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## Pericytes of the neurovascular unit: Key functions and signaling pathways

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### Abstract

Pericytes are vascular mural cells embedded in the basement membrane of blood microvessels. They extend their processes along capillaries, pre-capillary arterioles, and post-capillary venules. The central nervous system (CNS) pericytes are uniquely positioned within the neurovascular unit between endothelial cells, astrocytes, and neurons. They integrate, coordinate, and process signals from their neighboring cells to generate diverse functional responses that are critical for CNS functions in health and disease including regulation of the blood-brain barrier permeability, angiogenesis, clearance of toxic metabolites, capillary hemodynamic responses, neuroinflammation, and stem cell activity. Here, we examine the key signaling pathways between pericytes and their neighboring endothelial cells, astrocytes, and neurons that control neurovascular functions. We also review the role of pericytes in different CNS disorders including rare monogenic diseases and complex neurological disorders such as Alzheimer's disease and brain tumors. Finally, we discuss directions for future studies.

### Keywords

Pericytes; neurovascular functions; signal transduction pathways; neurological disorders

The neurovascular unit (NVU) is comprised of vascular cells (pericytes, vascular smooth muscle cells (VSMCs), endothelial cells), glial cells (astrocytes, microglia, oligodendrocytes), and neurons<sup>1–3</sup>. Pericytes are centrally positioned within the NVU between endothelial cells, astrocytes, and neurons (Figure 1a). They receive signals from their neighboring cells and generate functional responses that are essential for proper central nervous system (CNS) functioning<sup>2,4–6</sup> (Figure 1b).

Endothelial cells form the blood-brain barrier (BBB) that sanctions entry of macromolecules, cells, and pathogens from blood into the CNS. Brain endothelium also regulates CNS transport of energy metabolites, nutrients, and ions and clearance of neurotoxic metabolites<sup>1,7</sup>. The BBB integrity is maintained chiefly by pericytes<sup>8–10</sup>.

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Endothelial tight junctions and lack of fenestrae contribute to a physical barrier<sup>5,7-11</sup>, which prevents transport of peptides and proteins into the brain<sup>12,13</sup> unless they have specific carriers and/or receptors in brain endothelium<sup>14,15</sup>. The BBB integrity is vital for normal CNS functions as illustrated by rare genetic human diseases where specific gene defects in pericytes, endothelial cells, or astrocytes lead to NVU disruption and neurological disorders<sup>7</sup>. Pericyte degeneration and BBB breakdown are found in complex neurological disorders such as Alzheimer's disease (AD)<sup>1,3,16</sup>. Additionally, pericytes contribute to CNS tumor angiogenesis and growth<sup>5</sup>.

Here, we review the functions and signal transduction pathways in CNS pericytes in health and disease.

## Pericytes: Characterization, function, and dysfunction

### Characterization

Pericytes are embedded in the basement membrane of small blood vessels including capillaries, pre-capillary arterioles, and post-capillary venules<sup>2</sup>. Originally described by Charles Rouget, the term 'pericytes' was coined by Zimmermann in 1923 who proposed several subtypes of pericytes based on their morphology, location within the vascular network, and function<sup>17,18</sup>. Pericytes express several contractile and cytoskeletal proteins (e.g.,  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), vimentin, desmin, myosin, nestin)<sup>5,6,19-21</sup> and cell surface antigens (e.g., transmembrane chondroitin sulfate proteoglycan NG2, platelet-derived growth factor receptor- $\beta$  (PDGFR $\beta$ ), aminopeptidases A and N (CD13), regulator of G-protein signaling-5 (RGS5), CD146)<sup>5,18,21,22</sup>, some of which are also found on VSMCs<sup>2,21,22</sup>.

Recent studies of the cortical angioarchitecture in mice expressing fluorescent proteins under control of the NG2 and PDGFR $\beta$  promoters have identified several pericyte subpopulations including VSMC-pericyte hybrid on pre-capillary arterioles, thin strand helical mid-capillary pericyte, and mesh pericyte with a stellate morphology on post-capillary arterioles and venules<sup>19,20</sup>. Future single-cell RNA-seq and proteomic studies as used successfully to characterize subpopulations of cortical progenitor cells<sup>23</sup> and drug resistant tumor cells<sup>24</sup>, may also contribute to better understanding of different pericyte subtypes.

### Function

Pericytes regulate BBB permeability, angiogenesis, clearance, cerebral blood flow (CBF), neuroinflammation, and stem cell activity (Figure 1b).

**BBB permeability**—Pericytes control the expression of endothelial BBB tight and adherens junction proteins, the alignment of tight junction proteins, and bulk flow transcytosis of fluid-filled vesicles across the BBB<sup>8-10</sup>. The molecular pathways between endothelial cells and pericytes that can be manipulated to open the BBB 'on demand' for delivery of neuropharmaceuticals and/or to reverse BBB breakdown in neurological disorders<sup>7</sup>, remain at present, however, largely unknown.

**Angiogenesis**—Pericytes regulate angiogenesis, microvascular stability, and angioarchitecture during CNS development and vascular remodeling<sup>2,5,25</sup>.

**Clearance**—Pericytes can act as perivascular tissue macrophages to clear tissue debris and foreign proteins injected systemically and/or locally in the CNS<sup>2,9,10,26,27</sup> and participate in clearance of Alzheimer's amyloid- $\beta$  (A $\beta$ ) toxin, as shown in AD mice<sup>26</sup>.

**Cerebral blood flow**—VSMCs control dilation and constriction of arterioles and small brain arteries<sup>28,29</sup>. However, recent studies in cortical and cerebellar brain slices and retinal explants and *in vivo* studies of cortical, retinal, olfactory bulb, and ear microcirculation have demonstrated that capillaries contribute to hemodynamic responses<sup>19,30–34</sup>. It has been shown that pericytes regulate capillary tone and diameter<sup>6,18,35</sup>, as discussed in greater detail below. Some recent studies failed to show the role of pericytes in capillary blood flow regulation<sup>36</sup>, but the controversy has been attributed to a drift in pericyte definition, particularly renaming the mid-capillary pericytes into VSMCs<sup>19</sup>.

**Neuroinflammation**—Studies using transgenic pericyte-deficient mice have shown that pericytes control endothelial-mediated leukocyte adhesion and transmigration into the CNS<sup>37</sup>, and studies in wild type mice demonstrate enhanced leukocytes trafficking in microvascular regions lacking pericyte coverage<sup>38,39</sup>. The role of pericytes in neuroinflammation has also been suggested by *in vitro* studies<sup>40,41</sup>. Altogether, these findings suggest that immune activation of brain pericytes may contribute to communicating inflammatory signals within the NVU.

**Stem cell activity**—*In vitro* studies have suggested that cultured pericytes have multipotent stem cell potential<sup>17,42</sup>. Moreover, primary murine pericytes isolated from brain following ischemic stroke exhibited multipotential stem cell activity and differentiated into neural and vascular lineage cells<sup>42</sup>.

## Dysfunction

Pericyte degeneration leads to BBB breakdown causing brain accumulation of blood-derived neurotoxic molecules<sup>10,43–45</sup>. Pericyte ischemic injury results in contractile rigor and obstruction of capillary blood flow<sup>30,46</sup>. Pericyte-specific genetic defects lead to primary familial brain calcification or Fahr's disease<sup>47,48</sup>. Pericytes degenerate and likely play a role in cerebrovascular dysfunction in complex neurological diseases such as AD<sup>26,49,50</sup>, amyotrophic lateral sclerosis (ALS)<sup>51</sup>, and type 2 diabetes mellitus (T2DM)-related microangiopathy and retinopathy<sup>52–54</sup>. Pericyte dysfunction has been also associated with human immunodeficiency virus (HIV)-related dementia<sup>55</sup>, epilepsy<sup>56</sup>, cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL)<sup>57</sup>, and brain cancer<sup>58,59</sup>.

## Pericyte-endothelial signal transduction

Some of the key pathways between pericytes and endothelial cells are discussed below.

## PDGF-BB/PDGFR $\beta$ pathway

**Pathway characterization**—The platelet-derived growth factor (PDGF) family has four ligands (A-D) and two receptors ( $\alpha$  and  $\beta$ )<sup>59</sup>. PDGF receptor tyrosine kinases (PDGFR) form three active conformation dimers –  $\alpha\alpha$ ,  $\beta\beta$  and  $\alpha\beta$ . The PDGF ligands differentially bind PDGFRs, specifically i) PDGFR- $\alpha\alpha$  ligands are PDGF-AA, -CC, -AB, -BB; ii) PDGFR- $\alpha\beta$  ligands are PDGF-AB, -BB, -CC, -DD; and iii) PDGFR- $\beta\beta$  ligands are PDGF-BB, -DD; the underlined ligands denote high affinity ligand-receptor interactions<sup>59</sup>. Here, we will focus on endothelial-secreted PDGF-BB and PDGFR $\beta$ , a receptor on pericytes<sup>5,60,61</sup>.

Precise spatial and temporal regulation of PDGF-BB signaling is achieved via its “retention motif,” a region of positively charged amino acids at the C terminus, which binds to negatively charged heparin sulfate proteoglycans of the extracellular matrix (ECM) resulting in retention of PDGF-BB that generates a concentration gradient<sup>5</sup>, as shown by *in vitro* studies. PDGF-BB binds to PDGFR $\beta$  causing non-covalent dimerization and autophosphorylation of the receptor on up to 13 cytoplasmic tyrosine (Y) residues, which activates PDGFR $\beta$ <sup>62</sup> (Figure 2). Once activated, distinct phosphorylated Y residues of PDGFR $\beta$  are bound by specific Src homology 2 (SH2) domain-containing proteins, including phospholipase C $\gamma$  (PLC $\gamma$ ), Src family kinase (SFK), Grb2, phosphatidylinositol 3-phosphate (PI3K), GTPase activating protein (GAP), SH2 tyrosine phosphatase (SHP-2) and Stat5, to induce downstream signaling, which promotes pericyte survival, proliferation, migration, and recruitment to the vessel wall<sup>2,62</sup>.

RasGAP binding to phospho-PDGFR $\beta$  causes simultaneous binding of the Grb2-SOS1 complex to Ras that offsets the activation of Ras and reduces the downstream activity of the extracellular signal-regulated protein kinases 1 and 2 (Erk1/2), as shown *in vitro*<sup>63</sup>. Activation of Notch ligands and/or Notch receptors and activation of a Notch signal integrator transcription factor, CSL, can modulate PDGFR $\beta$  signaling independently of PDGF-BB, as shown using *in vitro* cultures and Notch3-deficient mice<sup>64</sup> (Figure 2).

**Functional importance**—Developmental studies using *Pdgfb* and *Pdgfr $\beta$*  transgenic mouse models<sup>60,62</sup> have shown that PDGF-BB/PDGFR $\beta$  signaling is important for endothelial-mesenchymal communication and CNS blood vessel development and stabilization<sup>5</sup>. Both *Pdgfb* and *Pdgfr $\beta$*  null mice are embryonic lethal and lead to development of CNS microvascular instability, endothelial hyperplasia, microaneurisms, and blood vessel ruptures with microhemorrhages, whereas deletion of the single allele does not result in an apparent vascular phenotype in the developing CNS.

In contrast, during postnatal development, adulthood and aging brain pericytes may fulfill a different regulatory role, as suggested by recent work in mouse models with partially disrupted PDGF-BB/PDGFR $\beta$  signaling due to either mutation in the PDGF-BB retention motif (*Pdgfb*<sup>ret/ret</sup>) or deficient PDGFR $\beta$  signaling which both result in age-dependent BBB breakdown and accumulation of blood-derived neurotoxic proteins in the neuropil and brain interstitial fluid (ISF)<sup>8-10</sup>. Deficient PDGFR $\beta$  signaling also leads to microvascular reductions, which in parallel with BBB breakdown may contribute to secondary neurodegeneration<sup>10,43</sup>.

In animal models of diabetic retinopathy, hyperglycemia leads to diminished PDGFR $\beta$  signaling resulting in pericyte apoptosis<sup>52</sup>, whereas studies of tumor angiogenesis have shown that pericyte loss may lead to endothelial apoptosis<sup>65</sup>. PDGFR $\beta$  signal transduction in pericytes also mediates proinflammatory responses at the BBB by transcriptional regulation of several chemokines that promote endothelial expression of monocyte chemoattractant protein-1 (MCP-1), nitric oxide (NO), interleukins IL-1, IL-6, IL-12, and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), resulting in transvascular trafficking of macrophages and leukocytes into the brain, as shown in pericyte-deficient *Pdgfr $\beta$ <sup>+/-</sup>* mice<sup>37</sup>. Disrupted PDGF-BB/PDGFR $\beta$  signaling upregulates vascular endothelial growth factor (VEGF)-A that accelerates vascular abnormalities, as shown *in vivo* in *Pdgfb* and *Pdgfr $\beta$*  deficient mice<sup>66</sup>.

### PDGF-BB/PDGFR $\beta$ signaling in neurological disorders

Dysfunction in the PDGF-BB/PDGFR $\beta$  signaling pathway contributes to various CNS pathophysiologies, as discussed below.

**Fahr's disease**—Fahr's disease is characterized by migraines, mood swings, motor symptoms (e.g., Parkinsonism), and dementia, and its etiology includes loss-of-function mutations in *PDGFB* and *PDGFR $\beta$*  genes<sup>47,48</sup> implicating involvement of pericytes. *PDGFB* mutations in humans and *Pdgfr<sup>ret/ret</sup>* pericyte-deficient transgenic mice with mutation in the retention motif of PDGF-BB<sup>9</sup> lead to calcifications in capillaries and small microvessels occurring mainly in the basal ganglia, which correlates with the degree of pericyte deficiency and BBB breakdown as shown in the murine model of this disease<sup>47</sup>.

Loss-of-function mutations in *SLC20A2* gene encoding the type III sodium-dependent phosphate transporter 2 (PiT-2) are also associated with Fahr's disease<sup>67,68</sup>, and likely involve changes in phosphate transport at the BBB that promote regional brain accumulation of inorganic phosphate that subsequently cause calcium phosphate deposition<sup>67</sup>.

**Alzheimer's disease**—Pericytes degenerate in AD, as shown by post-mortem brain tissue studies in humans<sup>50,69–71</sup> and animal models of AD<sup>26,72,73</sup>. Moreover, plasma PDGF-BB levels are increased in AD patients<sup>74</sup> and soluble PDGFR $\beta$  (sPDGFR $\beta$ ) levels, reflecting pericyte injury<sup>75</sup>, are increased in cerebrospinal fluid (CSF) of subjects with mild dementia and transgenic AD and pericyte-deficient murine models<sup>73</sup>, suggesting dysfunction in PDGF-BB/PDGFR $\beta$  pathway compared to control subjects and in experimental models.

In transgenic mice, deficient *Pdgfr $\beta$*  signaling leads to pericyte loss causing BBB disruption and microvascular reductions followed by neurodegenerative changes independently of A $\beta$ <sup>10</sup>. However, studies in mice overexpressing A $\beta$ -precursor protein (*APP*) crossed with pericyte-deficient *Pdgfr $\beta$ <sup>+/-</sup>* mice, i.e., *APP<sup>S<sup>w</sup>/0</sup>;Pdgfr $\beta$ <sup>+/-</sup>* mice, indicate that defective PDGF-BB/PDGFR $\beta$  signaling leads to faulty A $\beta$  clearance from brain ISF by diminishing low-density lipoprotein receptor-related protein 1 (LRP1)-mediated A $\beta$  clearance on pericytes<sup>26</sup>. Compared to control *APP<sup>S<sup>w</sup>/0</sup>* mice that develop a moderate pericyte loss<sup>26,72</sup>, *APP<sup>S<sup>w</sup>/0</sup>;Pdgfr $\beta$ <sup>+/-</sup>* mice have an earlier onset of cerebral amyloid angiopathy (CAA) and A $\beta$  load, and increased A $\beta$ 40 and A $\beta$ 42 levels in the brain<sup>26</sup>. Interestingly, accelerated pericyte degeneration in *APP<sup>S<sup>w</sup>/0</sup>;Pdgfr $\beta$ <sup>+/-</sup>* mice also leads to tau pathology and neuronal loss, which is not normally seen in *APP<sup>S<sup>w</sup>/0</sup>* mice<sup>26</sup>. These data suggest that a double vascular-A $\beta$

hit is needed for the development of full-spectrum AD pathology in mice. Whether the same double-hit contributes to the pathogenesis of late onset AD in humans, which is characterized by pericyte degeneration<sup>50,69–72</sup>, is unclear at present.

Mutations in *SORL1* and *SORCS1-3* genes encoding proteins containing vacuolar protein sorting-10 (Vps10) domains, namely sorL1 (also known as sorLA) and sorCS1-3, are risk factors for sporadic AD<sup>76,77</sup> and diabetes<sup>78</sup>. Under normal conditions, sorL1 and sorCS1-3 interact with the retromer complex to facilitate intracellular trafficking, recycling, sequestration, and metabolism of different proteins including APP<sup>79</sup>. Single nucleotide polymorphisms (SNPs) in these proteins have been suggested to promote either aberrant APP clearance and/or processing<sup>76</sup>. PDGF-BB binds to sorL1, sorCS1, and sorCS3<sup>80,81</sup>, which may influence its interaction with and/or downstream signaling from PDGFR $\beta$  that in turn might lead to pericyte dysfunction and/or degeneration as seen in late-onset AD<sup>50,69–71,73</sup>. In addition to PDGF-BB, sorL1 binds other LRP1 ligands similarly to LRP1<sup>81</sup>, which may influence LRP1-mediated BBB clearance<sup>82</sup>. More studies are needed to evaluate the effects of sorL1-PDGF-BB and sorCS1/3-PDGF-BB interactions on downstream PDGFR $\beta$  signaling in pericytes and whether these Vps10 proteins can provide a molecular link between AD and diabetes pathogenesis.

Interestingly, presenilin-1 (*PSEN1*) and *PSEN2* mutations, the most frequent cause of autosomal-dominant AD (ADAD)<sup>83,84</sup>, both result in reduced PDGFR $\beta$  mRNA and protein levels, reduced PDGF-BB binding sites, and reduced PDGFR $\beta$  activation and autophosphorylation that consequentially suppress the downstream MEK/ERK and PI3K/Akt signaling pathways, as shown in *PSEN1* and *PSEN2* knockout cells<sup>85</sup>. These changes may lead to pericyte degeneration and BBB disruption that has been reported in post-mortem brain tissue analysis of ADAD patients<sup>84</sup> and *PSEN1* transgenic mutants<sup>86</sup>. Elucidating the exact mechanism by which *PSEN1* and *PSEN2* mutations impair PDGF-BB/PDGFR $\beta$  signaling would importantly inform how pericyte and microvascular dysfunction contributes to ADAD pathophysiology.

Several dominantly inherited rare vasculotropic *APP* mutations within A $\beta$  21 to 23 residues (e.g., Dutch, Flemish, Iowa, Arctic) primarily affect the cerebrovascular system leading to BBB breakdown, CAA, and hemorrhages with recurrent strokes, as recently reviewed<sup>87</sup>. Although *APP* mutations lead to degeneration of mural cells, whether deficient PDGF-BB/PDGFR $\beta$  signaling might contribute to loss of pericytes, rupture of blood vessels, and/or BBB disruption is currently unknown.

A possible mechanism illustrating how deficient PDGF-BB/PDGFR $\beta$  pathway causing pericyte dysfunction and degeneration can contribute to dementia and AD pathology in late-onset and early inherited familial cases is illustrated in Figure 3.

**Amyotrophic lateral sclerosis**—Microvascular pathology is present in sporadic and familial ALS cases carrying superoxide dismutase 1 (*SOD1*) mutations<sup>1,51</sup>. Pericyte degeneration in ALS patients coincides with BBB and blood-spinal cord-barrier (BSCB) breakdown in motor neuron dense regions of the spinal cord and motor cortex<sup>51</sup>. Consistently, ALS transgenic *SOD1* mutants develop BSCB disruption, pericyte deficiency,

and erythrocyte extravasation prior to motor neuron dysfunction<sup>43,51,88</sup>. Moreover, preventing BSCB breakdown delays the onset of motor neuron disorder in *SOD1* mutants<sup>44</sup>. Whether ALS patients with the most frequent genetic cause of ALS, e.g., expanded hexanucleotide repeat of GGGGCC in a noncoding region of the C9orf72 gene<sup>89</sup>, also develop pericyte degeneration and BBB breakdown and/or disrupted PDGF-BB/PDGFR $\beta$  signaling is presently unknown.

**Type 2 diabetes mellitus and diabetic retinopathy**—Hyperglycemia influences the downstream PDGFR $\beta$  signal transduction cascade to induce pericyte apoptosis, as shown *in vivo* in rats and *in vitro* in retinal cultures<sup>52–54</sup> (Figure 2). Hyperglycemia-induced apoptosis *in vivo* can be prevented by inhibition of the PKC $\delta$ -p38 $\alpha$ -SHP1 pathway<sup>52</sup> and AGE-induced apoptosis by PDGFR $\beta$  downstream activation of Akt and NF $\kappa$ B<sup>54</sup>.

**HIV-1-induced neurocognitive deficits**—HIV-1-positive individuals can develop HIV-associated neurocognitive disorders (HAND) and HIV-associated dementia (HAD). Increased BBB permeability and pericyte loss, present in HAND and HAD, is thought to promote neurological impairments via increased transport of the HIV-1 viral neurotoxic protein, Tat101, into the brain<sup>55</sup>. Interestingly, Tat101 increases PDGF-BB expression and PDGF-BB/PDGFR $\beta$  signaling, specifically via MAPK and NF $\kappa$ B activation, resulting in elevated pericyte migration and deficiency<sup>55</sup>.

**Brain cancer**—The exact role of PDGFR $\beta$  signaling in pericytes in regulating growth and maturation of blood vessels in brain tumors remains still largely unexplored. Angiogenesis studies in the transgenic mouse model of pancreatic islet carcinogenesis (RIP1-Tag2) found that the broad spectrum receptor tyrosine kinase inhibitor SU6668, which preferentially targets PDGFR $\beta$ , leads to regression of blood vessels by detaching pericytes from tumor vessels that results in restricted tumor growth<sup>90</sup>. Similarly, SU6668 diminishes pericytes in xenograft tumors and restricts tumor growth<sup>91</sup>. Tumor-derived PDGFR $\beta$ <sup>+</sup> perivascular progenitor cells (PPCs) can differentiate into mature pericytes eliciting vascular stabilization, maturation, and survival<sup>65</sup>. Moreover, specific inhibition of PDGFR $\beta$  signaling eliminates PDGFR $\beta$ <sup>+</sup> PPCs and mature pericytes around tumor vessels, leading to endothelial cell apoptosis in transgenic RIP1-Tag2 mice with pancreatic islet tumors<sup>65</sup>.

A recent study has shown that PDGFR $\beta$  regulates cell proliferation and invasion of medulloblastomas (MB) via JAG2<sup>58</sup>, suggesting that PDGFR $\beta$  could be a potential therapeutic target for MB. It remains unclear, however, whether MB cells have the potential to differentiate into pericytes, as shown for some other types of brain neoplastic cells that can generate pericytes to control blood vessel function during tumor growth<sup>92,93</sup>.

### TGF-P/TGFPR2 pathway

**Pathway characterization**—Transforming growth factor-3 (TGF- $\beta$ ) is expressed in a latent form by pericytes, endothelial cells, neurons, and glia. Studies using *in vitro* primary co-cultures of endothelial cells and pericytes indicate that TGF- $\beta$  activation at the BBB requires pericyte-endothelial cell interaction<sup>94</sup>, specifically formation of the gap junction connexin-43 hemichannels<sup>95</sup>. Activated TGF- $\beta$  binds TGF- $\beta$  receptor 2 (TGF $\beta$ R2) on

pericytes and endothelial cells<sup>2,5</sup>. TGF- $\beta$ /TGF $\beta$ R2 signals downstream via activin receptor-like kinase 5 (Alk5) in both pericytes and endothelial cells or Alk1 only in endothelial cells, as shown in primary bovine retinal cultures<sup>96</sup> (Figure 4). In pericytes, Alk5-Smad2/3/4 signaling inhibits proliferation and promotes differentiation<sup>2,5</sup> (Figure 4, *left*). For example, murine embryos with reduced TGF $\beta$ R2-Alk5-Smad2/3 signaling exhibit enhanced pericyte proliferation<sup>97</sup> and depleting TGF- $\beta$  attenuates mural cell differentiation in murine embryonic mesenchymal cells and endothelial cell co-cultures<sup>98</sup>. In endothelial cells, Alk5 and Alk1 induce opposing effects via distinct downstream signal transduction events<sup>2,5,96</sup>. Specifically, *in vitro* studies in murine embryonic endothelial cells reveal that TGF- $\beta$ /TGF $\beta$ R2 signals downstream via i) Alk5-Smad2/3/4 to induce PDGF-BB expression and activate RBP-J $\kappa$  transcription factor to express N-cadherin to promote attachment; ii) Alk1-Smad1/5/8 to promote proliferation; and iii) PI3K-Akt pathway to promote survival and transcriptionally induce proliferation<sup>2,99,100</sup> (Figure 4, *right*).

**Functional importance**—TGF- $\beta$ /TGF $\beta$ R2 signaling promotes cell differentiation, maturation, proliferation, migration, and attachment of endothelial cells and pericytes, as shown by *in vitro* studies of endothelial cells and pericytes of murine embryos or bovine retinas<sup>96–98</sup> and confirmed *in vivo* in murine models<sup>99</sup>. Studies using transgenic mice with disrupted TGF- $\beta$ /TGF $\beta$ R2 downstream signaling, as for example *Smad1*, *Smad2*, and *Smad4* knockout mice<sup>100,101</sup>, indicate that TGF- $\beta$ /TGF $\beta$ R2 downstream pathways regulate BBB formation, particularly endothelial-to-mesenchymal transition (EndMT) during normal development and vascular stabilization, and that aberrant TGF- $\beta$ /TGF $\beta$ R2 signaling within this pathway leads to development of brain hemorrhages<sup>99</sup>.

Recent studies have shown that the forkhead transcription factor Foxf2 is specifically expressed in brain pericytes, and that Foxf2 knockout embryos develop intracerebral hemorrhage, perivascular edema, thinning of the vascular basal lamina, an increase in luminal endothelial caveolae, and BBB breakdown<sup>97</sup>.

### TGF- $\beta$ /TGF $\beta$ R2 signaling in neurological disorders

**Neonatal intraventricular hemorrhage (IVH)**—IVH is a major cause of premature infant mortality where hemorrhages are caused by vascular instability resulting from pericyte deficiency<sup>100</sup>. TGF- $\beta$  signaling induces N-cadherin expression to promote BBB integrity, whereas regional disruption in TGF- $\beta$  signaling leads to the development of IVH as shown in transgenic mice with endothelial cell-specific *Smad4* knockout<sup>100</sup>. Mice are protected from IVH via perinatal glucocorticoid administration that increases TGF- $\beta$  signaling, resulting in increased pericyte coverage<sup>102</sup>.

**Cerebral cavernous malformation (CCM)**—CCM is characterized by raspberry-like lesions of microvessels that exhibit pericyte deficiency and enlarged endothelial cells with pinocytotic vesicles and poorly developed tight and adherens junctions, making the vessels prone to hemorrhaging<sup>99,103</sup>. This phenotype is present in sporadic and familial CCM including loss-of-function mutations in *CCM1*- $\beta$ <sup>104</sup>. An analogous vascular phenotype develops in mice with endothelial cell-specific inducible ablation of *Ccm1* (*iCcm1*<sup>EC/EC</sup>)<sup>99,103</sup> and *Ccm2*- $\beta$ <sup>99</sup>. Disrupted TGF $\beta$  signaling at the BBB is reported in



studies using *iCcm1*<sup>EC/EC</sup> mice, human brain tissue of CCM subjects, and cultured human brain vascular cells<sup>99,103</sup> and contributes to CCM by inducing structural instability of capillaries. Human CCM3 also binds to paxillin, a scaffolding protein, and CCM3 and paxillin co-localize in mouse cerebral pericytes<sup>105</sup>.

**Brain cancer**—Several studies indicate the role of TGF- $\beta$  signaling<sup>93,106,107</sup> and pericytes<sup>93,108</sup> in cancer growth and tumor-associated blood vessel function. For example, high TGF- $\beta$ /Smad activity was found in aggressive, highly proliferative gliomas, which confers poor prognosis in patients with glioma<sup>106</sup>. The transcriptomic analysis of primary cultured patient-derived gliomas and human glioma biopsies indicate that the TGF- $\beta$ /Smad pathway promotes proliferation through the induction of PDGF-B in gliomas with an unmethylated PDGF-B gene<sup>106</sup>.

More recent studies revealed that self-renewing tumorigenic glioma stem cells (GSCs) give rise to pericytes to support vessel formation and tumor growth<sup>92,93</sup>. GSCs are recruited to endothelial cells by the stromal-derived factor-1 (SDF-1), an alpha-chemokine that binds to G-protein-coupled CXCR4, and are induced to become pericytes predominantly by TGF- $\beta$ <sup>93</sup>. TGF- $\beta$  signaling has also been shown to regulate vascular phenotype in gliomas, and was sufficient to invoke many of the changes found in a gene signature associated with pathologically altered vessels in human glioblastoma (GBM) grade IV<sup>107</sup>.

**Ischemic stroke**—Multiple studies support the role of TGF- $\beta$ /TGF $\beta$ R2 signaling in mediating angiogenic responses to brain injury due to ischemia or hypoxia<sup>109</sup>, as TGF- $\beta$  signaling stabilizes newly formed microvessels<sup>110</sup>.

## Notch pathway

**Pathway characterization**—Pericytes express Notch3 and endothelial cells express Notch1/4 receptors (Figure 4). Notch ligands such as delta-like ligand 4 (Dll4) and Jagged, activate the receptor to induce cleavage of the Notch intracellular domain (NICD). NICD translocates to the nucleus and interacts with a Notch signal integrator transcription factor, the DNA-binding protein CSL (i.e., CBF-1, RBP-J $\kappa$  suppressor of hairless, Lag-1), to induce transcription of downstream target genes, as shown in *Notch3* knockout mice<sup>111</sup>.

In endothelial cells, the Notch pathway is modulated by canonical Wnt signaling to influence vascular sprouting and remodeling<sup>112</sup>. The Wnt/ $\beta$ -catenin pathway in brain endothelium regulates differentiation of the brain vasculature, angiogenesis, and BBB integrity, as shown by *in vitro* studies using primary murine and rat brain microvascular endothelial cells and endothelial cells with conditional activation of  $\beta$ -catenin, and/or *in vivo* studies using *Pdgfb-iCreERT2*;  *$\beta$ cat*<sup>lox/lox</sup> mice, *Pdgfb-iCreER*; *Nrarp*, *Cttnb*<sup>lox</sup> mice, TOP-Gal Wnt reporter mice, and zebrafish (as reviewed<sup>112,113</sup>).

**Functional importance**—The Notch pathway regulates angiogenic sprouting and microvascular remodeling<sup>111</sup>. During sprouting angiogenesis, specialized endothelial tip cells lead the outgrowth of blood vessel sprouts towards gradients of VEGF-A<sup>114,115</sup>. Studies using *Dll4*<sup>+/-</sup> mice and mice with inducible endothelium-specific inactivation of *Notch1* (VEcad-CreER<sup>T2/R26R/Notch1</sup><sup>floxed/floxed</sup> mice) as well as Notch inhibition by  $\gamma$ -

secretase inhibitors or activation with soluble Jagged-1 peptide, have demonstrated that Dll4-Notch signaling between endothelial cells within the angiogenic sprout serves to restrict tip-cell formation in response to VEGF<sup>116</sup>. This model offers not only an explanation for the dose-dependency and haploinsufficiency of the *Dll4* gene<sup>117,118</sup>, but also suggests that  $\gamma$ -secretase inhibitors and/or modulators of *Dll4* or Notch signaling developed originally for AD, could also be used as important pharmacological regulators of angiogenesis.

### Notch signaling in neurological disorders

**Cerebral cavernous malformation**—Several studies using transgenic models, human tissue, and/or cultured brain vascular cells report CCM is associated with disruptions in TGF $\beta$  and Notch signaling at the BBB<sup>99,103</sup>.

**Neonatal intraventricular hemorrhage**—Similar to disrupted TGF- $\beta$  signaling, dysfunction in Notch signaling is associated with IVH as observed in *Rbpj* (encodes the Notch-related RBP-J $\kappa$  transcription factor) knockout mice<sup>100</sup>.

**Brain cancer**—Activating Notch1 signaling in cultured GBM stem cells induces a vascularization switch and causes the GBM stem cells to differentiate into pericyte-like cells (PDGFR $\beta^+$ , NG2 $^+$ ,  $\alpha$ -SMA $^+$ ) with upregulated expression of angiogenic factors including cytokines, matrix metalloproteinase-9 (MMP-9), and adhesion molecules<sup>92</sup>.

### VEGF-A/VEGFR2 pathway

**Pathway characterization**—Pericytes (paracrine signaling) and endothelial cells (autocrine signaling) secrete VEGF-A that activates the VEGFR2 pathway to increase expression of anti-apoptotic Bcl-2, survivin, and X-linked inhibitor of apoptosis protein (XIAP), as shown by *in vitro* studies of human brain pericyte and endothelial cell co-cultures<sup>119</sup> (Figure 4, *right*). For intracrine signaling, pericytes secrete vitronectin (in a PDGF-BB/PDGFR $\beta$ -dependent process) that activates integrin  $\alpha_V$ -NF $\kappa$ B signaling in endothelial cells to upregulate VEGF-A, which signals intracellularly via VEGFR2 to promote Bcl-w expression and cell survival, as shown by *in vitro* studies of human brain pericyte and endothelial cell co-cultures<sup>119</sup> (Figure 4, *right*).

**Functional importance**—VEGF-A/VEGFR2 signal transduction promotes cell survival, angiogenesis, and vascular permeability<sup>2,119</sup>.

### VEGF-A/VEGFR2 signaling in neurological disorders

**Ischemic brain injury and hypoxia**—*In vivo* studies in cats have shown that brain pericytes migrate away from capillaries rapidly following a hypoxic insult<sup>120</sup> and pericyte-endothelial cell ratio in brain is reduced one week following hypobaric hypoxia in rodents<sup>121</sup>. During hypoxia, VEGF levels are upregulated in pericytes within 24 hours and upregulated in astrocytes after 4 days<sup>121</sup>. Inhibiting VEGF in chronic mild hypoxia (1% O<sub>2</sub>) promotes angiopoietin-1 (Ang1)-mediated endothelial cell tight junction stabilization, whereas severe hypoxia (0.1% O<sub>2</sub>) promotes apoptosis of endothelial cells, as shown in *in vitro* endothelial and pericyte co-cultures<sup>122</sup>. Hypoxia activates hypoxia-inducible factor

(HIF)-1 $\alpha$  that translocates to the nucleus and binds hypoxia-response elements (HRE) to induce target gene transcription that promotes angiogenesis by upregulating VEGF-A and MMPs, anaerobic metabolism, apoptosis, cell survival, and proliferation<sup>123</sup>.

**Brain cancer**—Inhibition of VEGF-induced angiogenesis suppresses growth of human GBM cells *in vivo* in mice<sup>124</sup>. Clinical trials with a monoclonal antibody against VEGF-A, i.e., Bevacizumab, in GBM patients have shown it slows tumor growth but growth was inevitably recurrent<sup>125</sup>. Treatment with an anti-VEGF antibody was least effective in human subjects with GBM exhibiting heightened CBF and angiogenesis<sup>125</sup>. VEGF/VEGFR2 signaling can also function as a negative regulator of receptor tyrosine kinases including PDGFR $\beta$  in pericytes<sup>126</sup> and mesenchymal epithelial transition (MET) receptor in GBM neoplastic cells<sup>127</sup>. Pericytes *in vitro* are also reported to express VEGFR2, and VEGF ablation in tumor cells disrupts PDGFR $\beta$ /VEGFR2 heterocomplex formation and downstream PDGFR $\beta$  signaling in pericytes, and increases tumor vessel maturation<sup>126</sup>. MET was more abundantly expressed in human GBM tissue of patients that exhibited more resistance to Bevacizumab<sup>127</sup>, suggesting that VEGFR2's association with receptor tyrosine kinases in pericytes may play an important role in promoting cancer cell maturation and/or tumor growth.

### Ang/Tie2 pathway

**Pathway characterization**—Ang1, secreted by pericytes, binds to the endothelial-specific Tie2 receptor tyrosine kinase and activates downstream PI3K-Akt pathway in endothelial cells<sup>5</sup>. Angiopoietin-2 (Ang2), expressed by endothelial cells, was originally shown to inhibit Ang1-mediated phosphorylation of Tie2 as well as cellular responses during embryonic development<sup>5</sup>. Ang2 also has proangiogenic activities in adult tissues and cultured endothelial cells, independent of Ang1. For example, in the absence of Ang1, Ang2 binds endothelial Tie2 and activates PI3K-Akt pathway in cultured endothelial cells acting as a Tie2 agonist, but when Ang1 is present, Ang2 dose-dependently inhibits Ang1-induced Tie2 phosphorylation and endothelial cell survival acting as a Tie2 antagonist<sup>128</sup>. Ang2 also binds to endothelial integrins (i.e.,  $\alpha_v\beta_3$ ,  $\alpha_v\beta_5$ ,  $\alpha_5\beta_1$ ) to induce phosphorylation of the integrin adaptor protein, focal adhesion kinase (FAK), and Rac1 activation, which promotes endothelial cell migration and sprouting, as demonstrated in cultured Tie2-silenced endothelial cells and *in vivo* human xenotransplanted endothelial cells in mice<sup>129</sup>.

**Functional importance**—*Ang1* knockout mice exhibit pronounced angiogenic deficits in brain resulting in embryonic lethality, similar to *Tie2* knockout mice<sup>130</sup>. Studies using *in vivo* assays demonstrate significantly attenuated vascular responses to histamine, bradykinin, and VEGF in *Ang2*<sup>-/-</sup> mice<sup>131</sup>. Recombinant Ang1 attenuates retinal vascular disruptions caused by pericyte loss<sup>132</sup>, whereas overexpressing Ang2 in the retina results in pericyte loss and aberrant retinal angiogenesis<sup>133</sup>. Furthermore, cytokine-induced intracellular calcium influx was impaired in *Ang2* null endothelioma cells, consistent with reduced phospholipase activation *in vivo*<sup>131</sup>. Thus, Ang/Tie2 system has a critical role in regulating angiogenesis and vascular permeability, but Ang1 and Ang2 exert diverse effects on endothelial cell functions depending on experimental conditions and models.

### Ang/Tie2 signaling in neurological disorders

**Type 2 diabetes and ischemic stroke**—In db/db transgenic model of diabetes, ischemic stroke leads to enhanced BBB breakdown associated with increased expression of Ang2, and decreased expression of Ang1, Tie2, and tight junction endothelial proteins<sup>134</sup>.

**Brain cancer**—Ang2 is upregulated in tumors and thought to denote the onset of angiogenic sprouting<sup>135</sup>.

### MFSD2A pathway

**Pathway characterization**—The major facilitator superfamily domain-containing 2a (MFSD2A) is a sodium-dependent BBB transporter of long chain fatty acids that is expressed in the brain exclusively in the endothelium, as suggested by studies in mice and humans<sup>136,137</sup>. Studies in transgenic *Mfsd2a* null mice have shown that MFSD2A facilitates transport of docosahexaenoic acid (DHA), an essential omega-3 fatty acid, into the brain<sup>136</sup>. Its expression appears to depend on the presence of pericytes<sup>137</sup>, but the exact molecular pathway remains to be elucidated.

**Functional importance**—MFSD2A exhibits dual functions at the BBB by regulating the formation and maintenance of BBB integrity<sup>137</sup> and delivery of essential fatty acids to the brain as shown *in vitro* in endothelial cell cultures and *in vivo* in murine models<sup>136,137</sup>.

### MFSD2A signaling in neurological disorders

**Microcephaly**—*Mfsd2a* deficient mice develop a significantly reduced brain size, termed microcephaly<sup>136</sup>. Microcephaly syndrome in humans was recently shown to be caused by inactivating mutations in *MFSD2A*, and the syndrome's severity correlated with the degree of functional inactivation of the MFSD2A protein<sup>138,139</sup>.

### Ephrin/Eph pathway

The ephrin receptor tyrosine kinases and their membrane-tethered ephrin ligands provide critical guidance cues at points of cell-to-cell contact<sup>140</sup>. Ephrin B receptors (EphB) and ephrin B ligands play a critical role in the regulation of developmental angiogenesis and pericyte-endothelial interactions during vascular assembly, as shown *in vivo* by genetic loss-of-function studies<sup>140</sup>. Ephrin-B ligands and EphB receptors mark angiogenic vessels *in vivo* in the developing murine retina<sup>141</sup>. Studies in transgenic *Pdgfrb-Cre; Efnb2<sup>lox/lox</sup>* mice reveal that ephrin-B2/EphB4 signaling controls pericyte directional migration and adhesion to maturing vessels<sup>142</sup>. Studies using a genetically engineered mouse in which the lacZ coding region substitutes and reports for the ephrin-B2 coding region, have shown that ephrin-B2 is expressed in brain pericytes and endothelial cells<sup>143</sup>. Moreover, EphB4 was shown to control blood vascular morphogenesis during postnatal angiogenesis<sup>144</sup>. Thus, ephrin-B2 and its receptor EphB4 can participate in vascular remodeling and in different aspects of NVU formation. The ephrin-B/EphB pathway is activated bidirectionally<sup>143</sup>, where ephrin-B binding to EphB receptors causes phosphorylation of both the ephrin-B transmembrane ligand and its EphB receptor<sup>141</sup>.

## Other cell-cell communication pathways

Signaling between integrin  $\alpha_4\beta_1$  expressed by proliferating but not quiescent endothelial cells and VCAM-1 expressed by proliferating but not quiescent pericytes is critical for cell-cell adhesion events required for survival of endothelial and mural cells during vascularization, as shown both by *in vitro* and *in vivo* studies<sup>145</sup>.

Blocking N-cadherin function leads to defective pericyte adhesion, increased pericyte recruitment, and disturbed vascular morphogenesis as shown in chicken embryos<sup>146</sup>. Studies using *in vitro* pluripotent embryonic stem cells demonstrate that N-cadherin is required for the maturation of endothelial sprouts by interacting with pericytes<sup>147</sup>.

Endosialin is not expressed in the normal human adult brain<sup>148,149</sup> or the adult mouse brain<sup>150</sup>, but is abundantly expressed in brain pericytes in the developing murine CNS<sup>150</sup> and tumor vessel-associated pericytes<sup>149</sup>, suggesting that its expression in sites of active tissue remodeling and neovascularization might have implications for angiogenesis, tumor growth, and metastasis.

## Pericyte-astrocyte signal transduction

### CypA-NF $\kappa$ B-MMP-9 pathway

**Pathway characterization**—Astrocyte-secreted apolipoprotein E (APOE) interacts with the cell-surface LRP1 on pericytes that regulates, in an isoform-specific manner (i.e., APOE4 but not APOE2 and APOE3), activation of the proinflammatory BBB-degrading cyclophilin A (CypA)-NF $\kappa$ B-MMP-9 pathway<sup>11</sup> (Figure 5).

**Functional importance**—Increased MMP-9 activity by APOE4, but not APOE2 and APOE3, in the vessel wall leads to degradation of endothelial tight junction and basement membrane proteins causing BBB breakdown<sup>11</sup>, which in turn leads to brain accumulation of blood-derived neurotoxic molecules and erythrocytes and secondary neurodegenerative changes.

### CypA-NF $\kappa$ B-MMP-9 signaling in neurological disorders

**Alzheimer's disease**—The cerebrovascular contributions to dementia and AD are particularly salient in individuals carrying *APOE4* gene, the major genetic risk factor for late-onset sporadic AD<sup>83</sup>. Increases in CypA and MMP-9 CSF levels were recently reported to correlate with BBB breakdown in human *APOE4* carriers, but not age-matched *APOE2* or *APOE3* carriers that have an intact BBB<sup>49</sup>. Additionally, post-mortem analysis in *APOE4*-positive AD patients compared to non-carriers revealed increased CypA and MMP-9 protein levels in hippocampal and cortical pericytes as well as pericyte degeneration<sup>50,151</sup>.

Animal studies have demonstrated BBB breakdown in *APOE4* transgenic mice, but not in *APOE3* or *APOE2* transgenic mice<sup>11,152,153</sup>, resulting in neuronal injury and neurodegeneration<sup>11</sup>.

### Arachidonic acid (AA) pathway

Astrocytes have been proposed to regulate pericyte tone via some of the same signaling pathways as shown for astrocyte-mediated regulation of VSMCs tone<sup>6,30</sup> (Figure 5). The arachidonic acid (AA) pathway has been shown to have a critical role in the regulation of pericyte tone and capillary diameter in studies using rat brain slices and retinal explants<sup>35</sup>. *In vivo* studies in the murine cortex have shown that AA is metabolized in astrocytes into prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) via cyclooxygenase-1 (Cox1), which has been shown to regulate hemodynamic responses<sup>154</sup>. Studies using rat cerebellar slices and retinal explants have shown that PGE<sub>2</sub> activates EP4 receptors in pericytes leading to pericyte relaxation after addition of glutamate to slices<sup>30</sup>. AA secreted by astrocytes is metabolized to 20-hydroxyeicosatetraenoic acid (20-HETE) in pericytes via membrane-bound cytochrome P450 4A, as shown in rat cerebral arterial microsomes and brain slices<sup>30,155</sup>. In turn, 20-HETE leads to contraction of pericytes in cerebral slices and retinal explants<sup>30,35</sup>. Although the role of the AA pathway in astrocytes in controlling pericyte tone and capillary diameter is supported by studies in brain slices, the *in vivo* evidence is still lacking.

### [Ca<sup>2+</sup>]<sub>i</sub>-CaM-MLC pathway

Intracellular Ca<sup>2+</sup>, [Ca<sup>2+</sup>]<sub>i</sub>, increases in response to voltage-gated Ca<sup>2+</sup> channels as shown *in vitro* in primary rat brain pericytes<sup>156</sup> and isolated rat retinal microvascular pericytes<sup>157</sup>, and in response to ROS as shown *in vitro* in human microvascular pericytes<sup>158</sup>, *ex vivo* in rat cerebellar slices<sup>30</sup>, and murine pericytes *in vivo* following ischemic stroke<sup>46</sup>. An increase in extracellular [K<sup>+</sup>] activates voltage-gated Ca<sup>2+</sup> channels resulting in [Ca<sup>2+</sup>]<sub>i</sub> increases and depolarization and contraction of primary rat brain pericytes<sup>156</sup>. Increased [Ca<sup>2+</sup>]<sub>i</sub> in pericytes is shown to promote contraction, possibly via downstream signaling through calmodulin (CaM) and myosin light chain kinase (MLCK) to phosphorylate myosin light chain (MLC) and induce contraction, by analogy to events described in VSMCs of isolated rat cerebral arteries<sup>159</sup>. Pericytes were recently shown to express *My19*, which encodes myosin light chain regulatory polypeptide 9, in single-cell RNA-seq analysis of the murine cortex<sup>160</sup>. Treating isolated rat retinal microvascular pericytes with a cyclic guanosine monophosphate (cGMP) analog inhibits voltage-gated Ca<sup>2+</sup> channels, decreases [Ca<sup>2+</sup>]<sub>i</sub>, and inhibits Ca<sup>2+</sup>-gated Cl<sup>-</sup> channels, which functionally decreases whole-cell Ca<sup>2+</sup> and Cl<sup>-</sup> currents and promotes pericyte relaxation<sup>157</sup>.

### Pericytes-neuronal interactions

Neuronal innervation of pericytes covering brain capillaries is not as well understood as neuronal innervation of VSMCs surrounding arterioles and small arteries<sup>161</sup> despite that capillaries are more numerous and more densely spaced than arterioles, as shown for example in the mouse cortex<sup>162</sup>. Moreover, the average distance between a neuron and a capillary is 8-23 μm in the mouse hippocampus, while the average distance between a neuron and an arteriole is 70-160 μm<sup>163</sup> suggesting that pericytes and capillaries are well positioned to receive chemical transmitters from activated neurons and are likely to respond earlier to changes in neuronal activity than VSMCs.

## Neurotrophic factors

Cultured human brain pericytes express low but comparable levels of neurotrophic factors as cultured astrocytes<sup>164</sup>, but the functional importance of these findings remains unknown. A recent study using a murine hypothalamic GT1-7 cell line has shown that only pericyte-derived media, but not astrocyte or VSMC media, increased the insulin-stimulated phosphorylation of Akt in GT1-7 cells and insulin-dependent tyrosine phosphorylation of insulin receptor  $\beta$ <sup>165</sup>, suggesting that pericytes rather than astrocytes and VSMCs can increase insulin sensitivity in hypothalamic neurons by releasing soluble factors. Given the strategic location of pericytes within the NVU, it is intriguing to speculate whether pericyte-derived molecules can be distributed within the NVU and reach their neuronal targets by para-arterial CSF-ISF flow<sup>166</sup> or peri-vascular flow<sup>167</sup>.

## Neurotransmitters

Studies using bovine retinal pericytes<sup>168</sup> and *ex vivo* rat cerebellar slices and retinal explants<sup>30,35</sup> have shown that norepinephrine leads to pericyte contraction and reduction of capillary diameter. Neurotransmitters that lead to pericyte relaxation include gamma-aminobutyric acid (GABA) as shown *ex vivo* in rat cerebellar slices<sup>35</sup>, adenosine as shown *in vitro* in rat retinal pericytes<sup>169</sup>, glutamate as shown *ex vivo* in rat cerebellar slices<sup>30</sup>, and dopamine as shown *ex vivo* in rat retinal pericytes<sup>170</sup>. Studies in rat cerebellar slices and retinal explants have also shown that glutamate suppresses pericyte contractility through PGE<sub>2</sub><sup>30,35</sup> and that NO blocks 20-HETE-induced pericyte contraction by inhibiting AA conversion to 20-HETE resulting in capillary dilation<sup>30</sup> (Figure 5).

Recent *in vivo* studies have provided important evidence for neuronal control of capillary circulation by showing that capillaries dilate ahead of arterioles in the mouse cortex in response to whisker stimulation<sup>30</sup>.

## Integrating pathways: Towards a systems biology approach

Recent studies also focused on modeling the BBB, NVU, and pericyte functions in blood vessels<sup>171–173</sup>. A computational model predicting disruption of blood vessel development incorporates endothelial, inflammatory, and mural cells (i.e. pericytes)<sup>172</sup>. A physical three-dimensional, multi-compartment, organotypic microphysiological system representative of the NVU – a NVU on a chip – recapitulates all critical barriers in the brain including BBB, brain-CSF and blood-CSF barrier<sup>171</sup>. It has also been suggested that *in silico* modeling might even more rapidly enhance our understanding of the NVU compared to *in vitro* cell-based modeling<sup>173</sup>, but requires a critical level of biological understanding to successfully bridge the logical connectivity of molecular pathways with the computational integration systems<sup>174</sup>.

Here, we schematically illustrate a multi-layered model of the NVU with: i) an interconnected system of pathways within endothelial cells, pericytes, and astrocytes (NVU cells layer); ii) converging points of key signaling pathways in CNS pericytes and between pericytes-endothelial cells and pericytes-astrocytes (Interactive signaling layer); and iii) potential impact of such convergent pathways to neurological disorders (Disorders layer)

(Figure 6). Since all these processes may be species, strain, disease, or context dependent, we provide Figure 6 as an all-inclusive data source and encourage the readers to explore and critically examine the specifics in the existing literature.

## Future directions

Several functions of pericytes such as capillary contractility, neuroinflammation, and multipotent stem cell activity remain still to be fully characterized. It is also unclear how each pericyte subtype contributes to pericyte function, as for example control of CBF versus control of BBB integrity. Developing novel genetic models with mural cell-specific ablation along the vascular tree combined with RNA-seq and proteomic analyses would greatly facilitate the study of CNS pericytes and other mural cell subpopulations.

Many studies have pointed towards pericytes as potentially an attractive cellular target in rare genetic neurological disorders such as Fahr's disease, IVH, CCM, and CADASIL. A growing body of evidence also indicates that targeting pericytes could be an important treatment option to control growth of brain tumors including GBM, as well as to improve vascular remodeling and stabilization of the BBB in AD and possibly other neurodegenerative diseases. The potential of targeting pericytes to open BBB on demand and/or to stabilize dysregulated CBF is another important area for future studies because of pericytes' emerging role in brain diseases affecting BBB and CBF such as stroke and AD.

The systems biology approach can facilitate addressing some emerging questions in the field as, for example, whether some of the pathways identified in rare monogenic neurological diseases caused by genetic defects in pericytes, endothelial cells, or astrocytes that lead to NVU disruption have converging points with complex neurological disorders such as sporadic AD, ALS, and others associated with pericyte and BBB dysfunction. Computational modeling of CNS pericytes combined with high-throughput screening of diverse libraries of compounds is also likely to advance discovery of novel therapeutics for neurological disorders based on correcting aberrant signaling and function of pericytes and their neighboring cell types within the NVU.

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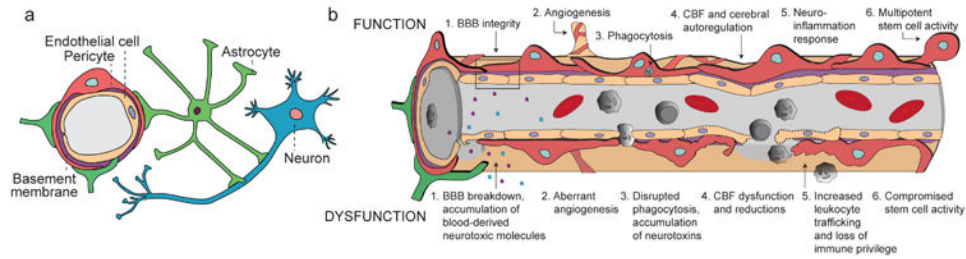
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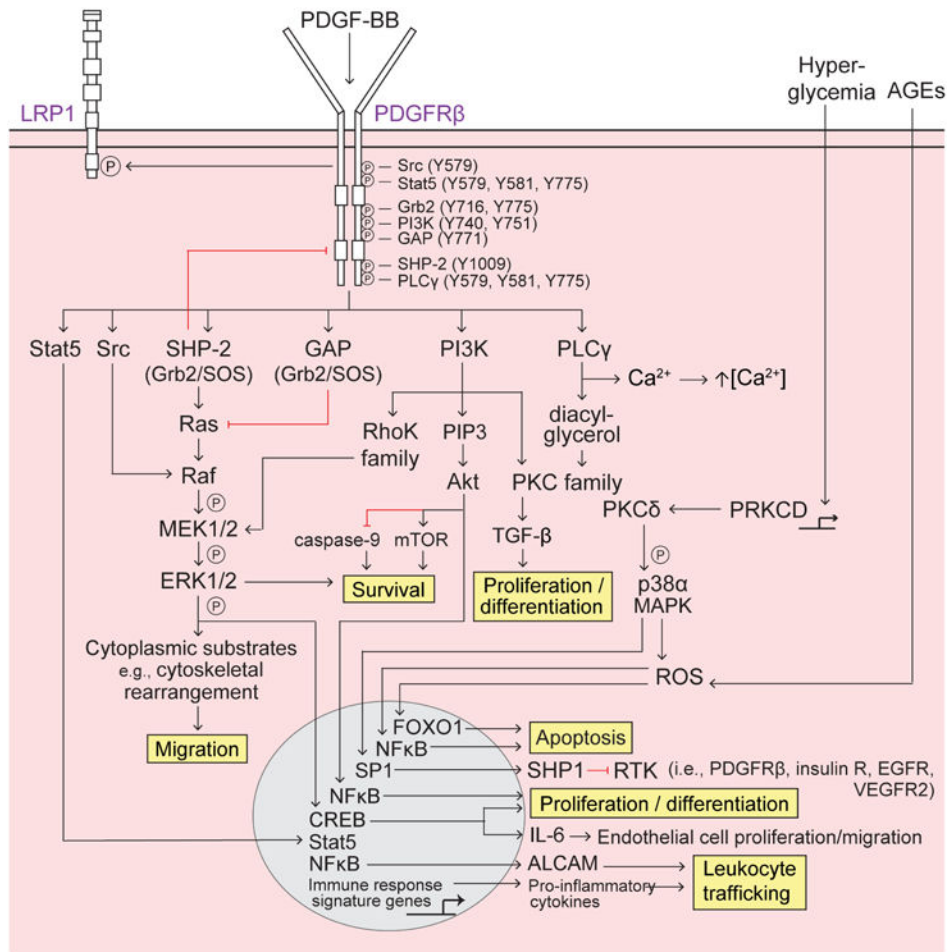
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**Figure 1. The multi-functional role of CNS pericytes at the neurovascular unit (NVU)**

(a) A simplified NVU diagram showing the interactive cellular network at the level of brain capillaries that comprises vascular cells (e.g., pericytes and endothelial cells), glial cells (e.g., astrocytes), and neurons. Intricate cell-cell communication and signal transduction mechanisms of NVU cell types are highly controlled to regulate numerous functions in the CNS. (b) Under physiological conditions (top row), pericytes regulate **1**) blood-brain barrier (BBB) integrity, i.e., tight/adherens junctions and transcytosis across the BBB; **2**) angiogenesis, i.e. microvascular remodeling, stability, and architecture; **3**) phagocytosis, i.e., clearance of toxic metabolites from the CNS; **4**) cerebral blood flow (CBF) and capillary diameter; **5**) neuroinflammation, i.e., leukocyte trafficking into the brain; and **6**) multipotent stem cell activity. Pericyte dysfunction (bottom row) is characterized by **1**) BBB breakdown causing leakage of neurotoxic blood-derived molecules into the brain (e.g., fibrinogen, thrombin, plasminogen, erythrocyte-derived free iron, and anti-brain antibodies); **2**) aberrant angiogenesis; **3**) impaired phagocytosis causing CNS accumulation of neurotoxins; **4**) CBF dysfunction and ischemic capillary obstruction; **5**) increased leukocyte trafficking promoting neuroinflammation; and **6**) impaired stem cell-like ability to differentiate into neuronal and hematopoietic cells. Pericyte dysfunction is present in numerous neurological conditions and can contribute to the disease pathogenesis.



**Figure 2. PDGF-BB/PDGFR $\beta$  signaling in pericytes**

Platelet-derived growth factor-BB (PDGF-BB) secreted by endothelial cells binds to the PDGF receptor- $\beta$  (PDGFR $\beta$ ) on pericytes, causing receptor dimerization, autophosphorylation, and activation. Several Src homology 2 (SH2) domain-containing proteins (Src, Stat5, Grb2, phosphatidylinositol 3-phosphate (PI3K), GTPase activating protein (GAP), SH2 tyrosine phosphatase (SHP-2), and phospholipase C $\gamma$  (PLC $\gamma$ )) bind to distinct phosphorylated (P) tyrosine (Y) residues. SH2 domain-containing proteins bound to PDGFR $\beta$  differentially activate downstream signaling pathways to regulate pericyte survival, migration, apoptosis, proliferation and differentiation, as well as leukocyte trafficking, described as follows: **Survival** – promoted via PI3K-Akt activation of mammalian target of rapamycin (mTOR) and inhibition of caspase-9 and the SHP-2-mediated MAPK pathway; **Migration** – SHP-2-mediated MAPK pathway promotes cytoskeletal rearrangement and cell migration. Src activated Raf synergistically activates the MAPK pathway whereas GAP inhibition of Ras decreases MAPK signaling; **Apoptosis** – Extracellular advanced glycation endproducts (AGEs) induce intracellular reactive oxygen species (ROS) and FOXO1-mediated apoptosis, and PRKCD transcriptional expression of protein kinase C- $\delta$  (PKC $\delta$ ) activates p38 $\alpha$  MAPK to induce downstream production of ROS and mitochondrial cytochrome c release, resulting in apoptosis; **Proliferation and differentiation** – promoted by the PI3K pathway, specifically via PKC-TGF- $\beta$  and PIP3-Akt transcriptional activation of

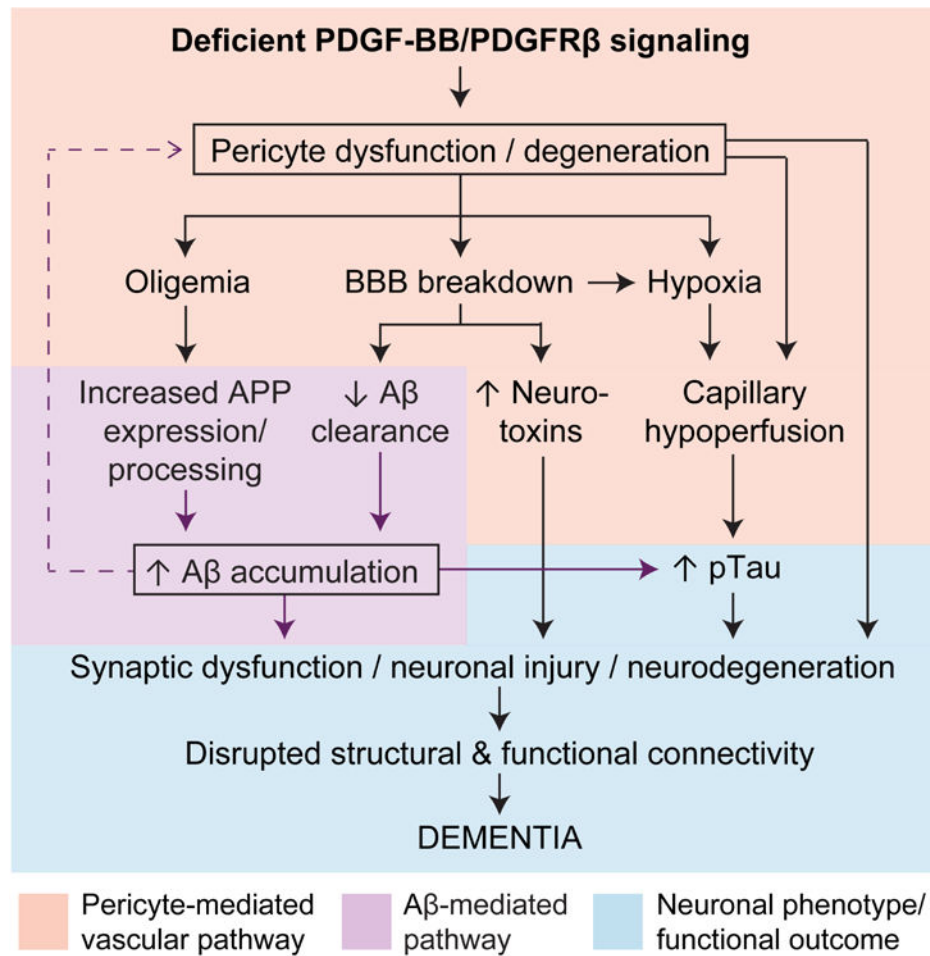
NF $\kappa$ B; Leukocyte trafficking – PDGFR $\beta$  regulates pro-inflammatory responses, e.g., peripheral leukocyte trafficking into the CNS, via transcriptional expression of immune response signaling genes (e.g., cytokines and chemokines) and also via Akt-induced activation of NF $\kappa$ B and transcriptional expression of the novel activated leukocyte cell adhesion molecule (ALCAM).

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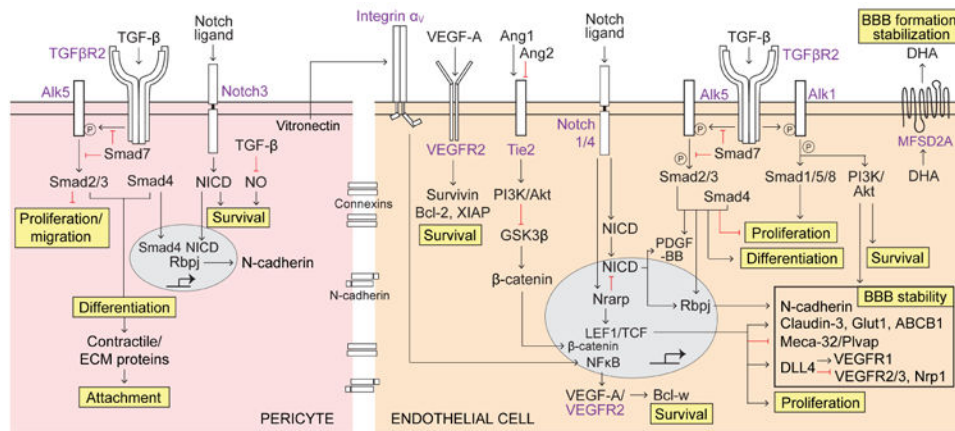
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**Figure 3. Deficient PDGF-BB/PDGFR $\beta$  signaling in pericytes: A possible role in promoting neuronal dysfunction and degeneration in Alzheimer's dementia**

In the *amyloid- $\beta$  (A $\beta$ )-independent pathway* (pink box), a deficient PDGF-BB/PDGFR $\beta$  signaling leads to pericyte dysfunction and/or degeneration resulting in microvascular and cerebral blood flow (CBF) reductions and oligemia (brain hypoperfusion), from one hand, and blood-brain barrier (BBB) breakdown with accumulation of blood-derived toxic products in the brain, from the other. BBB breakdown leads to capillary edema contributing to capillary hypoperfusion and hypoxia. In the *A $\beta$ - pathway* (purple box), oligemia leads to increased A $\beta$  production, whereas BBB breakdown and deficient PDGFR $\beta$  signaling can both lead to faulty A $\beta$  clearance, which in turn promotes A $\beta$  accumulation in the brain. Synergistic action of A $\beta$ -independent and A $\beta$  pathway lead to accelerated tau hyperphosphorylation, formation of neurofibrillary tangles, synaptic dysfunction and loss, and neuronal degeneration, which altogether promotes behavioral deficits and dementia (blue box).



**Figure 4. TGF- $\beta$ /TGF $\beta$ R2, Notch, VEGF-A/VEGFR2, Ang/Tie2 and MFSD2A signaling pathways**

**Pericytes** (left, pink) – Tumor growth factor- $\beta$  (TGF- $\beta$ ) secreted by endothelial cells and pericytes binds to TGF- $\beta$  receptor 2 (TGF $\beta$ R2) that phosphorylates activin-like kinase 5 (Alk5) (inhibited by Smad7) activating the downstream Smad signaling cascade. Activated Smad2/3 inhibits pericyte proliferation and migration. Recruitment of Smad4 to Smad2/3 complex transcriptionally promotes pericyte differentiation, expression of contractile/extracellular matrix (ECM) proteins, and pericyte attachment. TGF- $\beta$  also inhibits nitric oxide (NO) generation promoting survival. Activated Notch3 receptor cleaves its Notch intracellular domain (NICD), which promotes survival. Notch-NICD pathway works cooperatively with TGF- $\beta$ /TGF $\beta$ R2-Smad-4 pathway to stimulate Rbpj-mediated expression of N-cadherin that increases blood-brain barrier (BBB) stability. **Endothelial cells** (right, orange) – VEGF-A/VEGFR2 autocrine/paracrine pathway promotes survival via increased expression of anti-apoptotic Bcl-2, Survivin, and X-linked inhibitor of apoptosis protein (XIAP). Vitronectin secreted by pericytes acts on integrin  $\alpha_V$  on endothelial cells resulting in NF $\kappa$ B-mediated transcriptional expression of VEGF-A and intracrine-mediated VEGF-A/VEGFR2-dependent Bcl-w expression promoting survival. Pericyte-derived Ang1 acts on endothelial Tie2 receptor, and endothelial-secreted Ang2 blocks Ang1 binding to Tie2, acting as a Tie2 antagonist. Ang1/Tie2 activates phosphatidylinositol 3-phosphate (PI3K)/Akt pathway resulting in inhibition of glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ), an inhibitor of  $\beta$ -catenin; this leads to  $\beta$ -catenin nuclear translocation resulting in activation of TCF/LEF transcription factors that control expression of several proteins promoting BBB stability (shown in a box). Notch1/4 contributes to  $\beta$ -catenin-mediated BBB stability via the Notch-regulated ankyrin repeat protein (Nrarp), which increases  $\beta$ -catenin nuclear signaling by inhibiting LEF1 degradation and decreases Notch signaling via NICD destabilization. Additionally, Notch1/4-NICD pathway stimulates PDGF-BB expression and RBP-J $\kappa$ -mediated expression of N-cadherin contributing to BBB stability. As in pericytes, TGF- $\beta$ /TGF $\beta$ R2 pathway in endothelium similarly activates **i)** Alk5-Smad2/3/4 complex to transcriptionally promote differentiation, inhibit proliferation, and induce RBP-J $\kappa$ -mediated expression of N-cadherin, **ii)** Alk1-Smad1/5/8 to promote proliferation, and **iii)** Alk1-PI3K/Akt pathway to promote survival and BBB stability. The major facilitator superfamily domain-containing protein 2a (MFSD2A) facilitates luminal-to-abluminal transport of

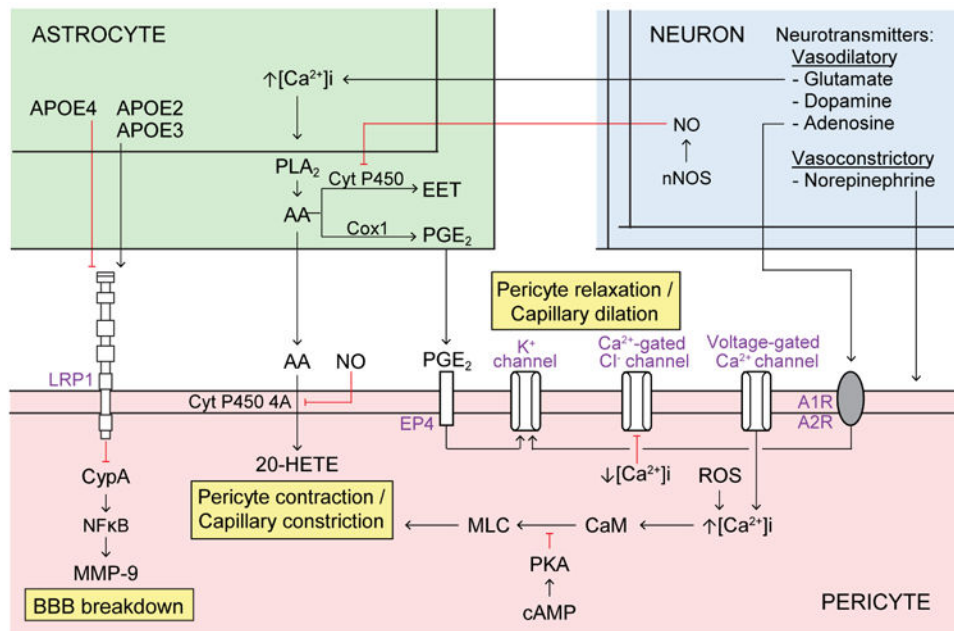
docosahexaenoic acid (DHA), an essential omega-3 fatty acid, and controls formation of the BBB; its expression depends on the presence of pericytes.

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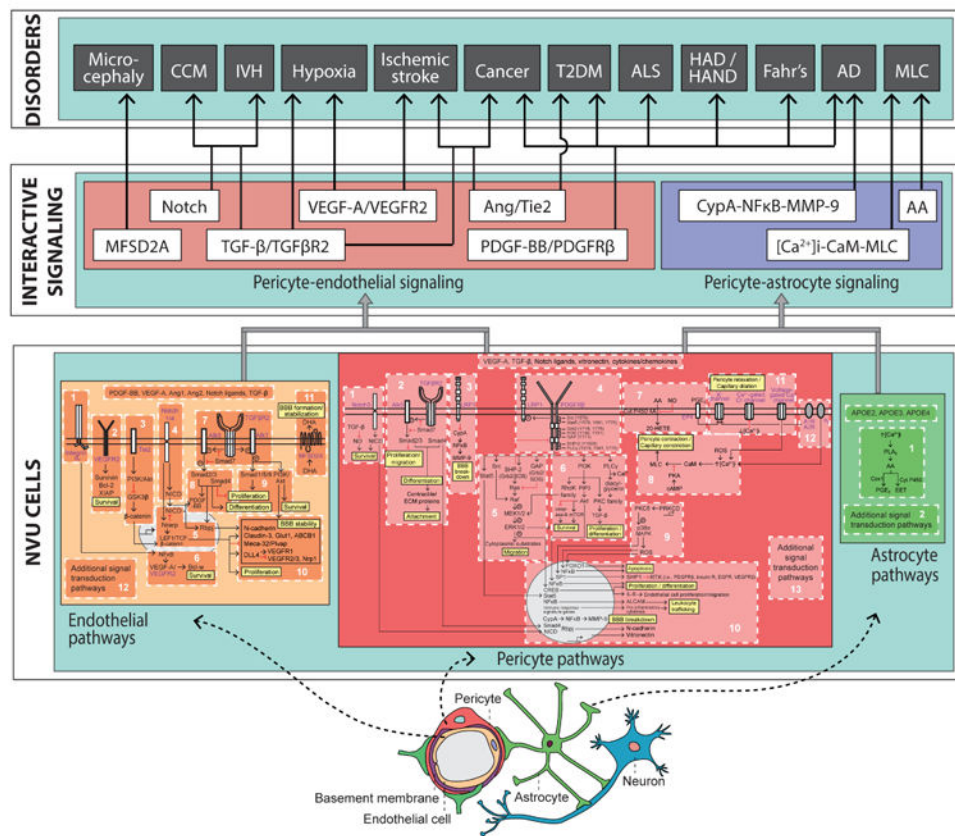
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**Figure 5. Pericyte-astrocyte and pericyte-neuron signaling pathways**

Astrocytes (top left, green) secrete apolipoprotein E (APOE) 2 and 3 that bind to pericyte lipoprotein LRP1 receptor (bottom, orange) to inhibit downstream CypA-NF $\kappa$ B-MMP-9 pathway. In contrast, APOE4 binds weakly to LRP1, which activates the pro-inflammatory CypA-NF $\kappa$ B-MMP-9 cascade leading to blood-brain barrier (BBB) breakdown. Astrocyte intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) increases in response to neuronal factors, for example glutamate, which promotes phospholipase  $\text{A}_2$  ( $\text{PLA}_2$ )-mediated arachidonic acid (AA) generation. In astrocytes, AA is metabolized into prostaglandin  $\text{E}_2$  ( $\text{PGE}_2$ ) via cyclooxygenase-1 (Cox1), as well as into epoxyeicosotrienoic acids (EET) via cytochrome P450. Astrocytic AA is metabolized into 20-HETE in mural cells via membrane-bound cytochrome P450 4A, which promotes pericyte contraction.  $\text{PGE}_2$  from astrocytes binds to pericyte EP4 receptor, which alters  $\text{K}^+$  conductance and promotes pericyte relaxation. Nitric oxide (NO) generated by neurons inhibits cytochrome P450 in astrocytes and cytochrome P450 4A in pericytes to prevent AA to EET and AA to 20-HETE metabolism, respectively. In pericytes, increased cyclic adenosine monophosphate (cAMP) signals via protein kinase A (PKA) to inhibit myosin light chain (MLC) phosphorylation and prevent pericyte contraction. Additionally, pericyte  $[\text{Ca}^{2+}]_i$  increases in response to voltage-gated  $\text{Ca}^{2+}$  channels and reactive oxygen species (ROS). Increased  $[\text{Ca}^{2+}]_i$  in pericytes is shown to promote contraction, possibly via downstream signaling through calmodulin (CaM) and MLC kinase (MLCK) which phosphorylates MLC to induce contraction, as shown in VSMCs. Conversely, decreasing  $[\text{Ca}^{2+}]_i$  in pericytes inhibits  $\text{Ca}^{2+}$ -gated  $\text{Cl}^-$  channels which promotes relaxation. Furthermore, neurotransmitters promote pericyte relaxation (e.g., glutamate, dopamine, and adenosine) or contraction (e.g., norepinephrine). For example, adenosine signals through adenosine A1 and A2 receptors (A1R, A2R) on pericytes to alter  $\text{K}^+$  conductance and promote pericyte relaxation.



**Figure 6. Integrated pathways between pericytes, endothelial cells and astrocytes within the neurovascular unit (NVU)**

A proposed three-layered model of the NVU. The first layer, ‘NVU cells,’ is the foundational layer of cell type-specific systems, each of which consists of integrated modular molecular pathways. Here, we show the endothelial cell (yellow) partitioned into 11 pathways, the pericyte (pink) partitioned into 12 pathways, and the astrocyte (green) partitioned into 1 pathway. Each major signal transduction pathway within each NVU cell type also provides for the modular addition of new signaling pathways, denoted as “additional signal transduction pathways.” The second layer, ‘Interactive Signaling,’ instantiates the converging points of interactions of key signaling pathways between pericytes-endothelial cells and pericytes-astrocytes. The pericyte-endothelial signaling (coral box) at the second layer consists of 6 major signaling pathways: MFSD2A, Notch, TGF- $\beta$ /TGF $\beta$ R2, VEGF-A/VEGFR2, Ang/Tie2, and PDGF-BB/PDGFR $\beta$ . The pericyte-astrocyte signaling (purple box), also at the second layer, consists of 3 major signaling pathway: CypA-NF $\kappa$ B-MMP-9, arachidonic acid (AA), and [Ca<sup>2+</sup>]<sub>i</sub>-CaM-MLC. The third layer, ‘Disorders,’ proposes major signaling pathways of CNS pericytes with neighboring NVU cell types (i.e., endothelial cells and astrocytes) that are suggested to contribute to pericytes dysfunction in neurological disorders, including: Microcephaly, cerebral cavernous malformation (CCM), intraventricular hemorrhage (IVH), hypoxia, ischemic stroke, cancer, type 2 diabetes mellitus (T2DM), amyotrophic lateral sclerosis (ALS), HIV-associated dementia (HAD) and HIV-associated neurocognitive disorders (HAND), Fahr’s disease,



Alzheimer's disease (AD), and megalencephalic leukoencephalopathy with subcortical cysts (MLC).

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