors containing a signal peptide, directing secretion or localisation to the plasma membrane. MMP catalytic activities have mainly been studied in the extracellular environment and at the cell surface (Fanjul-Fernandez et al., 2010). MMP activity, (as well as activation in some cases), is inhibited by the four Tissue Inhibitors of Metalloproteinases (TIMPs) (Larochelle et al., 2011). In humans, 23 different MMPs have been classified according to differences in their domain structure and original ECM substrate specificity (Fig. 2) (Yong, 2005; Rivera et al., 2010). The pioneering proteomic approaches taken by Chris Overall and several other groups over the last 20 years have revealed that MMPs can cleave many novel substrates, including

cell-cell junction components, chemokines, cytokines and their receptors as well as growth factors (reviewed in Schlage and auf dem Keller, 2015). These findings have informed much of the recent research regarding MMPs in BBB breakdown. In addition proteomic approaches reveal that MMP action intracellularly is of emerging importance (reviewed in Jobin et al., 2017), but will not be further considered here. MMPs are tightly regulated at three main levels: at the transcriptional level (eg cytokine-induced transcription), by proteolytic activation of their initial inactive form exposing their active catalytic domain and through inhibition by TIMPs. Additionally, MMP activity can also be controlled by post-translational modifications, substrate availability and cellular localization (reviewed in Yong, 2005).

A major consequence of neuroinflammation is the up-regulation of MMPs (Rempe et al., 2016). Components of endothelial cell TJs and AJs as well as the extracellular matrix surrounding ECs and pericytes, can be cleaved by members of this large family of proteinases (Fanjul-Fernandez et al., 2010; Eisenach et al., 2012; Liu et al., 2012a,b). Additionally, several studies using broad spectrum metalloproteinase inhibitors points at metzincins as main mediators of BBB pathological disruption. Administration of a broad spectrum (M)MPs inhibitor in a rat model of meningitis (Paul et al., 1998) or stroke (Pfefferkorn and Rosenberg, 2003), and a murine model of MS (Gijbels et al., 1994) resulted in suppression of induced BBB permeability. Interestingly, the ability of LPS to trigger BBB damage and the ability of the tested broad spectrum (M)MPs inhibitors to block LPS-induced BBB disruption was mouse strain-dependent, suggesting that genetic background could be playing an important role (Rosenberg et al., 2007).

MMP induction is associated with inflammation and brain injury: early studies revealed high levels of MMP9 together with a reduction in inhibitor levels (TIMP1) in serum from MS patients (an autoimmune disorder in which early BBB breakdown is likely to precede invasion of autoreactive immune cells into the CNS) (Lee et al., 1999; Waubant et al., 1999). During ischemia MMP3 expression levels can be elevated triggering loss of BBB integrity (Rosenberg et al., 2001; Gurney et al., 2006). Observed dysregulation of MMP levels may initiate BBB damage since several members of this family are capable of cleaving TJ and basement membrane elements (summarised in Table 1). Claudins, occludins and ZO-1 proteins can be directly cleaved by MMP1 (Wu et al., 2015), -2 (Yang et al., 2007), -9 (Yang et al., 2007; Bauer et al., 2010) and -13 (Lu et al., 2009) amongst others. Membrane anchored MMPs also activate other MMPs, as well as degrading basement membrane elements such as collagen IV and laminin (Itoh, 2015). In murine model, MMP2 and -9 can cleave dystroglican (a transmembrane receptor responsible for astrocyte foot processes anchorage to the basement membrane) promoting leukocyte infiltration, discussed in more detail later (Agrawal et al., 2006). Additionally, pro-inflammatory

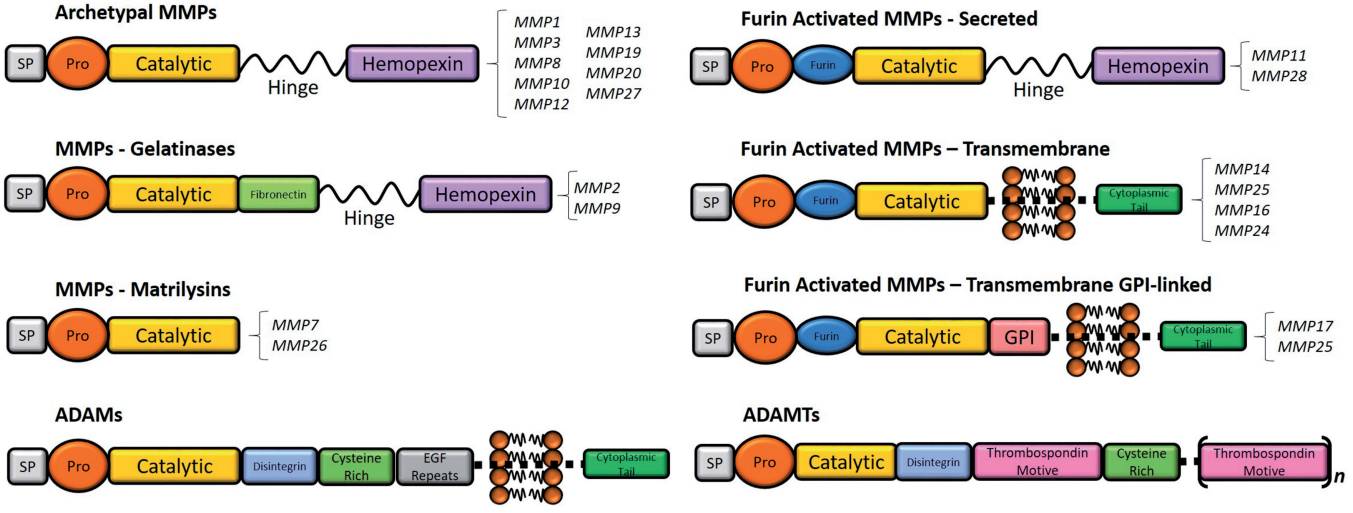

Fig. 2. Schematic representation of Metzincin structural domains. Metalloproteinases exhibit a high structural homology with a catalytic domain followed by a highly conserved methionine residue; a linker or hinge peptide; and a hemopexin domain with a calcium binding site. Differences in this consensus structure have been used to classify this family members in the above specified subgroups. The presence of a signal peptide (SP) directs their secretion or transmembrane anchorage. These metalloproteinases are activated proteolytically through cleavage of the pro-domain. Although MMPs are secreted, the presence of a transmembrane or glycosyl phosphatidylinositol (GPI) domain allows some members of this family to anchor to the cellular membrane. ADAMs have a disintegrin-like domain involved in cell adhesion together with a cysteine-rich region and Epidermal Growth Factor (EGF-) like repeats. Structurally similar to ADAMs, ADAMTS also contain a disintegrin-like and cysteine-rich regions (separated by a thrombospondin motive) but lack a transmembrane domain and are thus secreted.

cytokines can also be proteolytically activated by mentzincines, suggesting the existence of a feedback loop mechanism between (M)MPs and pro-inflammatory agents: TNFα can be cleaved and activated by ADAM17 as well as by MMPs7, -12, -14 and -17; IL-1β can be proteolytically activated by MMPs2, -3 and -9; and MMP9 can mediate interferon-β inactivation (reviewed in Rodriguez et al., 2010). Interestingly, ADAMs 8 and -17 can shed TNFα receptor (TNFR), releasing soluble form of TNFR which can sequester extracellular TNFα ameliorating inflammation (Reddy et al., 2000; Bartsch et al., 2010). IL-1R and IL-6R can be shed by ADAM10 and/or -17 (Reddy et al., 2000; Schumacher et al., 2015), releasing a soluble fraction capable of stabilising IL-1 or IL-6 and exacerbate the inflammatory response. Metzincins can also target a wide range of chemokines, regulating the recruitment of activated immune cells: MMPs 8 and 9 can cleave IL-8 enhancing their chemotactic activity (Van den Steen et al., 2000; Van Den Steen et al., 2003; Tester et al., 2007). Studies in MMP8 null mice showed an impaired recruitment of activated immune cells following LPS stimulation, corroborating the *in vitro* data (Tester et al., 2007). MMP12 participates in the termination of inflammation through the cleavage and inactivation of most members of the CXCL chemokine family (Dean et al., 2008). Thus, there is complex duality underlying mentzincines roles in the processing of chemokines (reviewed in Rodriguez et al., 2010). Metzincins cannot only modulate the infiltration of activated immune cells through the direct opening of the BBB, but also through their recruitment and activation via chemokine processing. For further discussion of MMPs in brain disease see Rempe et al. (2016) (Table 1).

ADAMs

The ADAM family shares great structural similarities with MMPs (Fig. 2), and has also been implicated in BBB disruption (reviewed in Reiss and Saftig, 2009). ADAMs have major roles in shedding molecules from the cell surface (for a new comprehensive sheddome database see Tien et al., 2017). Within the ADAM family, ADAM10 and ADAM17 are the principal shedding enzymes and during inflammatory conditions may promote BBB leakage through the shedding of adhesion molecules expressed by ECs (reviewed in Dreymueller et al., 2012a). In addition, the expression of ADAM17 has been found to be up-regulated in active lesions during MS (Plumb et al., 2006). Recent evidence indicates that Natalizumab (an anti-alpha 4 integrin antibody) treatment in patients with multiple sclerosis, which blocks leukocyte adhesion to VCAM-1 on inflamed endothelial cells also results in reduction in circulating soluble Vascular Cell Adhesion Molecule 1 (sVCAM-1) levels (Petersen et al., 2016). VCAM-1 shedding is ADAM17-dependent (Garton et al., 2003; Singh et al., 2005). Whether Natalizumab effects on VCAM-1 levels are ADAM17-mediated remains to be studied, although it could be speculated that Natalizumab blockade of activated immune cell recruitment could lead to a local decrease in secreted cytokines, leading to a downregulation in ADAM17 activation and a possible reduction in VCAM-1 shedding.

ADAMTS

A Disintegrin and Metalloproteinase with Thrombospondin motifs (ADAMTS) proteinases are secreted extracellular enzymes with a characteristic thrombospondin type 1 sequence repeat (TSR) motif that share the same catalytic domain as MMPs and ADAMs (Fig. 2) (Kelwick et al., 2015). Several members of this family have been implicated in BBB disruption: genetic linkage studies in Multiple Sclerosis patients have associated ADAMTS14 (a procollagen aminopropeptidase) with this disease (Goertsches et al., 2005) whereas studies in an ischemic murine model have reported that ADAMTS13 blocks tissue Plasminogen-induced BBB disruption after cerebral stroke (Wang et al., 2013) (Table 1).

Despite recent advances in our understanding of metalloproteinases, the precise underlying mechanisms by which metzincins regulate BBB stability remain uncertain. Many pathways can regulate BBB integrity during adulthood. In this review we aim to highlight signalling pathways which are already implicated in metzincin-mediated regulation of BBB integrity as well as those which are deserving of further study.

Pathways implicated in metzincin regulation of BBB integrity

Several pathways have been implicated in development of the BBB, some of which have also been explored in the context of disease. For each pathway, we consider the pathway's role in development and/or maintenance of the BBB and indicate where metzincins have established or emerging roles. We also make reference to other systems where metzincin interaction is of importance and which may provide useful pointers for future BBB research.

Hedgehog pathway

The importance of astrocyte-secreted Shh in BBB maintenance has emerged in recent years. Compelling evidence for the role of hedgehog signalling in BBB maintenance in adult mice has been provided by Alvarez and colleagues (Alvarez et al., 2011b). In the hedgehog pathway the absence of Shh, the cell surface receptor patched (Ptch) functions as a constitutive inhibitor of Smoothened (Smo). Upon Shh-Ptch binding, Smo initiates the Shh signalling cascade, resulting in the activation of the Gli family of transcription factors (Gli1, Gli2 and Gli3) (Benson et al., 2004; Choudhry et al., 2014). By selectively deleting Smo in mouse brain ECs,

a decrease in junctional proteins (claudins -3 and -5, occludin and ZO-1) accompanied by a fragmented basement membrane and higher BBB permeability *in vivo* was observed (Alvarez et al., 2011b). Studies in ECs show that Shh promotes neovascularization through upregulation of pro-angiogenic factors (including VEGF) as well as MMP2 and 9 (Renault et al., 2010; Yi et al., 2016). Interestingly, Renault et al. suggest that a non-classical, alternative pathway involving Rho could mediate Shh-induced angiogenesis and MMP9 upregulation in Human Umbilical Vein Endothelial Cells (HUVECs) (Renault et al., 2010). In a disease-

Table 1. Key findings regarding metalloproteinases effects in BBB integrity and BBB related disease.

	OBSERVATION	STUDIED IN	REFERENCES
MMP1	Cleaves claudins and occludins but not ZO-1 proteins	Co-culture of mBMEC with human breast cancer cells	Wu et al., 2015
MMP2	Required by monocytes, dendritic cells and activated T cells to induce BBB breakdown	EAE	Graesser et al., 2000
	Resistance to EAE in MMP-2 and MMP-9 in double knockout mice	EAE	Agrawal et al., 2006
	Positive feedback mechanism: cytokines produced by leukocytes induce MMP-9 and -2, which in turn can promote further infiltration of immune cells	EAE	Agrawal et al., 2006
	Cleaves claudin-5	Cerebral artery occlusion and reperfusion in rats	Song et al., 2015
	Increase susceptibility to EAE in MMP2$^{-/-}$ mouse due to a compensatory increase in MMP9 levels	EAE	Yang et al., 2007
MMP3	LPS intracerebral injection showed reduced BBB opening and neutrophil infiltration in MMP3$^{-/-}$ mouse	Knockout mouse	Esparza et al., 2004
	Increased expression during brain ischemic insult	Ischemic rat brain	Gurney et al., 2006
	mRNA levels elevated during Relapsing Remitting Multiple Sclerosis	MS patients	Rosenberg et al., 2001
	Increased transcriptional up-regulation in a murine virus induced model of Multiple Sclerosis	Murine MS model	Larochelle et al., 2011
MMP7	Increased in lesions from post-mortem Multiple Sclerosis brains	Post-mortem brain	Hansmann et al., 2012
	mRNA levels elevated during Relapsing Remitting Multiple Sclerosis	MS patients	Lindberg et al., 2001
MMP8	Serum levels increased in Multiple Sclerosis patients	MS patients	Larochelle et al., 2011
	Up-regulated expression levels in the CNS of a mouse model of Multiple Sclerosis (EAE)	EAE	Larochelle et al., 2011
MMP9	Required by monocytes, dendritic cells and activated T cells to induce BBB breakdown	EAE	Toft-Hansen et al., 2004
	Resistance to EAE in MMP-2 and MMP-9 in double knockout mice	EAE	Agrawal et al., 2006
	Positive feedback mechanism: cytokines produced by leukocytes induce MMP-9 and -2, which in turn can promote further infiltration of immune cells	EAE	Graesser et al., 2000
	Higher levels in MS patients and associated with relapse. High serum levels correlated with BBB disruption in MS patients	MS patients	Agrawal et al., 2006
	Gene knockout is associated with a reduction in infarction and attenuation of BBB opening after focal cerebral ischemia	Transient focal ischemia in mice	Song et al., 2015
	Cleavage of ZO-1, occludin and claudin-5	Cerebral hypoxia mice. Brain artery occlusion rats	Lee et al., 1999
	Reduced susceptibility to EAE, BBB damage and infarcts susceptibility in MMP9$^{-/-}$ mouse	Knockout mouse	Waubant et al., 1999
	Polymorphisms in its promoter have been linked to increased susceptibility to Multiple Sclerosis	MS patients	Asahi et al., 2001
MMP10	Up-regulated expression levels in the CNS of a mouse model of Multiple Sclerosis (EAE)	EAE	Bauer et al., 2010
MMP12	Up-regulated expression levels in the CNS of a mouse model of Multiple Sclerosis (EAE)	EAE	Yang et al., 2007
	Increased susceptibility to EAE in MMP12$^{-/-}$ mouse	EAE	Asahi et al., 2001
MMP13	Can enhance BBB permeability through ZO-1 fragmentation	Primary rat astrocytes and ARBECs co-culture	Dubois et al., 1999
MT-MMPs	Cleavage of BBB basement membrane components such as laminin and collagen IV	*In vitro* studies	Fiotti et al., 2004
	Serum levels (MT-MMP1) can be elevated in Multiple Sclerosis patients	MS patients serum	Toft-Hansen et al., 2004
TIMP1	Low serum levels correlated with BBB disruption in Multiple Sclerosis (MS) patients	MS patients serum	Toft-Hansen et al., 2004
ADAM10	Promote BBB leakage through the shedding of adhesion molecules	HUVECs	Weaver et al., 2005
ADAM15	ADAM15 depletion can decrease endothelial permeability. This can be reversed by its overexpression	HUVECs	Lu et al., 2009
ADAM17	Promote BBB leakage through the shedding of adhesion molecules. Expressed in blood vessels of MS lesions	HUVECs MS patients	Itoh, 2015
ADAMTS13	Capable of blocking tPA induced BBB disruption after cerebral ischemia in mice	Ischemia mouse model	Larochelle et al., 2011
ADAMTS14	Associated to Multiple Sclerosis through genetic linkage	MS patients	Waubant et al., 1999

mimicking context, cytokine IL1β suppresses Shh expression in murine astrocytes whilst elevating levels of a number of chemokines (Wang et al., 2014). Conditioned media from untreated astrocytes was, as expected, important in promoting barrier formation in endothelial cells whilst medium from cytokine-treated astrocytes abrogated this effect (Wang et al., 2014). Very recently, conditioned medium from mycobacterium tuberculosis (MTb)-infected monocytes was shown to up-regulate MMP9 expression in astrocytes and, as well as cleaving type IV collagen, induced MMP9 had an additional role in preventing Shh delivery from astrocytes to endothelial cells (Brilha et al., 2017). Protein levels of claudin-5, occludin and ZO-1 were all reduced but mechanisms underpinning these observations remain unexplored. The fact that Shh can induce MMPs in endothelial cells raises a potential tension between BBB formation and breakdown mediated by this pathway, which is worthy of further investigation.

Notch pathway

The Notch pathway has been implicated in vascular barrier integrity (for review see (Cai et al., 2016)) but the underlying metalloproteinase involvement in the context of the BBB remains unclear. Upon binding to their ligands, Jagged and Delta-like (Dll), the Notch family of transmembrane receptors (Notch1 to 4 in mammals) are generally cleaved by ADAM10, enabling γ-secretase-mediated release of the Notch intracellular domain (NICD), which translocate to the nucleus. Here the NCID associates with CSL and Mastermind-like-1 (MAMIL) to regulate gene transcription (reviewed in (Siebel and Lendahl, 2017; Wetzel et al., 2017)). Interestingly, Notch shedding can be also be triggered in a ligand-independent manner, involving endosomal localisation/association (reviewed in Palmer and Deng, 2015; La Foya 2016)). Notch interaction with the wnt signalling pathway may impact on vascular barrier integrity through NCID/β-catenin regulation of gene expression or through triggering of β-catenin degradation (reviewed in La Foya et al., 2016).

Early studies showed roles for ADAM10 (Pabois et al., 2015; Zhuang et al., 2015) and ADAM17 (Broux et al., 2012) in the extracellular cleavage of Notch. Murine deletion studies demonstrated that ADAM10 plays a role in blood vessel development in several vascular beds, such as liver, bone and retina (Glomski et al., 2011). However, histopathological observations revealed that mice with an endothelial-specific ADAM10 do not develop major phenotypic defects in the brain (Alabi et al., 2016). This comprehensive study revealed that endothelial-specific deletion of Notch1 and Notch4 phenocopies the ADAM10-deleted mice. ADAM10/17 double knockout mice did not show any additional defects, demonstrating that ADAM10 is the key proteinase in these interactions. Further studies will determine whether ADAMs 10 or 17 are implicated in Notch signalling in relation to functional maintenance of the BBB in adult mice and in disease. The same study revealed that Notch 1 and Notch 4 have partially overlapping roles in vascular barriers (Alabi et al., 2016). Additionally, ADAM10 and Notch can respond to inflammatory stimuli in other vascular pathologies: in an *in vivo* model of vascular inflammation an increase in IL-6 was mediated by ADAM10-dependent shedding of Notch, suggesting that ADAM10 activation of Notch signalling could be involved in inflammation and recruitment of activated immune cells (Pabois et al., 2015).

Oxidative stress can synergistically trigger a reduction in Notch4 and ZO-1 protein levels in brain ECs isolated from rats (Manda et al., 2010). However, it remains to be determined whether the described loss in BBB stability is Notch4-dependent or a broader consequence of oxidative stress exposure. Notch3 (expressed by surrounding vascular smooth muscle cells (VSMCs)) plays a key role in BBB maintenance, as Notch3$^{-/-}$ mice exhibit enhanced BBB permeability *in vivo* (assessed by Evans Blue and Horseradish Peroxidase extravasation) (Henshall et al., 2015). Thus, members of the Notch family may exert different effects under the same conditions depending on the overall activity of the Notch pathway. Supporting this idea, *in vivo* and *in vitro* studies in human brain microvascular ECs (HBMECs) suggest a role for Notch1 rather than Notch3 (whose expression is specific to VSMCs) to be a key mediator in cocaine-induced BBB breakdown (Yao et al., 2011). Although EC to EC Notch signalling has been previously reported to be essential during angiogenesis and vascular homeostasis (Noseda et al., 2004) more recent studies suggest the existence of a complex Notch cross-talk between all the components of the NVU. A thorough characterization of Notch receptor and ligand expression among the components of the NVU shows a heterogeneous and complex distribution of these components: Notch 1 and 4 are expressed in ECs together with the ligands Jagged2 and Dll4; Notch 2 and 3 are present in astrocytes; Jagged 1 is located in pericytes and neurons express Dll1 (Yamamizu et al., 2017). Supporting this idea, neuron-derived Dll was reported to be needed for the complete differentiation of brain ECs in a complex co-culture *in vitro* model of the BBB (Yamamizu et al., 2017). Studies in mouse retina showed that Notch3 is required for pericyte attachment to ECs in an N-cadherin dependent manner (Liu et al., 2010).

One of the many consequences of Notch1 activation is the inhibition of Phosphatase And Tensin Homolog (PTEN), downstream of Notch, and the consequent activation of PI3K/p-Akt pathway (El-Habr et al., 2014; Song et al., 2015). The role played by the Serine/Threonine kinase Akts in BBB integrity remains to be fully clarified. Studies with Akt1$^{-/-}$/Akt2$^{-/-}$ double KO mice showed no altered EC survival, but a gradual loss of VSMCs due to reduced Jagged1/Notch signalling (Kerr et al., 2016).

Conflicting with the previously discussed studies in

Notch3$^{-/-}$ mice (Henshall et al., 2015), Akt1$^{-/-}$/Akt2$^{-/-}$ double KO mice exhibit an intact BBB (Kerr et al., 2016). However, the assessment of BBB properties in this study was performed through NaF permeability, a very low molecular weight molecule broadly used to measure basement membrane permeability to water and other small solutes rather than large molecules such as dextran. A mathematical prediction of BBB functions suggests that even when TJs are compromised, the basement membrane and astrocyte foot processes could theoretically maintain a low permeability to water and other small solutes (Li et al., 2010). Hence, it is possible that a functional basement membrane in the Akt1$^{-/-}$ and Akt2$^{-/-}$ double KO could mask TJ dysfunction when tested with NaF, explaining the apparently contradictory results.

Despite the uncertain role of Notch signalling in BBB stability, a role for secreted MMP9 and MMP2 in shedding extracellular Notch1 (consequently enhancing Notch1 signalling) has been suggested in a murine model of MS (experimental autoimmune encephalomyelitis, EAE). Song and colleagues showed an up-regulation of Notch1-specific transcription factors in MMP2- and MMP9-treated astrocytes (Song et al., 2015a,b). Additionally, *in vitro* experiments showed reduced T cell transmigration in the presence of primary Notch1$^{-/-}$ astrocytes exposed to a pro-inflammatory environment. Reduced astrocytic chemotactic activity and chemokine secretion could not be restored by addition of activated MMP2 or MMP9 (Song et al., 2015a,b). The key roles of MMP2 and MMP9 as mediators of leukocyte infiltration into the CNS have been shown in MMP2$^{-/-}$/MMP9$^{-/-}$ double knock-out mice, but levels of Notch proteolytic activation were not assessed in this context. MMP14 (or MT1-MMP) can also trigger Notch proteolytic activation but its impact in the context of the BBB remains to be clarified (Ma et al., 2014) (Fig. 3).

Pathways to cytoskeletal reorganization

TNFα stimulation of brain microvascular ECs results in the formation of actin stress fibres, loss of ZO-1 immunostaining and increased barrier permeability at early time-points, which can be reversed by MMP9 inhibition (Wiggins-Dohlvik et al., 2014). As previously

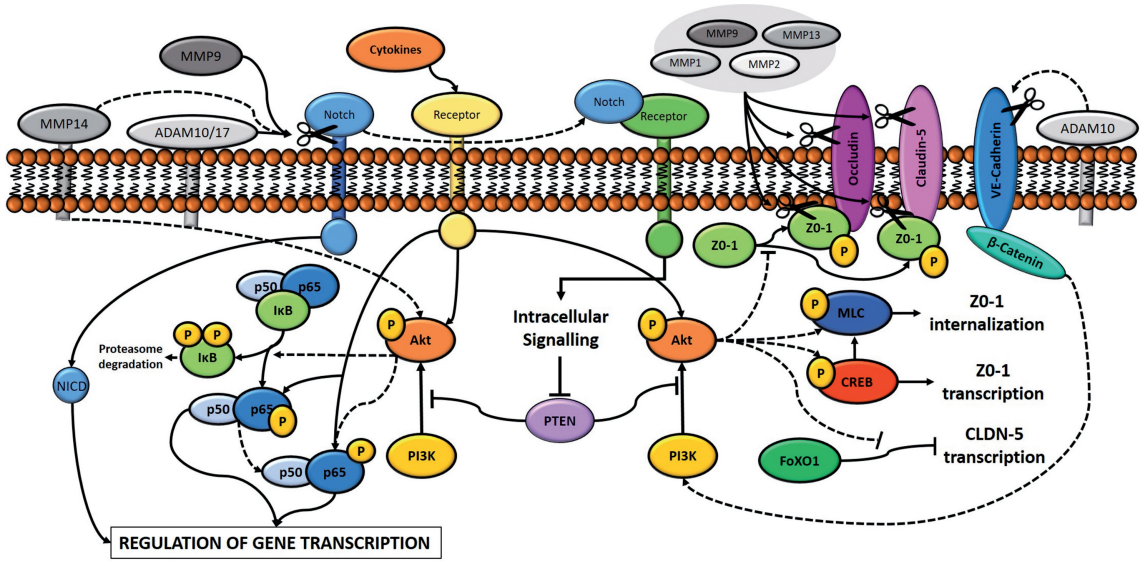

Fig. 3. Some of the possible intracellular mechanisms underlying BBB stability. Under inflammatory conditions cytokines are released to the extracellular environment. Endothelial cells (ECs) forming the blood-brain barrier (BBB) respond to these released factors through transmembrane receptors (such as TNFR or ILR) and trigger a cascade of intracellular events in which the PI3K/Akt pathway may function as a key node. Cytoskeletal changes triggered by myosin phosphorylation together with ZO-1 phosphorylation status, both potential Akt substrates, seem to determine ZO-1 intracellular location and consequent TJ formation. Additionally, Akt-mediated phosphorylation may be responsible for the activation of transcription factors such as CREB (which can trigger ZO-1 transcription), NFκβ (depicted p50/p65 and whose inhibitor degradation IκB can be also modulated by Akt) FoXO1 (in the absence of VE-cadherin, β-catenin is no longer sequestered in the membrane and can interact with FoXO1, inhibiting claudin-5 transcription). NFκβ modulates gene transcription of several genes, some of them implicated in BBB maintenance: represses TJ proteins expression while enhancing the transcription of metzincins such as MMP9, -14, ADAM10 and -17. Interestingly, (M)MPs have not only been described to directly cleave TJ proteins and basement membrane components but also cleave/activate notch, which in turn may further enhance Akt-mediated effects through inhibition of PTEN, a repressor of the PI3K/Akt pathway. After Notch cleavage, Notch intracellular domain (NICD) is free to translocate into the nucleus, where it can regulate gene transcription and impact BBB stability. Additionally, ADAM15 can also impact on barrier stability not only through cleavage but also by promoting VE-cadherin phosphorylation and consequent AJ disassembly through intracellular signalling cascade triggered with its cytoplasmic tail (not depicted). Interactions validated in the BBB are represented by solid lines, while dotted lines refer to connections described in a different cellular context.

mentioned, ZO proteins act as a link between transmembrane components of the TJ (occludins and claudins) and the actin cytoskeleton (Fanning et al., 1998; Itoh et al., 1999), similar to the role of catenins during AJ formation (Yap et al., 1997). Thus, ZO proteins work as adaptors between the dynamic cytoskeleton and the stable TJ, providing ECs with a barrier which can adjust to different cellular requirements. Under certain environmental conditions, actin filaments can change their usual conformation (distributed across the ECs as short filaments and monomers) and polymerize into large structures known as stress fibres (Burridge and Wittchen, 2013). *In vitro* studies using isolated mouse brain capillaries and HBMECs have shown that stress fibres can increase cytoskeletal tension leading to an impaired TJ formation through a RhoA/Rho kinase (ROCK)-mediated mechanism (McKenzie and Ridley, 2007; Shi et al., 2016). This actin-mediated disassembly of TJs could result in the opening cell-cell contacts, exposing other junctional proteins and ECM components to the degradation by surrounding (M)MPs.

Actin contractility is induced by the phosphorylation of myosin light chain (MLC), which is in turn activated by the ROCK/myosin-light chain kinase (MLCK) pathway (Hathaway et al., 1981). This impairment of TJ formation leads to an increased permeability to small molecules and activated immune cells in a well-established *in vitro* model of the BBB (Shi et al., 2016).

Studies in immortalised human brain microvascular ECs (hCMEC/D3) report an actin-mediated nuclear translocation mechanism for ZO-1 after activation of the ROCK/MLCK pathway that will result in ZO-1 internalization and TJ impairment. This research also demonstrated that Rho activation triggered cAMP Response Element Binding Protein (CREB) phosphorylation, which in turn could enhance MLC activation (Zhong et al., 2012). CREB is a transcription factor that can recognize and bind to a CREB responsive element (CRE) present in the promoter region of many cAMP-responsive genes (Johannessen et al., 2004) including ZO-1 (Chen et al., 2008; Zhong et al., 2012) . Akt can phosphorylate CREB on Ser133 leading to its activation (Du and Montminy, 1998). Thus, it could be proposed that Akt-mediated phosphorylation of CREB can enhance DNA binding activities leading to an increase in ZO-1 transcription, which would enhance barrier properties in the context of the BBB (Fig. 3).

Akt is frequently activated down-stream of BBB disrupting agents including inflammatory cytokines, and attention has turned more recently to its role in localisation of TJ proteins: in the bEND.3 cell line cytokine treatment results in early loss of paracellular claudin-5 localisation via the PI3K/Akt pathway (Camire et al., 2014; Machida et al., 2017). In a co-culture model of rat pericytes and ECs, thrombin can induce pericyte release of MMP9 through PAR1/Akt activation (Machida et al., 2017).

It is also possible that Akt could be mediating TJ formation through direct phosphorylation of ZO-1 (Furuse et al., 1999). Whereas ZO-1 tyrosine phosphorylation has been correlated with both increased and decreased barrier permeability, depending on the cell type, in epithelial cells serine/threonine phosphorylation has mainly been described to impair TJ function through the internalization of ZO-1 (Harhaj and Antonetti, 2004). Akt mediated phosphorylation can also enhance ZO-1-dependent TJ formation in diabetic mouse retina explants (likely in the vasculature) (Liu et al., 2012a,b). Thus, studies of direct effects of Akt-driven phosphorylation of ZO-1 in the context of the BBB may be warranted.

Overall, it seems that the Akt/CREB pathway could mediate opposing effects on BBB stability: inducing ZO-1 internalization and TJ disruption or directly promoting ZO-1 gene expression. Thus, the Akt/CREB pathway may be acting as an intracellular node that enhances or impairs TJ stability depending on the environmental conditions (Fig. 3). Interestingly, McKenzie and Ridley (2007) reported that a TNFα effect on cytoskeletal rearrangements, through ROCK/MLCK pathway, was enough to cause TJ distribution at early time-points but insufficient to trigger long-term effects on EC permeability. These observations suggest that TJ distribution and stability could be responding to internal mechanisms depending on the time-points studied (McKenzie and Ridley, 2007).

Overall, regulation of ZO-1 cellular localization seems to play an essential role in BBB stability by affecting TJ assembly directly. In addition, and as previously mentioned, as well as being a substrate for MMP9 (and other MPs potentially), ZO-1 re-distribution may result in exposure of other junctional proteins, opening cell-junctions to allow access by (M)MPs to basement membrane components, resulting in their subsequent cleavage.

NFκβ pathway

NFκβ is a heterodimeric transcription factor which has long been established to activate transcription of several MMPs (Richmond, 2002). A number of inflammatory cytokines trigger the NFκβ pathway resulting in loss of TJ integrity, and consequent BBB disruption (Chen et al., 2011; Aslam et al., 2012; Coelho-Santos et al., 2015). NFκβ can be sequestered in the cytoplasm by the Inhibitor of κβ (Iκβ), whose phosphorylation on specific serine residues leads to its ubiquitination and proteasome degradation, freeing NFκβ to enter the nucleus, bind to its specific promoter elements and activate gene transcription (Fig. 3). Despite the fact that Iκβ phosphorylation is not directly regulated by Akt, it has been suggested that Akt can mediate the activation of the Iκβ kinase complex (IKK) resulting in Iκβ phosphorylation and consequent degradation (Li and Stark, 2002; Bai et al., 2009). *In vitro* studies in Human Umbilical Vein ECs (HUVECs) suggest Akt could be a mediator of NFκβ nuclear transduction in the context of

the vascular endothelium, although if Akt's effects on NFκβ cellular location are direct or indirect remains to be determined (Yu et al., 2014).

The NFκβ family of transcription factors can regulate the expression of a wide range of genes implicated in various cellular mechanisms, and some are intimately involved in the formation of TJs: in ECs NFκβ directly represses claudin-5 expression by binding to its promoter (Aslam et al., 2012) and binding regions have been identified in MMP14 promoter (Haas et al., 1999). Studies in several cell types point to many possible BBB-related genes that could be also regulated by NFκβ, yet to be studied in an EC-BBB context. For example, NFκβ binding regions have been identified in the occludin promoter (Wachtel et al., 2001; Kimura et al., 2008); characterization of the MLCK human promoter showed various NFκβ responsive elements (Graham et al., 2006) (which will lead to TJ internalization as previously discussed). NFkβ activation also results in the transcription of several (M)MPs (such as MMP1, 3, -9, -10, -12 and -13 (Lee et al., 2007; Akhtar et al., 2010; Fanjul-Fernandez et al., 2010; Nakayama, 2013; Yun et al., 2014) and some ADAMTS proteinases (Li et al., 2015; Sun et al., 2015). Recent *in vitro* studies with a human brain microvascular EC line (bEnd.3) has shown that under hypoxic conditions NFκβ can mediate BBB disruption through enhanced MMP9 expression (Won et al., 2015).

Of particular interest is the already discussed role of MMP9 in Notch1 activation, since it raises the possibility of a positive feedback loop between the NFκβ and Notch pathways. MMP9-mediated Notch activation will lead to PTEN inhibition and the consequent Akt activation, now able to enhance NFκβ DNA binding ability and the transcription of those NFκβ responsive genes, such as MMP9 further compromising BBB integrity (Fig. 3).

Additional complexity in the NFκβ pathway is provided by membrane-type matrix metalloproteinases (MT-MMPs) some of which have a cytoplasmic tail that can trigger signal transduction cascades (Itoh, 2015). For example in macrophages, MMP14 (also known as MT1-MMP) can induce the PI3K/Akt signalling cascade in a proteinase-independent manner (Ohtake, 2006). Studies in a human breast cancer cell line confirm MMP14 activation of Akt, and also showed that Akt inhibition triggered a reduction in MMP14 levels (Eisenach et al., 2010) supporting the previously discussed idea that Akt may be mediating (M)MPs expression by ultimately modulating the activity of particular transcription factors (such as NFκβ). A number of studies have explored the roles of MMP14 in endothelial cell signalling (reviewed in Ohkawara et al., 2015) showing a TNFα-dependent reduction in Akt phosphorylation and an association of cytoplasmic MMP14 with Akt which can modulate NFκβ responses (Findley et al., 2007). More recent studies show that under oxidative stress (a well described activator of NFκβ), the presence of a broad spectrum (M)MP inhibitor could reverse induced occludin loss and intercellular gap formation, although barrier function was not restored. These results suggest that in addition to (M)MPs, there is a complex effect of oxidative stress in barrier permeability (Lischper et al., 2010).

Thus (M)MPs may modulate signalling pathways underlying BBB integrity as well as acting as proteolytic enzymes responsible for direct BBB disruption but the cellular context/stimulus is key.

Adherens junctions in metzincin-mediated BBB integrity

The impact of metzincins on Adherens Junctions (AJs) is also of a great interest in the context of the BBB. Recent studies point at VE-cadherin (a main component of AJs) as a possible metalloproteinase target. Microvascular ECs isolated from lungs of TIMP3 knockout mice showed a reduction in barrier ability associated with a disrupted expression of membrane VE-cadherin. Both barrier function and VE-cadherin expression were rescued in the presence of a broad-spectrum metalloproteinase inhibitor (Arpino et al., 2016). It is important to consider that TIMP3 not only inhibits MMPs, but also several members of the ADAM family, including ADAM10. Interestingly, Reyat and colleagues described a significant increase in VE-cadherin surface expression in ADAM10 siRNA knockdown cells (Reyat et al., 2017). Thus, ADAM10-mediated VE-cadherin cleavage could have a direct impact on BBB stability, although it is important to consider that other metalloproteinases could also be involved in this degradation. In order to clarify these observations further studies are needed in the context of the BBB. Junctional Adhesion Molecule A (JAM-A) is also shed by ADAM17 in cultured endothelial cells treated with pro-inflammatory cytokines (Koenen et al., 2009) and LPS-induced JAM-A cleavage was reduced after ADAM17 silencing in human lung microvascular endothelial cells (Dreymueller et al., 2012b). Interestingly, studies in human brain microvascular ECs exposed to HIV-infected monocytes revealed that JAM-A shedding was not exclusive to ADAM17, since normal levels of JAM-A were restored after treatment with an MMP9 specific inhibitor (Huang et al., 2009).

Interestingly, a crosstalk between AJs and TJs has been suggested in the literature (Tietz and Engelhardt, 2015). One of the key elements of this junctional crosstalk is VE-cadherin, which modulates TJ stability through regulation of claudin-5 transcription (Taddei et al., 2008).

Despite the lack of a complete characterization of the claudin-5 promoter, bioinformatic analysis has predicted paired binding regions for Forkhead box protein O1 (FoxO1) and β-catenin suggesting an interaction between these two transcription factors (Taddei et al., 2008). Chromatin immunoprecipitation (ChIP) together with luciferase assays showed that a direct interaction between β-catenin and FoxO1 is

needed to stabilise FoxO1's binding to the claudin-5 promoter and enhance its repressor activity (Taddei et al., 2008). When forming AJs, transmembrane VE-cadherin forms a complex with and sequesters β-catenin at the plasma membrane, impairing its nuclear translocation. Due to this intracellular redistribution, FoxO1 repressor activity can no longer be enhanced by β-catenin interaction, resulting in increased claudin-5 expression and consequent TJ stabilization (Taddei et al., 2008).

Additionally, FoxO1 activity and cellular localization is determined by its phosphorylation status: DNA binding activity is truncated by phosphorylation at Ser256, whereas nuclear exclusion is promoted by phosphorylation at Thr24 (Daitoku et al., 2011). Taddei et al. demonstrated that clustering of VE-cadherin at the plasma membrane can lead to activation of the PI3K/Akt pathway and consequent phosphorylation of FoxO1, which can no longer repress claudin-5 (Taddei et al., 2008). *In vivo* and *in vitro* studies with Akt1 null mice or with Akt-depleted human microvascular ECs (HMECs) stimulated with pro- and anti-vascular agents confirm the effect of Akt on FoxO -dependent claudin-5 expression and endothelial barrier integrity (Gao et al., 2016). Complementary to these studies, mice expressing a truncated form of VE-cadherin lacking the β-catenin-binding cytoplasmatic tail showed that a cadherin-catenin complex is required for PI3K recruitment and activation of the PI3K/Akt pathway (Carmeliet et al., 1999).

Supporting the hypothesis of VE-cadherin/β-catenin mediated regulation of claudin-5 expression, *in vitro* studies with brain microvascular endothelial cells not only corroborate the previously discussed mechanisms but also suggest an essential role for non-muscle myosin light chain kinase (nm-MLCK) in the pathway (Beard et al., 2014). Importantly this study assessed VE-cadherin modulation of TJ stability under inflammatory conditions; hence, nm-MLCK role in VE-cadherin/β-catenin regulation of claudin-5 expression needs to be interpreted in this context, while other intermediates may vary depending on the extracellular environment.

Metalloproteinase effects on AJs should thus not only be considered from the perspective of a direct impact on barrier stability: VE-cadherin cleavage will not only impair intercellular contact formation but will also release membrane-bound β-catenin, now free to modulate TJ stability through claudin-5 transcription. Thus, AJ-TJ crosstalk mechanisms should be explored further in future BBB studies.

Metzincins as indirect promoters of BBB integrity

It is important to consider the possibility that metzincins may play a key role in limiting leukocyte recruitment (one of the first steps of neuroinflammation) at the BBB. MMP2 (together with MMP13 and 14), can terminate the inflammatory response through the cleavage of Macrophage chemoattractant protein 3 (MCP-3) (McQuibban et al., 2000). Full-length MCP-3 promotes the recruitment of active monocytes and leukocytes, but once cleaved it can antagonize chemokine receptors attenuating the inflammatory response (McQuibban et al., 2000). MMP2 may play dual roles during inflammation since studies in $MMP2^{-/-}$ mice showed earlier onset and more severe EAE due to a compensatory increase in MMP9 (Esparza et al., 2004). In contrast to this data, Agrawal et al. did not report any variation in EAE course or severity in their $MMP2^{-/-}$ mice when compared to wild type littermates, since EAE resistance was exclusively observed in MMP2 /MMP9 double knockout mice (Agrawal et al., 2006). Additionally, fractalkine (a pro-inflammatory chemokine) can be cleaved by MMP2 into a soluble antagonist (Dean and Overall, 2007). Further studies will be needed to define the exact role of MMP2 during the inflammatory response in the biology of the BBB.

MMPs often interface closely with the serine proteinase plasminogen activator family. Tissue Plasminogen Activator (tPA) has been shown to induce BBB disruption by promoting MMP9 (Wang et al., 2003) and NFκβ activation (Cheng et al., 2006). Protease-activated receptor 1 (PAR-1) is an intermediate in the tPA/NFκβ/MMP9 pathway (Cheng et al., 2006) that can be cleaved and consequently activated by MMP1 (Boire et al., 2005). Although the impact of PAR-1's MMP1-mediated activation on the NFκβ/MMP9 axis has not yet been elucidated, several studies have described MMP1- mediated shedding of PAR-1 in endothelial cells (Goerge et al., 2006; Blackburn and Brinckerhoff, 2008; Tressel et al., 2011; Nugent et al., 2016). However, the impact of this pathway in the context of the BBB and inflammation remains to be fully studied.

von Willebrand factor (VWF) is a multimeric glycoprotein with an essential role in thrombus formation and the only known substrate for ADAMTS-13. Although the underlying mechanisms are not fully understood, *in vivo* studies in a murine stroke model have revealed that tPA can increase VWF plasma levels leading to BBB disruption. ADAMTS-13 may mitigate this pathway though VWF degradation (Wang et al., 2013). Interestingly, studies in VWF-null mice reveal that, unchallenged, these mice do not have a BBB phenotype but are far more susceptible to hypoxia/reperfusion insult. Although the status of ADAMTS13 in VWF-null mice was not studied, an increase in claudin-5 levels was noted (Suidan et al., 2013). Alternatively, tPA can also induce BBB disruption by upregulating the VEGF/MMP9 pathway (Kanazawa et al., 2011). In this scenario, ADAMTS13 might protect BBB stability by abrogating the VEGF pathway in a dose-dependent manner, though the molecular mechanisms are yet unknown, co-immunoprecipitation experiments point to a direct ADAMTS13/VEGF interaction (Lee et al., 2012). However, it is possible ADAMTS13 also has detrimental effect on BBB integrity by antagonising the positive

effect of VEGF on angiogenesis, opposite to the effect of MMP9 (Bergers et al., 2000).

Conclusions and future directions

It is clear that the BBB plays a pivotal role in a wide range of acute and chronic inflammatory neurological disorders, including multiple sclerosis and stroke. The importance and complexity of metzincin roles in BBB integrity is gradually being elucidated and becoming a more widespread field of study. However, it seems that metzincin effects on BBB stability could be the outcome of a complex network of intracellular mechanisms. In order to start untangling this highly intricate network in human cells, new models may be beneficial. Further refinement of human BBB models in which interactions between different pathways can be dissected is needed. A good example of this is the novel BBB *in vitro* model generated by Yamamizu et al. (Yamamizu et al., 2017) in which human induced pluripotent stem cells (hiPSC) were used to generate a co-culture of the 4 different cell populations integrating the BBB (ECs, pericytes, astrocytes and neurons) (Yamamizu et al., 2017).

Hence, a better understanding of the interplay between networks and signalling pathways underlying BBB maintenance could offer new insights on BBB pathological dysregulation, as well as help to identify novel therapeutic targets in a wide range of neurological disorders.

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