Cerebral blood flow regulation and neurovascular dysfunction in Alzheimer's disease

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Abstract

Cerebral blood flow (CBF) regulation is essential for normal brain function. The mammalian brain has evolved a unique mechanism for CBF control known as neurovascular coupling. This mechanism ensures a rapid increase in the rate of CBF and oxygen delivery to activated brain structures. The neurovascular unit composed of astrocytes, mural vascular smooth muscle cells and pericytes, and endothelia, regulates neurovascular coupling. This article examines the cellular and molecular mechanisms within the neurovascular unit contributing to CBF control, and neurovascular dysfunction in neurodegenerative disorders such as Alzheimer's disease.

Introduction

Brain receives one fifth of the cardiac output, and consumes one fifth of the body's oxygen (O_2) and glucose[1]. Both, O_2 and glucose are delivered to neurons by cerebral blood flow (CBF) and transport across the blood-brain barrier (BBB)[1–3]. Thus, brain functions depend on healthy blood vessels and cardiovascular system. If CBF stops, brain functions stop in seconds, and irreversible damage to neurons occurs in minutes[4].

The CBF is maintained by a coordinated action of interconnected blood vessels, which in human brain form a 400-mile long vascular network[1]. Within this network, cerebral arteries, arterioles and capillaries supply brain with O_2, energy metabolites and nutrients. The cerebral venous return removes carbon dioxide (CO_2) and metabolic waste products from brain into systemic circulation for clearance by lungs, kidney and liver, respectively.

The mammalian brain has evolved a unique mechanism for regional CBF control known as neurovascular coupling or functional hyperemia[5]. This mechanism ensures a rapid increase in the rate of CBF to activated brain structures[2,5]. Under physiological conditions, the capacity of increased CBF and O_2 delivery exceeds metabolic demand and oxygen consumption by activated brain sites providing a large gradient for oxygen diffusion to brain cells furthest from capillaries[2,6,7]. Different cell types within the neurovascular unit (NVU) including astrocytes, mural cells, such as vascular smooth muscle cells (VSMCs) and pericytes, and endothelial cells, contribute to neurovascular coupling[1–3,5]. The NVU cellular composition differs along the vascular tree (Figure 1). However, within the NVU, mural

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cells of the vessel wall are the ones with contractile properties that allow direct control of the vessel diameter and blood flow.

Because of the tight interactions between CBF and neuronal activity, the CBF provides important insights into how the brain works. This forms the basis for functional imaging to evaluate the brain's functional networks[8,9] and responses to different tasks[10–12]. Disrupted functional connectivity and neurovascular uncoupling resulting from a mismatch between CBF, O_2 delivery and neuronal activity, are found in the early stages of many neurological disorders including Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS) and stroke[1,3,5]. Therefore, understanding the cellular and molecular mechanisms underlying physiological and/or pathophysiological CBF responses is critical for understanding brain functions in health and disease.

In this Review, we discuss CBF regulation by the NVU, and neurovascular dysfunction in neurodegenerative disorders with a focus on AD, because neurovascular deficits are well documented in this disorder compared to other neurodegenerative diseases. First, we examine the role of astrocytes, mural cells, and endothelia in neurovascular coupling, and their interactions with neurons, in the arterial, arteriolar and capillary segments of brain microcirculation. Next, we examine cell-signaling pathways mediating dilation and constriction of cerebral blood vessels resulting in CBF increases and reductions, respectively. Finally, the article considers the link between neurovascular dysfunction and neurodegeneration in AD.

Arterial and arteriolar component

The vast network of arteries, arterioles, and capillaries deliver O_2 and nutrients to the brain in a highly regulated manner. The arteries that feed the brain split off the pial arteries that run along the surface of the brain, diving down into the parenchyma, narrowing and branching into arterioles and capillaries. As the size and level of branching change, so too does the cellular composition of the NVU (Figure 1). In this section, we will focus on CBF regulation by arteries and arterioles, role of VSMCs in regulating arteriolar and arterial diameter, and signal transduction pathways within the NVU regulating VSMC tone.

Control of arterial and arteriolar blood flow by VSMCs

Where arteries begin their dive into the brain parenchyma, the NVU is composed of endothelial cells making up the blood vessels surrounded by one to three layers of VSMCs, astrocyte endfeet forming glia limitans that separates cerebrospinal fluid (CSF)-containing Virchow-Robin spaces from brain interstitial fluid (ISF), and neuronal afferent projections[13–15]. In the brain parenchyma, the arteries narrow into arterioles with a single layer of VSMCs and closer apposition of astrocyte endfeet (Figure 1). Arteries and arterioles ensure blood delivery to the brain at a fairly uniform pressure, such that the pulsatile effect of heartbeat is less pronounced in the downstream cerebral microvessels.

During functional hyperemia, arterioles dilate and this dilation propagates at a high-speed in a retrograde direction to upstream arteries, including branches of pial arteries[12,14,16–19]. Studies in rats have shown that dilation may extend more than 1 mm away from the center of

the responding brain region[12]. In addition to regulating CBF delivery to the activated brain sites, studies in rats[20] and mice[21] have demonstrated that arterioles deliver the majority of O_2 during the resting state, which is considered to provide a safe margin of O_2 supply to cerebral tissue[22]. Recent studies in pericyte-deficient mice indicated, however, that capillaries and capillary pericytes also play a role in O_2 delivery to the brain[23]. More work is needed to determine the exact contributions of arteriolar versus capillary O_2 delivery under physiological and pathophysiological conditions, particularly in regards to the timing of arteriolar and capillary dilation in response to neuronal stimulus, which remains a topic of debate[12,3,19,23–27], as discussed below.

The key signaling pathways regulating arteriolar and arterial relaxation and contraction are illustrated in Figure 2, and described in detail below.

Astrocyte-mediated regulation of VSMCs tone

Astrocytes contribute to the regulation of neural circuit formation[28], synapse formation[29], neuronal calcium oscillation[30], plasticity and memory[31], and inflammation in neurodegenerative diseases[32]. However, whether astrocytes and astrocytic calcium, Ca^{2+}, play a role in the regulation of arteriolar tone, remains a controversial topic as examined below.

Earlier reports were not conclusive as to whether an increase in the intracellular Ca^{2+} in astrocytes leads to arteriolar constriction or dilation. Photolysis experiments with caged Ca^{2+} in astrocytes have shown that an increase in the intracellular Ca^{2+}, $[Ca^{2+}]_i$, in the astrocyte endfeet precedes arteriolar constriction in rat and mouse brain slices, which is mediated via the phospholipase A2 (PLA2)-arachidonic acid (AA) pathway in astrocytes followed by free diffusion of AA to VSMCs and conversion into 20-hydroxyeicosatetraenoic acid (20-HETE) via cytochrome p450 (p450) causing VSMCs depolarization and contraction[33] (Figure 2). Using similar rat brain slices another study, however, found exactly the opposite -- that an increase in $[Ca^{2+}]_i$ in astrocytes leads to arteriolar dilation, not constriction[34]. This study has shown that dilation and VSMC relaxation is mediated by metabotropic glutamate receptors (mGluR) and cyclooxygenase (Cox) product prostaglandin E2 (PGE2)[34] (Figure 2). The discrepancy between the two studies has been reconciled in part by acknowledging different experimental conditions, such as that slices in the earlier study were preincubated with N^G-nitro-l-arginine methyl ester (l-NAME) to block nitric oxide (NO) formation and preconstrict blood vessels[34], which has been shown to abolish arteriolar constriction by mGluR agonists leading to a moderate vasodilation[33].

The first *in vivo* study imaging the activity of astrocytes labeled with the Ca^{2+} indicator rhod-2 in the somatosensory cortex of adult mice found that photolysis of caged Ca^{2+} in astrocytic endfeet ensheathing small pial and penetrating arteries leads to vasodilation, which is blocked by Cox1 inhibitors[35]. This study confirmed the role of PLA2-AA pathway in astrocytes and showed that astrocytes *in vivo* can metabolize AA to epoxyeicosatetraenoic acids (EETs) or PGE_2 via cyp450 and Cox1, respectively[35]. Subsequently, EETs released from astrocytes activate large-conductance calcium-activated potassium (BK_{Ca}) channels in VSMCs, whereas PGE2 acts through cyclic adenosine monophosphate (cAMP), both leading to hyperpolarization and VSMCs relaxation[36].

A recent study using genetically encoded Ca^{2+} sensor in astrocytes also demonstrated that physiological activation of neuron terminals triggers $[Ca^{2+}]_i$ increases in astrocyte processes increasing local red blood cell (RBC) flow in brain capillaries[37]. Although suggestive of a major role of capillaries in functional hyperemia[37], this study did not directly show whether astrocytic Ca^{2+} regulates neurovascular coupling to capillary pericytes and/or VSMCs in arterioles, or to both. Another recent *in vivo* study found that astrocytic Ca^{2+} does not regulate arteriolar responses[26].

Several studies disputed the role of astrocytes and astrocytic $[Ca^{2+}]_i$ in regulation of arteriolar responses. For example, stimulus-induced arteriolar dilation persists in inositol 1,4,5-triphosphate (IP3) type-2 receptor (R2) knock-out (KO) mice, in which the primary mechanism of astrocytic $[Ca^{2+}]_i$ increase - the release of Ca^{2+} from intracellular stores following activation of an IP3-dependent pathway[38] (Figure 2) - is lacking[39]. Additionally, *in vivo* astrocytic Ca^{2+} increase in response to sensory stimulus was delayed relative to arteriolar dilation, suggesting that astrocytes do not play a role in arteriolar responses via a Ca^{2+}-dependent mechanism[39]. Others have demonstrated that G-protein-coupled receptors (GPCRs)-IP3R-dependent Ca^{2+} signal in astrocytes does not mediate arteriolar neurovascular coupling in mice implying that mGluR and purinergic receptors in astrocytes are not involved in regulation of arteriolar dilation[40]. Additionally, mGluR3, the only mGluR expressed in adult mouse astrocytes, did not generate Ca^{2+} elevations in response to agonists, suggesting glutamatergic signaling per se is insufficient to trigger astrocytic Ca^{2+} signaling[41]. These recent studies[39–41] questioned whether mGluR[33] and purinergic receptors[42] in astrocytes can sense glutamate and adenosine triphosphate (ATP), respectively, contributing to regulation of the arteriolar tone. More recent findings confirmed that astrocytes do not mediate neurovascular coupling to arterioles, and that dilation of arterioles depends on N-methyl-D-aspartate receptor (NMDA) activation and Ca^{2+}-dependent generation of NO by interneurons[26].

The non-selective transient receptor potential vanilloid channel 4 (TRPV4) have been shown to stimulate Ca^{2+}-induced Ca^{2+} release in astrocytic end feet, which amplifies neurovascular coupling[43,44] by activating the BK_{Ca} channels releasing K^+ into the extracellular space[36]. The moderate increase in extracellular $[K^+]$ activates inward rectifier potassium (K_{IR}) channels in VSMCs resulting in hyperpolarization and relaxation, as indicated by *ex vivo* and *in vivo* studies[45]. Interestingly, pathologically high levels of the extracellular $[K^+]$, as seen during cortical spreading depolarization, activate voltage-gated calcium channels (VGCCs) in VSMCs depolarizing the cells, which results in VSMCs contraction[46].

The metabolic state of the tissue influences both arteriolar dilation and constriction[47]. Under normoxic pO_2 conditions (normal 20% O_2), lactate levels in brain slices are elevated compared to hyperoxic pO_2 conditions (high 95% O_2), which inhibits PGE_2 reuptake by prostaglandin transporter in astrocytes leading to elevated PGE_2 levels in the extracellular space causing VSMCs relaxation[47]. In high pO_2, however, the AA is converted in VSMCs to 20-HETE leading to arteriolar constriction[47] (Figure 2). Although this O_2 modulatory effect has been shown using *ex vivo* preparations of brain slices and retina explants[47,48], it has not been confirmed in blood flow studies in hyperoxic animals[48], leaving open to question whether pO_2 has a role in blood flow regulation *in vivo*. In contrast, the effects of lactate on

modifying vessel dilation in slices[47] have been translated *in vivo* as demonstrated in miniature pig retina[49] and guinea pig cochlear organ[50], both showing vasodilation after systemic or local lactate administration.

The molecular pathways in astrocytes that generate signals to VSMCs as suggested by some initial studies, but not reproduced by more recent studies, are illustrated in Figure 2. Whether astrocytes are involved in neurovascular coupling to arterioles and arteries remains controversial topic that merits further investigation. Some questions to be addressed are: Can astrocytes contribute to neurovascular coupling by modifying neuronal synaptic activity? Do neurons differentially signal to astrocytes on arterioles and capillaries? Do different types of astrocytes communicate with VSMCs and pericytes?

Direct neuron-mediated regulation of VSMCs tone

Early studies have shown that activation of neuronal NMDA receptors in rabbits leads to concentration-dependent NO-mediated dilatation of pial arterioles[51]. Subsequent studies have demonstrated that mircroperfusion of NMDA in the striatum of newborn sheep[52] or functional stimulation of the somatosensory cortex of rats[53] both lead to CBF increases through NO production, establishing neuronal NO as a major regulator of the CBF[5]. Activation of purinergic P2XR[54] and P2YR[55] ATP-gated channels in VSMCs has been shown to lead to an increase in $[Ca^{2+}]_i$ causing VSMCs contraction. It has been suggested that ATP from both neurons[56] and astrocytes[57] can activate VCMCs purinergic receptors. Additionally, adenosine released from neurons[58] is a potent endothelium-independent vasodilator that acts as a direct VSMCs relaxant[2,5,23].

Neurotransmitters including vasoactive intestinal polypeptide, dopamine, substance P, serotonin, gamma-aminobutyric acid, noradrenaline, neuropeptide Y, somatostatin and acetylcholine have all been reported to mediate vascular changes[5,12]. Different types of inter-neurons have been suggested to control local CBF responses possibly through astrocytes or pericytes[10,13]. In addition to the role of cholinergic afferents modulating CBF via acetylcholine release[13], studies in mice[59] and neonatal rats[60] have demonstrated that noradrenaline release from locus coeruleus afferents generates vasoconstriction. Recent studies using a combination of optogenetic stimulation and mutiphoton imaging *in vivo* in mice, have shown both excitatory and inhibitory neurons can signal arteriolar dilation, but only inhibitory neurons releasing neuropeptide Y were able to cause arteriolar constriction[61]. Currently, more information is needed to determine the action and the exact function of each of the neurotransmitter systems, their afferents and effects on the cells that make up the NVU.

Endothelium-mediated regulation of VSMCs tone

Endothelial cells modulate vascular tone by retrograde propagation of vasodilation[16,17]. Endothelial hyperpolarization travels distances > 1 mm with limited attenuation, which leads to self-dilation via myoendothelial coupling to VSMCs through myoendothelial gap junctions[17] or via a putative endothelial-derived hyperpolarizing factor[62]. Additionally, endothelial release of NO leads to VSMCs relaxation and arteriolar dilation[12]. Shear stress on red blood cells (RBC) passing through the vessel can release ATP, which interacts with

P2XR and P2YR on endothelial cells resulting in generation of AA, EETs and cAMP[17,63]. cAMP generates prostacyclin (PGI_2), which acts on VSMCs much like PGE_2 causing relaxation[12].

Mechanical shear stress can also cause direct generation of AA and/or NO via endothelial nitric oxide synthase (eNOS) in endothelial cells to initiate the pathways described above[2,17]. Endothelial receptor targets besides acetylcholine and ATP include bradykinin, adenosine diphosphate, uridine triphosphate and adenosine[12]. Receptor binding activates phospholipase C (PLC) or PLA_2, which through diacyl-glycerol (DAG) produces EETs and PGI_2 that both contribute to VSMCs relaxation via cAMP[12] (Figure 2).

While these and many additional studies not included here have allowed us to piece together many aspects of endothelial-mediated arteriole regulatory mechanisms, much of this work comes from *in vitro* or *ex vivo* studies, and studies from peripheral tissues. More comprehensive *in vivo* studies in brain are needed to confirm these mechanisms in the functional regulation of brain arteriole vasculature.

As will be discussed later, the neurovascular coupling, VSMC contractility, and endothelial-dependent and independent mechanisms of arteriolar dilation become dysfunctional in models of neurodegenerative disorders including AD, and in individuals with mild cognitive impairment (MCI) and early stage AD.

Capillary component

Capillaries, the smallest vessels in the brain, branch off of the arterioles and form a rich microvascular network with the largest surface area of 120 cm^2 per gram of brain available for transport exchanges of molecules between blood and brain across the endothelium of the BBB[1]. The BBB is formed by a continuous monolayer of endothelial cells connected by tight junctions, which limits entry of large macromolecules, cells and pathogens into the brain. The BBB allows rapid diffusion of O_2 from blood-to-brain and of CO_2 from brain-to-blood according to their concentration gradients, and regulates transport of energy metabolites and nutrients into the brain, and clearance of metabolic end products from brain to venous circulation via numerous specialized carrier-mediated and receptor-mediated transport systems in the endothelium[15,64].

Brain capillaries are covered by pericytes, which share the basement membrane with endothelial cells[15] (Figure 1). Pericytes stretch their processes along and around capillaries, pre-capillary arterioles and post-capillary venules with more longitudinal processes in the middle of capillary bed, more circumferential processes at the arteriole end, and more stellate morphology at the venule end of capillaries[65–67]. Pericytes and endothelial cells make direct interdigitated or "peg-socket"-like contacts where cytoplasmic protrusions (pegs) of one cell type insert into pockets in the opposing cell membrane (socket) of the other cell type[68,69]. Although it is not clear which type of junctional proteins mediate *in vivo* interactions between pericytes and endothelial cells, immunostaining studies suggested involvement of N-cadherin during brain angiogenesis[70]. Several *in vitro* culture studies suggested that pericytes express connexin 43 (C×43)[71]. Expression of C×43 and C×37 in

pericytes has been confirmed by single-cell RNA-seq study of different cell types in the mouse cortex and hippocampus[72]. Whether these connexins mediate pericyte-endothelial, pericyte-pericyte and/or pericyte-astrocyte interactions remains, however, to be determined by future studies.

Pericytes regulate angiogenesis during brain development, formation and maintenance of the BBB, clearance of toxins, and neuroinflammatory and stem cells responses, as recently reviewed[73]. Studies using multiple models have suggested that pericytes regulate CBF and capillary vascular tone[24,66,74–78]. Below, we examine CBF control by capillaries, role of pericytes in regulating capillary diameter, and signaling pathways regulating pericyte tone.

Control of capillary blood flow by pericytes

The prevailing view is that pericytes contribute to regulation of capillary diameter and CBF[23,24,26,27,65,66,74–78]. This view has been challenged, however, by some recent studies[25,79]. What defines a pericyte and pericyte contractility is discussed in Box 1.

Several studies have shown Ca^{2+}-dependent contraction of pericytes in response to neurotransmitters (*e.g.*, norepinephrine), electrical stimulation or neuronal activity[23,24,26,27,74,77,78,80,81]. Recent studies demonstrated that astrocytes mediate neurovascular coupling to capillary pericytes but not to arterioles[26], and that glial cells in the retina, called Muller cells, also regulate capillary, but not arteriolar diameter in response to light stimulation of neurons[27]. Pericyte degeneration has been shown to lead to neurovascular uncoupling and reduced oxygen supply to brain, suggesting diminished hemodynamic responses in a pericyte-deficient transgenic model[23]. Optogenetic experiments indicated initially that pericytes do not contribute to CBF regulation in neural-glial antigen 2-driven *NG2-cre* channelrhodopsin (ChR2) mice[25], which has not been confirmed by another optogenetic study in platelet-derived growth factor receptor beta-driven *Pdgfrb-cre* ChR2 mice demonstrating that strong 2-photon stimulation leads to pericyte contraction, constriction of capillary lumens and inhibition of RBC flow *in vivo* (D.A. Hartmann, R.I. Grant and A.Y. Shih, Medical University of South Carolina; personal communication). Whether the differences seen in optogenetic studies are model-dependent and/or light source and duration-dependent, and whether different Cre drivers lead to differential ChR2 expression in different pericyte subpopulations, remains to be examined by future studies.

Recent *in vivo* studies in mice found that hindlimb stimulation (10s)[23,26] or whisker-pad stimulation (15s)[24] resulted in capillary dilation ahead of arterioles; this was correlated with pericyte coverage and could be caused by isotonic pericyte relaxation. A shorter stimulation time (2s) produced, however, RBC flow increase without vessel dilation[79], and could result from isometric relaxation of pericytes. An earlier study demonstrated that capillaries in the activated rat olfactory glomerulus, but not capillaries adjacent to this glomerulus, actively dilate in response to odor stimulation[82]. As arterioles do not form a single glomerulus-specific network, their contribution to odor response has not been determined in this model[82]. Dilations and constrictions of cerebral capillaries have been also observed in rat cortex after forepaw stimulation[83] and in response to hypercapnia[84]. During brain ischemia, pericytes contract and limit CBF by constricting capillaries and trapping blood cells in their

lumen, and later die, as shown in a murine model[85,86]. Pericyte contraction and cell death in response to ischemia has been confirmed *ex vivo* in brain and retina explants[24,77].

While a growing body of evidence supports the role of pericytes in CBF regulation, there is also a growing need to better define pericytes, particularly at the molecular level (Box 1). Future studies should also determine whether all or only some pericyte subpopulations regulate CBF dynamics and/or are more susceptible to ischemic and/or hypoxic insults.

Signaling in astrocytes and pericytes regulating capillary tone

Recent studies have demonstrated that astrocytes mediate neurovascular signaling to capillary pericytes, which involves a rise of $[Ca^{2+}]_i$ in astrocytes not by release from stores but by entry through ATP-gated channel - purinergic receptor P2XR, and that Ca^{2+} generates AA via phospholipase D2 and diacylglycerol kinase rather than PLA2[26] (Figure 3). The role of a Ca^{2+}-dependent glial cell signaling mechanism in regulating capillary but not arteriole diameter has also been shown in the retina *ex vivo* and *in vivo* upon physiological light stimulation[27].

Pericytes respond to the AA metabolites PGE_2 and 20-HETE to generate relaxation or contraction, respectively, but unlike VSMCs, they do not respond to EETs[24,77]. Pericytes express both P2X[87] and P2Y[88] purinergic receptors, which when activated by ATP lead to an increase in Ca^{2+}, depolarization and pericyte contraction. Activation of large- (BK_{Ca}) and small- (K_{Ca}) conductance potassium channels or ATP-sensitive potassium (K_{ATP}) channels results in potassium efflux and hyperpolarization of pericytes, decreasing Ca^{2+} entry through VGCCs[89].

Adenosine that is normally released from neurons and leads to VSMCs relaxation also binds A1 and A2 receptors on pericytes, and activates K_{ATP} channels releasing potassium from the cell leading to hyperpolarization and relaxation[89]. Glutamate-evoked capillary dilation was reduced by blocking NO synthase with L-NG-nitroarginine in brain slices suggesting a role for NO in pericyte relaxation and capillary dilation that was likely mediated by inhibiting the AA conversion to 20-HETE, which blocks depolarization and contraction[24]. It is unclear if pO2 plays as significant a role in modulating relaxation versus contraction at the capillary level as observed in arterioles[24].

Several endothelial-derived vasoactive mediators can also regulate pericyte contraction and relaxation likely via similar mechanisms as in VSMCs[89] (Figure 3). A number of questions regarding pericyte signaling *in vivo* remain, however, to be addressed by future studies including the proposed regulatory neurotransmitter and endothelial signaling pathways. As discussed below, pericytes degenerate in several neurodegenerative disorders including AD and ALS, and are injured during early stages of aging-MCI-AD spectrum. Thus, it is possible that their loss contributes to vascular dysregulation observed in neurological diseases associated with early neurovascular dysfunction as suggested by recent studies in pericyte-deficient mice[23].

Alzheimer's vascular dysfunction

In addition to amyloid-β (Aβ), tau pathology and neuron loss, AD is associated with early neurovascular dysfunction, which contributes to disease pathogenesis, as indicated by recent epidemiological, clinical, pathological and experimental studies[1,3,5,90–95]. Moreover, small vessel disease of the brain has been estimated to contribute wholly or partially to approximately 40% of all dementias worldwide including AD[3,96–99].

Multiple risk factors influence AD pathogenesis including genetics, vascular, environment, and lifestyle (Figure 4). According to the *two-hit* vascular hypothesis of AD[1,15], Aβ-independent (*hit 1*) and Aβ-dependent (*hit 2*) mechanisms interact and converge on blood vessels leading independently and/or synergistically to neuronal and synaptic dysfunction, neurodegeneration, and cognitive impairment (Figure 4). Besides direct negative effects of CBF reductions and dysregulation, and BBB breakdown and dysfunction on neuronal function and accumulation of Aβ in the brain (*hit 1*), Aβ-mediated vascular dysfunction (*hit 2*) could also be an early event in AD pathogenesis[5,100]. Notably, function of each of the NVU cell types (*e.g.*, VSMCs, pericytes, astrocytes, endothelia) regulating CBF and BBB integrity is affected during different stages of AD by either Aβ-independent or Aβ-dependent mechanisms, and/or both[3,5,73,92,100,101], contributing to dementia. Although reduced to two major disease pathways, the *two-hit* hypothesis considers multiple factors influencing AD.

Here, we will review neurovascular deficits during AD pathogenesis, focusing on CBF changes. First, we examine findings in animal models, and next neurovascular dysfunction in humans in relation to cognitive impairment and AD. Many of the molecular and cellular mechanisms underlying arterial, arteriolar and capillary components of CBF regulation that we discussed in the first part of this review have not been studied, however, in animal models of AD, or in humans at risk or diagnosed with AD. Therefore, in the sections below we will also attempt to identify some of the outstanding mechanistic gaps that should be addressed by future studies.

Aβ-independent vascular changes in animal models

Pericyte-deficient transgenic models with undetectable Aβ pathology develop early CBF reductions in gray matter[102] and aberrant CBF responses in the presence of initially normal neuronal activity, endothelial-dependent and independent vasodilation, and astrocyte numbers and coverage of the blood vessels[23]. Pericyte degeneration also leads to early reductions in O_2 supply to activated brain[23]. These vascular changes develop independently of Aβ, and precede neuronal dysfunction and neurodegeneration that develop months later. These findings indicate that pericytes could be an important target in neurological disorders associated with perciyte loss and neurovascular dysfunction. This incudes AD[91,103–108], ALS[109,110] and stroke[24,85,86,111]. It is also known that Aβ kills pericytes[106,112], which in turn might accelerate neurovascular dysfunction in AD transgenic models overexpressing amyloid precursor protein (*APP*)[105,106] and/or AD patients[3,5,100]. The relative contributions of Aβ-independent pericyte loss as shown in the ischemic brain[24,85,86,111] and Aβ-dependent pericyte degeneration[106] remains an important topic for future research of CBF dysregulation in AD.

Hypertension (HTN) is a risk factor for AD. A number of studies have shown that HTN alters functional hyperemia and endothelial function[113]. Angiotensin II (ANGII)-induced hypertension in rodents attenuates CBF responses to whisker stimulation[114] due to diminished endothelium-dependent vasodilation to acetylcholine[115]. Interestingly, cerebrovascular effects of ANGII are independent on the elevation of blood pressure[116–118]. Alterations in cerebrovascular responses to endothelium-dependent vasodilators have also been show in rodent models of chronic HTN[114,116,119]. Relatively little is known, however, about the role of other NVU cell types in the pathogenesis of HTN, and in relation to HTN role in AD pathogenesis. For example, age-dependent loss of pericytes has been shown in ANGII model of HTN[120], but the effects on neurovascular coupling and the underlying molecular and cellular mechanisms remain largely unexplored.

Studies in transgenic *Notch3^R169C* mouse model of cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) expressing the pathogenic *Notch3* mutation in VSMCs and pericytes, as in human CADASIL, have shown that both mural cell types accumulate toxic *Notch3* aggregates and degenerate[121–123]. Consistent with loss of mural cells, these mice develop impaired CBF autoregulation[124]. The exact contributions of VSMCs versus pericytes to CBF dysregulation and possible downstream molecular targets remain, however, presently unknown.

CBF reductions and vascular dysfunction were also found in apolipoprotein E4 (*APOE4*) transgenic mice with targeted replacement of murine *APOE* with human *APOE4* gene[125–127], the major genetic risk factor for AD. It has been also shown that vascular phonotype in *APOE4* mice precedes neuronal and synaptic dysfunction[125]. Whether the same cyclophilin A pathway in pericytes mediating the BBB breakdown in *APOE4* mice[125] is also involved in CBF dysregulation is not clear at present.

Together, these studies suggest that CBF dysregulation develops early in experimental models of pericyte, VSMCs or endothelial dysfunction, which can lead to neuronal dysfunction and loss independently of Aβ. Little is known, however, about signaling pathways in the NVU that lead to CBF dysregulation in these models. More work is needed to uncover the mechanistic links between dysregulated CBF in the Aβ-independent vascular models and astrocyte-pericyte signaling[26,27] and the roles of astrocytic Ca^{2+} [26,27] and neuronal and endothelial NO[5].

Aβ-dependent vascular changes in animal models

Early studies in the isolated rat aorta have shown that Aβ exerts vasoconstrictive properties[128]. The follow-up *in vivo* findings in transgenic mice expressing Aβ precursor protein (*APP*) Swedish mutation indicated that Aβ attenuates acetylcholine-mediated endothelium-dependent vasodilation[129]. Studies in young *Tg2576* mice expressing *APP* Swedish mutation demonstrated reduced cerebrovascular reactivity to endothelium-dependent vasodilators (*e.g.*, acetylcholine, bradykinin, calcium ionophore A23187) and increased response to vasoconstrictors acting directly on VSMCs (*e.g.*, thromboxane A2 analogue U46619)[130], as well as altered neurovascular coupling[131]. Collectively, these studies suggest that accumulation of low levels soluble Aβ prior to Aβ deposition leads to a global impairment of vascular responses. Aβ mediated generation of ET-1 through the

receptor for advanced glycation end products (RAGE) in brain endothelium that binds Aβ has been also shown to led to CBF reductions in *Tg2576* mice[132,133].

Additional investigations indicated that NADPH-oxidase is a key source of the radicals mediating neurovascular dysfunction caused by Aβ[134], which causes endothelial dysfunction by activating transient receptor potential melastatin-2 channels in endothelial cells via poly(ADP)-ribose polymerase pathway[135]. CD36, another scavenger receptor that binds Aβ, also leads to Aβ-mediated oxidative stress in cerebral blood vessels causing diminished neurovascular coupling[136]. Interestingly, RAGE regulates CD36 expression[136]. The exact relationship between CD36 and RAGE in Aβ-induced CBF dysregulation[132,133], however, awaits further exploration.

Consistent with these studies, findings in transgenic mice with vasculotropic Dutch and Iowa Aβ mutations crossed with the Swedish *APP* mutants, *i.e.*, *TgSwDI APP* mice, and in *Tg2576 APP* mice have shown that arterial VSMCs exhibit a greatly reduced ability to clear Aβ, which leads to Aβ accumulation in the vessel wall causing cerebral amyloid angiopathy (CAA) and impaired vascular reactivity[137]. This has been recently confirmed in older *APP* mice carrying Swedish and Indiana mutations (*i.e.*, *hAPPJ20*)[138].

Similar to AD patients, *TgSwDI APP* mice and *Tg2576 APP* mice express low levels of serum response factor (SRF) and myocardin (MYCD), the two transcription factors that control VSMCs differentiation, which leads to elevated expression of several SRF-MYCD-directed VSMCs contractile proteins including SMA, calponin and myosin heavy chain[139]. This in turn leads a hypercontractile VSMCs phenotype, and diminished endothelium-dependent and endothelium-independent relaxation causing attenuated CBF responses[139]. Additionally, genes that regulate Ca^{2+} homeostasis, such as myosin light-chain kinase, calsequestrin 1 and sarcoplasmic/endoplasmic reticulum calcium ATPase 2 are also elevated in AD VSMCs contributing to VSMCs hypercontractile phenotype[139]. Although, SRF-MYOCD-dependent regulation of VSMCs contractile proteins and Ca^{2+} homeostasis genes is Aβ-independent[139] these changes likely act synergistically with Aβ to accelerate CBF reductions in AD models, and possibly in AD. Finally, Aβ leads to pericyte cell death in human cultured pericytes[106,112] and Tg2576 *APP* mice[105,106], which might contribute to CBF dysregulation.

In summary, a body of evidence suggests that Aβ has vasoactive and vasculotoxic effects on cerebral blood vessels affecting different cellular components of the NVU that regulate CBF. Therefore, preventing Aβ accumulation will eliminate Aβ-dependent effects on CBF dysregulation. The question persists, whether removing Aβ will have the same beneficial effects on already damaged vessels. Recent studies have suggested, however, that counteracting the deleterious effects of Aβ after vascular depositions in aged *TgSwDI APP* mice is not effective in reversing the neurovascular dysfunction owing to VSMCs damage caused by aging and massive Aβ deposition[105]. Importantly, the BBB dysfunction of Aβ clearance receptors and reduced CBF both promote Aβ accumulation in the brain and vessel wall (*hit 1*) enhancing Aβ pathogenic effects (Figure 4).

Combined Aβ and vascular models

Here, we briefly illustrate with a few examples interaction of Aβ with some factors negatively affecting cerebral circulation. More detailed discussion on this topic could be found elsewhere[97,98].

Regarding HTN, it has been shown that ANGII-induced HTN in mice worsens Aβ-induced neurovascular dysfunction and promotes β-secretase activity increasing amyloidogenic APP processing, which may contribute to the pathogenic interaction between HTN and AD[115]. ANGII-induced HTN also impairs CBF, cognition and functional connectivity in *APP/PS1* mice and decreases functional connectivity in wild type mice[140], suggesting another possible mechanism for a link between midlife HTN, decreased cerebral hemodynamics and connectivity.

Cerebral hypoperfusion accelerates CAA in *TgSwDI APP* mice[141]. CAA, on the other hand, leads to a more severe cerebrovascular dysfunction than Aβ alone causing intra- and post-ischemic CBF deficits, which exacerbates cerebral infarction, as shown in Tg2576 mice[142].

Elevated levels of homocysteine in plasma, hyperhomocysteinemia (HHcy), have been shown to impair action and synthesis of endothelial NO leading to CBF dysregulation[143]. Induction of HHcy in wild-type mice models vascular dementia by inducing cerebral microhemorrhages and neuroinflammation[144]. Moreover, HHcy shifts Aβ deposition to the vasculature and exacerbates memory impairment in *APP/PS1* mice[145].

Cerebrovascular reactivity in Alzheimer's disease

Individuals with early stage, probable AD compared to cognitively normal controls have impaired cerebrovascular reactivity in response to hypercapnia induced by CO_2 inhalation and display large CBF fluctuations after repeated sit-stands with no changes in the systemic arterial blood fluctuations[146], suggesting that local CBF dysregulation develops early in AD in the presence of intact cardiovascular control of the arterial blood pressure.

Recent studies have shown that individuals carrying *APOE4* gene, the major genetic risk factor for AD, compared to non-carriers develop early impaired cerebrovascular reactivity to a memory task and during CO_2 inhalation[147]. Using CO_2 inhalation challenge, a larger study in cognitively normal *APOE4* carriers compared to *APOE4* non-carriers confirmed impaired CBF responses, suggesting that early CBF dysregulation contributes to cognitive impairment in *APOE4* carriers[148]. Using the CO_2 inhalation challenge and blood-oxygen level dependent (BOLD)-fMRI, another study found that cerebrovascular deficits in AD could be associated with amyloid deposits, as detected by positron emission tomography (PET) with ^{11}C-Pittsburg compound B[149].

Impaired cerebrovascular reactivity indicates damage to the cerebral blood vessels and CBF regulatory mechanisms, which may result in CBF reductions and/or neurovascular uncoupling, as discussed below. The molecular and cellular mechanisms involved remain, however, mostly unknown at present.

Cerebral blood flow reductions in Alzheimer's disease

An earlier large population-based study showed that diminished CBF velocity precedes cognitive decline and hippocampal atrophy[150]. Additionally, individuals exhibiting greater CBF velocity had larger hippocampal and amygdalar volumes[150].

Early studies in individuals with mild cognitive impairment (MCI) with memory loss (*e.g.*, amnestic MCI) have shown CBF reductions[151,152] in the posterior cingulate gyrus and precuneus using single positron emission tomography (SPECT), that is also confirmed in individuals with probable AD[153]. As the posterior cingulate gyrus and precuneus participate early in the pathophysiology of disrupted functional connectivity in AD[154], it is possible that initial vascular dysregulation may precede and/or trigger disrupted brain connectivity in these regions. Interestingly, a decrease in regional CBF in the posterior cingulate gyrus and precuneus in early stage AD occurs prior to a loss of gray matter volume[153,155] suggesting that CBF reductions precede brain atrophy. Consistent with lower CBF values, and findings demonstrating that glucose transport into the brain depends on the CBF[101,156], the same brain regions showing diminished CBF showed diminished brain glucose uptake in early stages of AD, as detected by ^{18}F-fluorodeoxyglucose (FDG)-PET[157].

Reductions in regional CBF in the bilateral parietal areas and the precuneus preceded conversion of MCI to AD[158,159]. Additionally, CBF decline in the frontal, parietal, and temporal cortices preceded the onset of cognitive decline in non-demented *APOE4* carriers compared to *APOE4* non-carriers[160]. Consistent with these findings, low glucose uptake by the posterior cingulate, parietal, temporal, and prefrontal cortex was found in young *APOE4* carriers compared to *APOE4* non-carriers[161]. More recent *in vivo* assessment of regional CBF via non-invasive arterial spin labeling (ASL)-MRI, relying on magnetic labeling of arterial blood water, and simultaneous ^{18}F-FDG-PET acquisition confirmed a high correlation between regional brain hypoperfusion and impaired glucose uptake by the brain of AD individuals compared to controls[162]. Collectively, these findings demonstrate that regional CBF reductions and diminished regional glucose delivery are amongst the earliest functional changes preceding cognitive decline in AD.

A recent large study in healthy controls and MCI and AD patients demonstrated that CBF changes determined by ASL-MRI, and vascular dysregulation are the initial events associated with cognitive decline before changes in classical AD biomarkers Aβ and tau occur[94]. Another recent study revealed that global CBF was lower in cognitively normal *APOE4*-carriers compared to *APOE4* non-carriers prior to development of amyloid deposits[163]. Moreover, *APOE4* subjects who developed amyloid deposition showed lower global CBF compared to those who were free of amyloid[163]. These data suggest that *APOE4* gene can diminish CBF in humans independently of Aβ, similar as shown in animal studies[125], but during AD progression *APOE4*-induced amyloid deposition results in even greater CBF reductions. A recent contrast-enhanced perfusion MRI study confirmed significantly reduced CBF in parietotemporal regions and the basal ganglia in MCI compared to healthy controls prior to any gray matter brain atrophy[164], suggesting that CBF alterations are driven by *APOE4* gene[164]. These recent studies support the role of reduced cerebral perfusion in the development of cognitive impairment prior to reductions in cortical thickness and/or hippocampal atrophy.

Neurovascular uncoupling in Alzheimer's disease

Studies using BOLD-fMRI detected changes in brain activation in AD in the hippocampus in response to visual stimuli and memory encoding tasks[165] and face-name association tasks[166]. BOLD-fMRI studies in cognitively normal individuals with genetic risk for AD (*i.e.*, at least one *APOE4* allele) revealed decreased brain activation in key areas engaged during naming and fluency tasks when compared to age-matched controls with no risk factors[167]. Changes in hemodynamic responses to visual stimuli were also found in patients with CAA compared to healthy elderly controls[168]. As the diminished BOLD-fMRI responses to different cognitive tasks are regionally specific, collectively BOLD-fMRI findings indicate multiple pathophysiological alterations in neurovascular coupling in early stage AD, and in individuals at genetic risk for AD, demonstrating important role of local CBF dysregulation during AD progression.

Because of the tight relationship between CBF and neuronal activity, the resting state fMRI, also called brain "default-mode network" (DMN), relies on local CBF to provide important insights into how the brain works[9,169]. DMN typically includes the medial prefrontal cortex, the posterior cingulate and precuneus, inferior parietal lobe, lateral temporal cortex, and hippocampus[169–171]. A decreased resting-state activity in the posterior cingulate and hippocampus was reported in subjects during early stages of AD when compared to age-matched elderly controls, suggesting disrupted connectivity between these two regions[172]. In addition, disrupted hippocampal connectivity was also confirmed in the medial prefrontal cortex and cingulate cortex in AD compared to cognitively normal controls[173].

A large cross-sectional study found a significant decrease in DMN functional connectivity by fMRI in autosomal dominant AD (ADAD) including presenilin 1 (*PSEN1), PSEN2* and *APP* mutation carriers before clinically evident dementia[174]. A decrease in functional connectivity with increasing dementia show similarity in ADAD and sporadic AD[175]. Early disrupted functional connectivity by fMRI in DMN regions including hippocampus, parahippocampus, precuneus, and cingulate, mediotemporal, and orbital cortices has been demonstrated in non-demented *APOE4* carriers compared to *APOE4* non-carriers prior to amyloid deposits[176], suggesting that early neurovascular dysfunction occurs prior to Aβ pathology.

Collectively, these studies provide strong evidence demonstrating that impaired cerebrovascular reactivity, CBF reductions, and CBF dysregulation, and BBB breakdown are early events in the AD pathophysiological cascade. How they relate to each other and disrupted brain connectivity remains to be determined by future studies (Box 2).

Conclusions and perspectives

Recent studies have demonstrated that signaling pathways in astrocytes, VSMCs, pericytes, and endothelia control CBF. Importantly, many of the same pathways are found in different NVU cell types, raising a possibility that targeting more common pathways might result in synergistic cellular responses contributing to CBF control. However, some outstanding questions remain as indicated throughout the review. There is also a large gap in understanding how the basic physiological findings of CBF regulation translate to animal

disease models, and humans with healthy brain and neurological disorders associated with neurovascular dysfunction such as AD, as well as between animal disease models and human neurological conditions.

To determine the relative contributions of different cell types and pathways in CBF control, the pathways should be targeted in a cell-specific manner taking advantage of advanced genetic engineering and pharmacological approaches. For example, using cell-specific ablation models and/or disrupting pathways in a cell-specific manner in VSMCs, pericytes, endothelia and astrocytes, coupled with a single-cell RNA sequencing and proteomic analyses, should be able to define more precisely the role of each NVU cell type in neurovascular coupling and maintenance of cerebrovascular integrity. *In vivo* CBF studies are also needed to better understand the neuronal component of CBF regulation, and the role of different neurotransmitter systems in neurovascular coupling.

Although we know that each cell type regulating CBF is affected in neurodegenerative disorders such as AD, the translation of knowledge from bench to bedside has been slow. Studies in the living human brain have established that aberrant cerebrovascular reactivity, CBF reductions and dysregulated CBF are a prominent feature during early stages across the aging-MCI-AD spectrum. However, the pharmaceutical industry and academia are still not reacting in a way to explore systematically whether treating neurovascular dysfunction will delay onset and/or slow down the neurodegenerative process. The question persists – are we missing an important opportunity by not utilizing the wealth of knowledge generated in the vascular field, and not focusing enough on vascular dysregulation as a major therapeutic target in neurodegenerative disease such as AD?

Acknowledgments

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Box 1

### Pericyte definition and the question of contractility

Zimmermann[177] included in the definition of pericytes several subtypes that differ by morphology, location within the vascular tree, and function[65,73,178]. Indeed, a recent morphological study[67] indicated that pericytes represent a heterogeneous cell population with different subtypes including "mid-capillary" in the majority of the capillary bed, "transitional" close to vascular smooth muscle cells (VSMCs), and "stellate" on post-capillary venules, as compared to arteriolar ringed VSMCs[65,67] (Box 1 figure). Whether different pericyte subtypes have different functions in brain microcirculation such as, for example, regulation of blood-brain barrier (BBB) permeability versus control of blood flow[65], remains to be determined.

When it comes to cell contractility, earlier studies have shown that transitional pericytes of pre- and post-capillary microvascular segments express α-smooth muscle actin (SMA)[179], a contractile protein typically found in VSMCs[25]. Using a highly-sensitive immunogold labeling at the ultrastrctural level, SMA expression has been shown in a significant number of mid-capillary pericytes in brain and retina[81], which has been corroborated by immunostaining by some studies[81,86], but not by others[25,79]. Besides SMA, other contractile proteins such as myosin and vimentin have been identified in mid-capillary pericytes in brain in situ[81]. A recent single-cell RNA-seq study demonstrated expression of several contractile proteins in pericytes derived from mouse cortex or hippocampus including skeletal muscle actin, vimentin, desmin, calponin, non-muscle myosin variants, and a low SMA expression[72]. Importantly, it would be unfortunate to limit one's thinking by assuming that one particular specialized cell type, like VSMCs, must necessarily reflect how the generally occurring phenomenon of contractility in other cell types within the neurovascular unit is achieved, including pericytes.

Although some studies failed to show pericyte contractility in vivo[25,79], multiple studies reported that perciytes regulate CBF[23,24,26,27,65,66,74–78]. Pericytes dilate in response to lactate[47,50,78], or contract in response to K^+, Ca^{2+},[74] or after application of the thromboxane A2 agonist U46619 in vivo[75]. They also contract after electrical stimulation or when exposed to neurotransmitters ex vivo in rat retina[77] and cerebellar slices[24]. Human pericytes in culture treated with endothelin-1 also contract[76]. In vivo studies measuring capillary diameter changes in cortex upon hind limb stimulation[23,26] and whisker stimulation[24], or in the retina after light stimulation[27] also demonstrated active capillary dilation and pericyte ability to regulate capillary tone.

Still, a number of outstanding questions remain to be addressed when it comes to a definition of a pericyte. Some of these questions are: Can a pericyte be molecularly defined by a single cell RNA-seq of a genome-deep quantitative transcriptional profiling compared to other cell types of the neurovascular unit? Do different pericyte subtypes exist in the same organ, and can they be molecularly distinguished from each other in the brain as suggested by morphological studies? Do pericytes have organotypic features, as

do endothelial cells, and are pericytes in brain different from pericytes in peripheral organs?
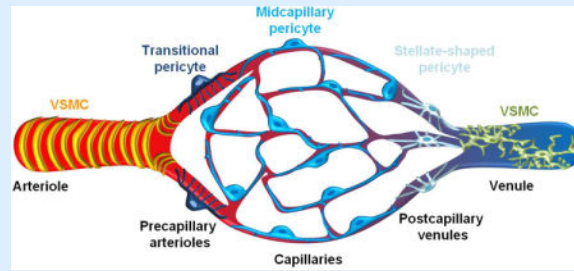
Box 2

Early imaging biomarkers of neurovascular and neuronal dysfunction

As recently reviewed[180], multiple imaging modalities and biomarkers in the living human brain have been used to evaluate neurovascular and neuronal dysfunction during early stages of Alzheimer's disease (AD) pathophysiology. Here, we will briefly examine some of these biomarkers and how they relate to each other. For example, early cerebral blood flow (CBF) studies using transcranial Doppler[150] or SPECT[151,155] and recent studies using arterial spin labeling (ASL)-MRI[94,163] and contrast-enhanced MRI[164] have suggested that CBF reductions in the hippocampus, cingulate cortex and precuneus precede cognitive decline, hippocampal and cortical atrophy and/or amyloid deposition during early stages of AD, and are observed in individuals with genetic risk for AD including APOE4 carriers. Similarly, recent studies using dynamic contrast-enhanced (DCE)-MRI have shown increased blood-brain barrier (BBB) permeability in the hippocampus during normal aging with no cognitive impairment (NCI), which worsens with mild cognitive impairment (MCI), but precedes changes in hippocampal volume[91]. DCE-MRI studies also confirmed BBB breakdown in the hippocampus and cortical and subcortical regions during early stages of AD[181,182] and in individuals with vascular cognitive impairment[183]. Some studies have suggested that neurovascular dysfunction occurs prior to amyloid deposition and tau-mediated neurodegeneration[94], and/or changes in Aβ and tau cerebrospinal fluid (CSF) biomarkers[91,92]. However, longitudinal studies are needed to establish the exact temporal pattern of neurovascular changes relative to brain atrophy, and how they relate to Aβ and tau CSF biomarkers, amyloid and tau lesions, and cognitive decline, particularly in individuals with genetic risk for sporadic AD (i.e., APOE4) and autosomal dominant AD (ADAD) (i.e., PSEN1, PSEN2 and APP mutation carriers). It is also unclear how CBF changes relate to BBB changes and white matter hyperintensities in early stages of NCI and aging-MCI-AD spectrum. It is also almost completely unclear how imaging biomarkers of neurovascular dysfunction relate to disrupted structural connectivity determined by diffusion tensor imaging (DTI)-MRI and DTI-based connectomics analysis[184] or functional connectivity by resting state fMRI in default-mode network regions[169,171].
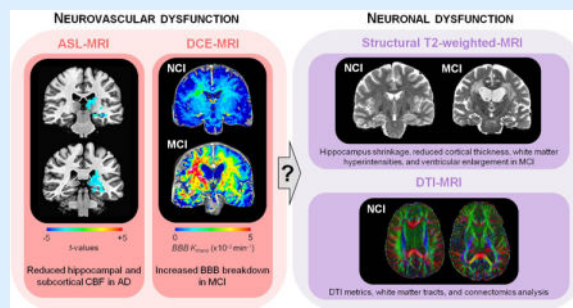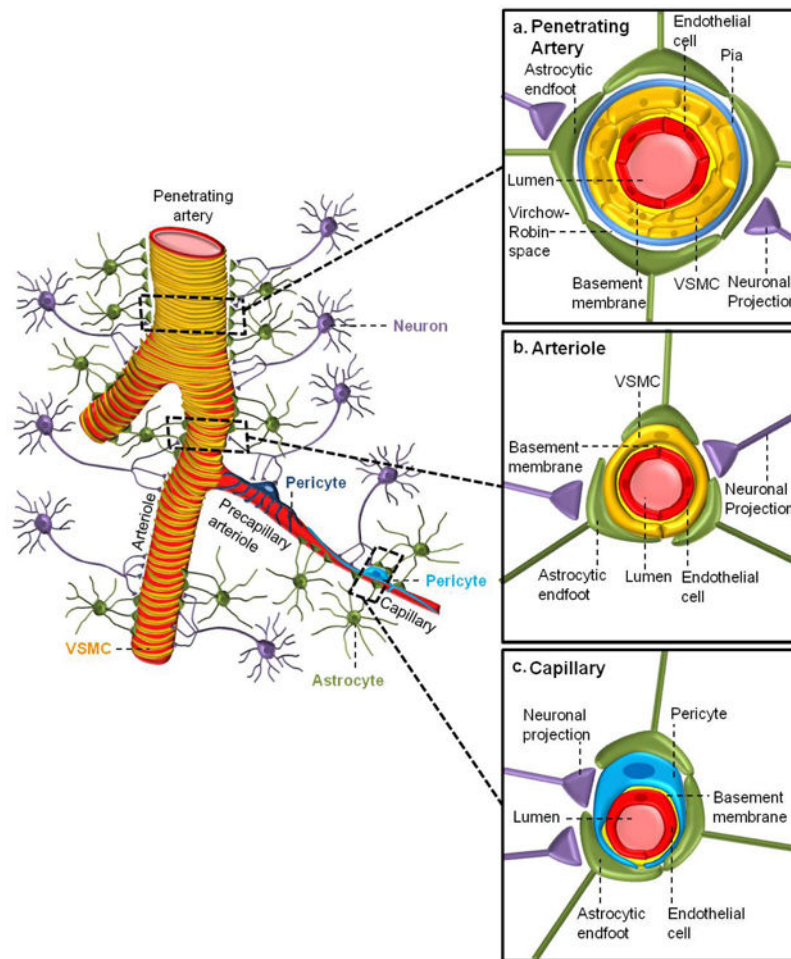
Figure 1. A schematic representation of the neurovascular unit showing cellular elements regulating cerebral blood flow along the vascular tree

Different cell types of the neurovascular unit (NVU) including neurons, astrocytes, mural cells – vascular smooth muscle cells (VSMCs) and pericytes, and endothelium, regulate cerebral blood flow at different levels of the vascular tree. The cellular composition of the NVU differs along the vascular tree, but the principal cellular components all remain represented, as illustrated here. **a)** At the level of penetrating arteries, the NVU is composed of endothelial cells making up the inner layer of the vessel wall, covered by a thin extracellular basement membrane and ringed by one to three layers of VSMCs, and ensheathed by pia. The Virchow-Robin space containing the cerebrospinal fluid is between pia and the glia limitans formed by astrocytic endfeet. Both VSMCs and astrocytes are innervated by local neurons. **b)** Arterioles differ in that there is only one layer of VSMCs, and astrocyte coverage and innervation of the vessel wall and endothelial inner layer display continuity with penetrating arteries, and brain capillaries, above and below the arteriole level, respectively. In addition to VSMCs, precapillary arterioles may also contain transitional pericytes, a cell type between pericyte and VSMCs. **c)** At the capillary level, the NVU is composed of endothelial cells that share a common basement membrane with pericytes. Pericytes stretch their processes along and around capillaries and make direct interdigitated or "peg-socket"-like contacts with endothelial cells. Pericytes and endothelial

cells are covered by astrocyte endfeet. Both astrocytes and pericytes are innervated by local neurons similar as shown for astrocytes and VSMCs in the upper segments of the vascular tree.
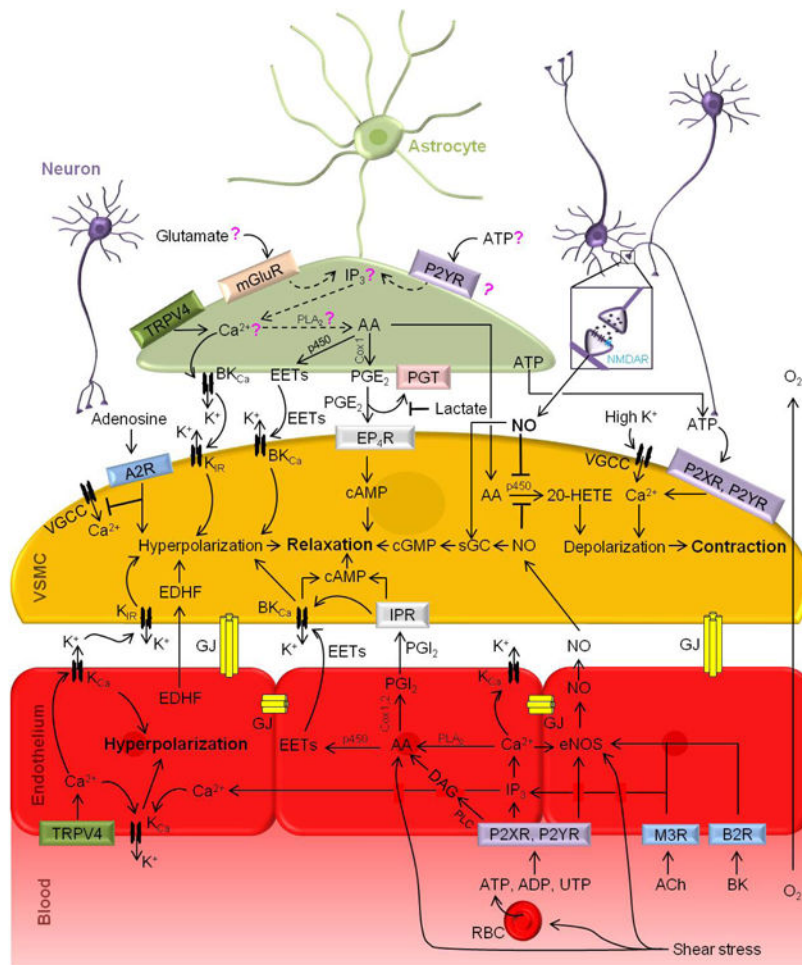
Figure 2. Arteriolar regulation of cerebral blood flow

Neurons. Nitric oxide (NO) is a major moderator of functional hyperemia. NO produced by neurons acts directly on VSMCs, leading to VSMC hyperpolarization and relaxation. Adenosine triphosphate (ATP) and adenosine released by neuronal activity can also act directly on VSMCs through purinergic P2XR and P2YR receptors resulting in constriction, or adenosine 2A receptors (A2R) resulting in relaxation, respectively. Neuronal-mediated large increases in extracellular [K+] activate VSMC voltage-gated calcium channels (VGCCs), resulting in VSMCs intracellular [Ca^{2+}] increases, leading to depolarization and contraction.

Astrocytes. The role of astrocytes in neurovascular coupling to arterioles is controversial. Glutamate or ATP released from neurons are postulated to act on metabotropic glutamate receptors (mGluR) or P2YR on astrocytes, respectively, to initiate 1,4,5-trisphosphate (IP_3)-dependent intracellular [Ca^{2+}] increase, which has been shown by some studies to contribute to neurovascular coupling, but disputed by others. According to some studies, intracellular [Ca^{2+}] rise launches signaling cascades in the astrocytes and release of vasoactive ions and molecules from astrocyte endfeet to VSMCs, mainly K+ ions from large conductance calcium- activated potassium channels (BK_{Ca}), arachidonic acid (AA), through phospholipase A2 (PLA_2) pathway, and AA metabolic products epoxyeicosatetraenoic acids (EETs), and prostaglandin E2 (PGE_2), via cytochrome P450 (p450) and cyclooxygenase 1

(Cox1), respectively. Extracellular Ca^{2+} intake from transient receptor potential vanilloid channel 4 (TRPV4) provides another means of increasing intracellular $[Ca^{2+}]$. Dashed lines and question marks indicate pathways for which there is limited or conflicting data in the literature.

VSMCs. EETs and moderate increases in extracellular $[K^+]$ both act on VSMC potassium channels including BK_{Ca} and inward rectifier potassium channels (K_{IR}), resulting in hyperpolarization and relaxation of the VSMCs (*left*). However, large increases in extracellular $[K^+]$ activate VGCCs, resulting in intracellular $[Ca^{2+}]$ increases leading to VSMCs depolarization and contraction (*right*). PGE_2, which acts through prostaglandin EP_4 receptors (EP_4R) on VSMCs, generates cyclic adenosine monophosphate (cAMP) from intracellular ATP, also producing hyperpolarization and relaxation. PGE_2 levels can be modulated by extracellular lactate levels, which can block reuptake of PGE_2 by prostaglandin transporters (PGT). Lactate levels depend on the oxygen content of the tissue. Conversely, AA taken in by VSMCs can be metabolized to 20-hydroxyeicosatetraenoic acid (20-HETE), a potent VSMC depolarizer, resulting in VSMC contraction. NO released by neurons or endothelial cells can block VSMC 20-HETE production, modulating VSMC contraction and favoring relaxation through facilitation of conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP) via soluble guanylate cyclase (sGC). Adenosine released by neurons acts on A2R and blocks VSMC VGCC activation leading to VSMCs hyperpolarization and relaxation. In contrast, neuronal release of ATP to VSMCs P2XR and P2YR can increase intracellular $[Ca^{2+}]$, resulting in depolarization and contraction.

Endothelium. Vasoactive compounds, including ATP, adenosine diphosphate (ADP), uridine triphosphate (UTP), acetylcholine (ACh), and bradykinin (BK), in the blood stream can bind their respective receptors (P2XR, P2YR, muscarinic M3R, and B2R) to initiate signaling pathways in endothelial cells similar to the pathways in astrocytes, with the addition of the diacyl-glycerol (DAG) AA pathway mediated by phospholipase C (PLC), generating vasoactive molecules that are released to VSMCs. Intracellular $[Ca^{2+}]$ increases initiated by receptor-mediated signaling of endothelium can produce endothelial AA, EETs, and prostacyclin (PGI_2), which act on VSMCs similarly to PGE_2, to generate VSMC hyperpolarization and relaxation. The intracellular $[Ca^{2+}]$ rise can also activate endothelial nitric oxide synthase (eNOS) leading to NO production, and endothelial K_{Ca} channels, releasing K^+ that can act on VSMCs as well as hyperpolarizing the endothelium. Endothelial-derived hyperpolarizing factor (EDHF) can also trigger VSMC hyperpolarization. Additionally, shear stress on the endothelial vessel walls and red blood cells (RBCs) due to blood flow triggers ATP release from RBCs and signaling pathways, including activation of endothelial eNOS, and direct production of AA and its metabolites. Endothelial and endothelial-VSMCs gap junctions (GJs) facilitate retrograde endothelial signal propagation and signaling to VSMCs.
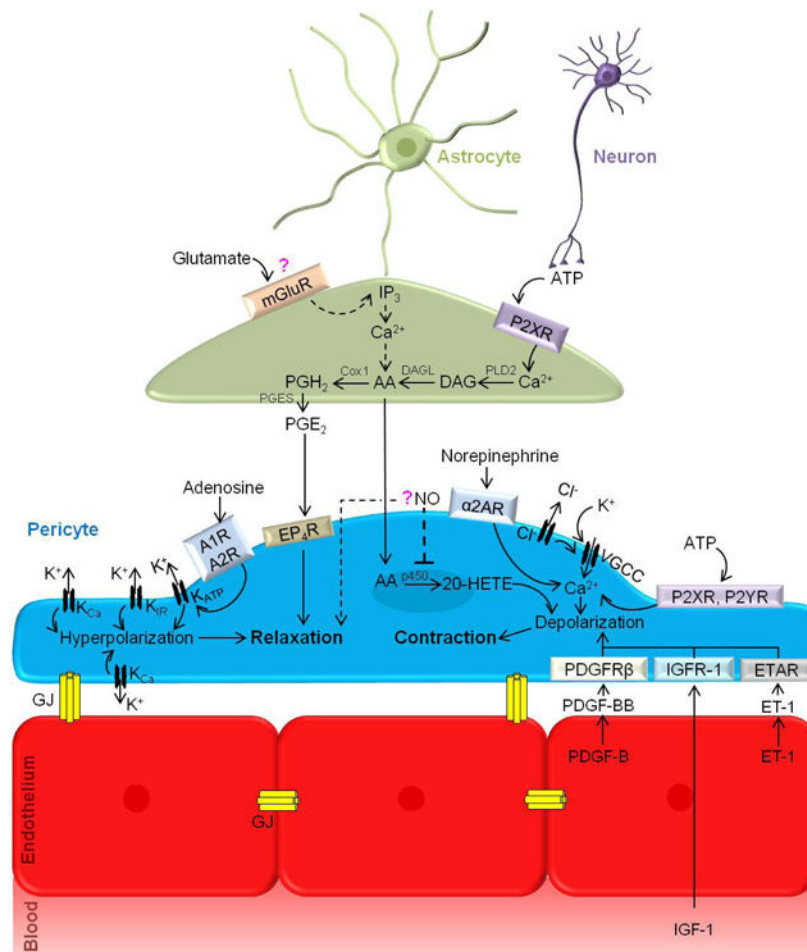
Figure 3. Capillary regulation of cerebral blood flow

Neuronal ATP can activate P2XRs on astrocytes to produce AA via phospholipase D 2 (PLD2)-mediated production of diacylglycerol (DAG) and subsequent metabolism by DAG lipase (DAGL). Cox1 can metabolize AA to produce PGE_2 via prostaglandin H2 (PGH2) and prostaglandin E synthase (PGES), triggering EP_4R on pericytes and leading to pericyte relaxation. AA produced in astrocytes can also diffuse into pericytes and form 20-HETE via p450 leading to pericyte depolarization and contraction. Neuronal activity releases glutamate, which has been shown to activate astrocytic mGluRs, triggering an IP_3– dependent increase in intracellular $[Ca^{2+}]$ leading to AA production via the PLA2 pathway. However, recent studies contradict this finding (indicated by dashed lines and question mark. Neurotransmitters adenosine, norepinephrine, and ATP have been demonstrated to alter pericyte contractile state. Specifically, adenosine binds A1 and A2 receptors (A1R, A2R) and activates KATP channels leading to hyperpolarization and relaxation. Also, activation of potassium channels including KCa and possibly KIR results in hyperpolarization of pericytes, decreasing Ca^{2+} entry through voltage-gated channels. Neuronal release of NO, which in pericytes inhibits AA metabolism to 20-HETE, can lead to pericyte relaxation. However, the role of NO in capillary dilation has been questioned (indicated by dashed lines and question mark). Norepinephrine acts through α2-adrenergic receptors (α2AR) leading to increased intracellular $[Ca^{2+}]$, depolarization and contraction. ATP activation of the

purinergic receptors P2XR or P2YR on pericytes induces depolarizing currents, and increases intracellular $[Ca^{2+}]$ and pericyte contraction. Additionally, neuronal-mediated large increases in extracellular $[K^+]$ activates VGCCs, resulting in pericyte intracellular $[Ca^{2+}]$ increases, depolarization and contraction. Furthermore, several vasoconstrictors, including endothelin-1 (ET-1), platelet-derived growth factor-B (PDGF-BB), both secreted by vascular endothelial cells, or blood-derived insulin-like growth factor (IGF-1), act on their respective receptors (endothelin A receptors (ETAR), Pdgfrβ and IGFR-1) leading to depolarization of pericytes and Ca^{2+} entry into the cell. Endothelial-endothelial and pericyte-endothelial gap junctions allow fast and direct exchange of small molecules.
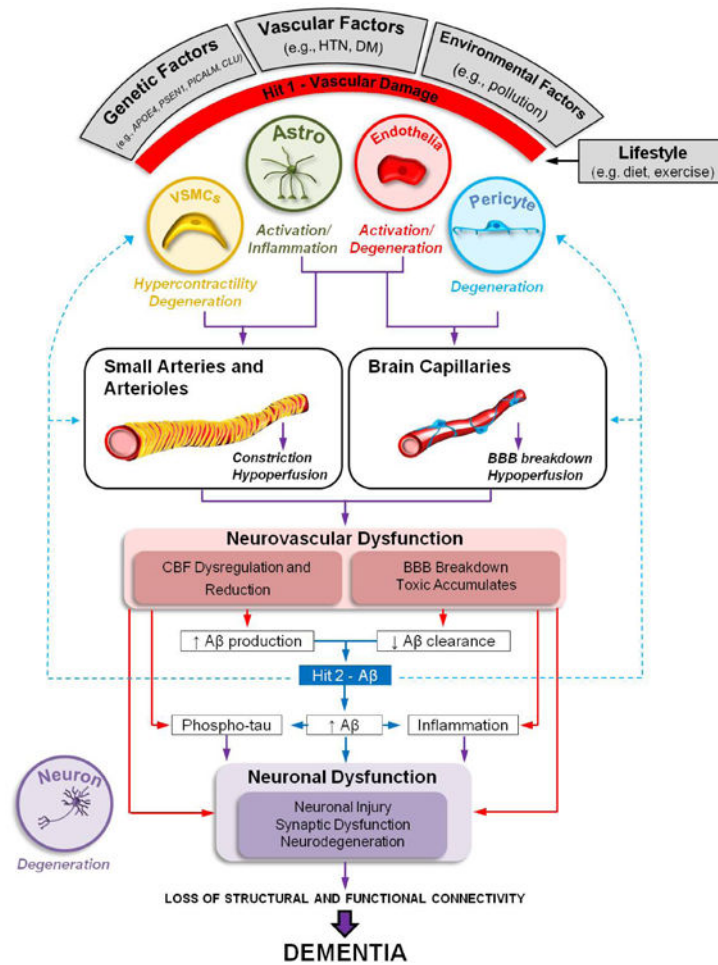
Figure 4. Neurovascular dysfunction in Alzheimer's disease: two-hit vascular hypothesis
Several *genetic risk factors* for Alzheimer's disease (AD) (*e.g.*, apolipoprotein E4 gene
(*APOE4*), Presenilin-1 mutations (*PSEN1*), Phosphatidylinositol Binding Clathrin Assembly
Protein (*PICALM*), Clusterin (*CLU*), *vascular factors* (*e.g.*, hypertension (HTN), diabetes
mellitus (DM)), and *environmental factors* (*e.g.*, pollution) lead to neurovascular dysfunction
and damage to small arteries, arterioles and brain capillaries via amyloid-β (Aβ)-
independent (*hit 1, red*) and/or Aβ-dependent (*hit 2, blue*) pathway. Both pathways interact
and converge on blood vessels, and can independently or synergistically (*purple lines*) lead
to neuronal injury, synaptic dysfunction and neurodegeneration contributing to dementia.
Lifestyle can modify the effects of these hits; for example a moderate exercise and diet have
beneficial effects on cardiovascular and cerebrovascular system.
AD affects different cell types of the neurovascular unit. For example, VSMCs
hypercontractility and degeneration leads to aberrant responses of small arteries and
arterioles, cerebral blood flow (CBF) dysregulation and reductions independently of Aβ or
in Aβ-dependent manner. In the Aβ pathway, damage to small arteries and arterioles are
often associated with amyloid angiopathy, and rupture of the vessel wall with
microhemorrhages. Degeneration of pericytes leads to loss of capillary dilation in response
to neuronal stimuli, hypoperfusion and blood-brain barrier (BBB) breakdown with
accumulation of blood-derived toxins and fluid in the perivascular spaces. Both, Aβ-

independent (e.g., hypoxia, ischemia) and Aβ-dependent mechanisms contribute to changes in capillary circulation. Endothelial damage leads to loss of endothelial-dependent vasodilation, CBF dysregulation and reductions. Activation of astrocytes and microglia mediates inflammatory response and release of vasoactive cytokines and chemokines, further comprising CBF regulation and BBB integrity.

Damage to blood vessels can initiate a cascade of events leading to Aβ accumulation in brain (*hit 1*), which accelerates the Aβ-dependent pathway of neurodegeneration (*hit 2*). For example, brain ischemic changes (*hit 1*) lead to increased Aβ production by stimulating expression of α and γ secretases, enzymes mediating Aβ generation, whereas BBB dysfunction in Aβ clearance receptors lipoprotein receptor and multidrug resistance protein 1 leads to faulty Aβ clearance and retention in brain.

Reduced CBF (*hit 1*) and elevated Aβ (*hit 2*) can each independently or synergistically lead to tau phosphorylation (Phospho-tau) and tau pathology in neurons, and worsen neuroinflammation. When combined, they accelerate neuronal damage and injury.