Diverse mechanisms regulating brain energy supply at the capillary level

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Highlights

* Capillary pericytes play a key role in regulating cerebral blood flow

* Enzyme-derived messengers and inorganic molecules may both contribute to capillary control of brain blood flow

* Interaction between capillary pericytes and endothelial cells can contribute to neurovascular coupling

Abstract

Neural information processing depends critically on the brain's energy supply, which is provided in the form of glucose and oxygen in the blood. Regulation of this supply occurs by smooth muscle and contractile pericytes adjusting the diameter of arterioles and capillaries, respectively. Controversies exist over the relative importance of capillary and arteriolar level control, whether enzymatically generated signals or K^+ ions are the dominant controller of cerebral blood flow, and the involvement of capillary endothelial cells. Here, we try to synthesise the relevant recent data into a coherent view of how brain energy supply is controlled and suggest approaches to answering key questions.

1. Introduction

The majority of brain energy expenditure is on neuronal information processing [1]. The fact that neural activity increases cerebral blood flow, to match energy supply to energy usage, was suggested 130-140 years ago by Mosso and by Roy and Sherrington (for a historical review see [2]), and it became increasingly important to understand this neurovascular coupling (NVC) process on realising that it underlies non-invasive brain imaging using Blood Oxygen Level Dependent functional Magnetic Resonance Imaging (BOLD fMRI). Researchers found enzyme-derived mediators of NVC by two distinct bodies of work recognised by Nobel Prizes: one in 1982 for the arachidonic acid-derived prostaglandins which were later recognised as also being generated by astrocytes, thus

providing a link between neurons and blood vessels [3], and another in 1998 for work showing that nitric oxide (NO) is an endothelium-derived relaxing factor in the cardiovascular system (including in the brain [4]).

In the intervening 40 years, a large number of experiments have demonstrated that NO and various arachidonic acid (AA) derivatives including prostaglandin E_2 (PgE2) and epoxyeicosatrienoic acids (EETs) contribute to NVC [5]. Strikingly, however, inhibiting the enzymes producing such messengers leads to at most a roughly 65% reduction of NVC [6]••. A possible mechanism acting in parallel to enzyme-derived messenger systems is neuronal activity-evoked extracellular K^+ concentration ($[K^+]_o$) rises. $[K^+]_o$ rises near blood vessels as a result of spatial buffering of K^+ through astrocytes to their vascular endfeet [7]. This rise of $[K^+]_o$ would hyperpolarise arteriolar smooth muscle cells (SMCs) or capillary pericytes by evoking an outward current through inward rectifier channels (see Potassium section below), thus evoking relaxation and an increase of blood flow. An initial test of this idea proved negative [8], but the concept was reinvigorated with the notion that neuronal activity-evoked intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) transients in astrocytes might spread to the astrocyte endfoot and trigger K^+ efflux through Ca^{2+}-sensitive K^+ channels. This would directly trigger contractile cell relaxation [9], or endothelial cell hyperpolarisation which could spread along the endothelium and through gap junctions to arteriolar SMC, decreasing their Ca^{2+} influx and causing them to relax [10]••. Another mechanism independent of enzyme-derived messengers involves neuronal activity-mediated local decreases of the oxygen concentration ($[O_2]$) which may also increase blood flow by making red blood cells (RBC) more deformable [11].

This review will address the conflict between the concepts of blood flow being mediated by enzyme-derived messengers versus inorganic K^+ ions or O_2 molecules. Do these systems act independently, what is their relative importance and do they interact?

The second issue we will address is the locus of control of CBF. Despite the award of the Nobel Prize to Krogh in 1920 for his discovery of contractile elements (now termed pericytes) on capillaries which act independently of SMCs on arterioles [12], until recently the majority

of neuroscientists assumed that cerebral blood flow (CBF) is controlled solely by arteriolar SMCs. It is now known, however, that most of the resistance of the brain's vascular system is located in capillaries rather than arterioles [13]••, and that pericytes are contractile and control CBF in health and in a range of diseases [14–19]. With this change of viewpoint, it is becoming crucial to understand at which level of the capillary bed pericytes can regulate blood flow, and the relative importance of pericytes versus arteriolar SMCs in that control. Moreover, we will address how an interaction between pericytes and capillary endothelial cells (cECs) may contribute to NVC.

It is remarkable that we still do not know for sure which messengers regulate CBF, or even at which level of the vascular bed they act. This review attempts to synthesise answers to these questions, and to propose experiments to gain further mechanistic insights.

2. The locus of the initiation of neurovascular coupling

Capillary dilation precedes arteriolar dilation [15,17,18,20], implying that capillaries actively dilate to increase CBF, rather than passively increasing diameter in response to a pressure rise produced by upstream arteriolar dilation. Capillary dilation is mediated by relaxation of pericytes, the only contractile cell in the capillary bed. In contrast, two groups argued that pericytes do not contribute to functional hyperaemia [11,21]. This discrepancy originates in a different definition of what is a pericyte, based on morphology and expression of the contractile protein alpha smooth muscle actin (αSMA) (reviewed by [22]). Recent work on cerebral precapillary "sphincters" increases the ambiguity of cell definition [17]. Sphincters are located at the junction between the penetrating arteriole (PA) and some 1st order capillaries, and constitute a narrowing established by contractile cells around the vessel wall – arguably pericytes – which act as a bottle neck for RBCs. Importantly, across labs there is consensus that the pericytes located on the first 4 branch orders of capillaries coming off the PA are involved in NVC, but are often mistaken to be SMCs [21,23].

These pericytes on capillaries just downstream of the penetrating arteriole (PA) have more circumferential processes around the capillaries and express higher levels of αSMA

than pericytes towards the distal end of the vascular bed which have more longitudinal processes and less αSMA. The αSMA content is interpreted as an indicator that pericytes can contribute to NVC. Indeed, 1st and 2nd branch order capillaries show the largest dilation to neuronal activity [15,24]. However, it has been shown that αSMA visualization in paraformaldehyde fixed tissue is hampered because of actin depolymerisation, and a faster cold methanol-based fixation technique allows detection of small quantities of αSMA in pericytes up to the 7th vessel branch order [25]. In addition, to αSMA-based contractility, Rho kinase signalling and actin polymerisation could alter contractile tone in pericytes with lower αSMA levels [26–28]. These higher order pericytes may also contribute to NVC [28], since they exhibit a delayed pericyte $[Ca^{2+}]_i$ reduction in response to neuronal activity [20]•, conceivably relaxing the vessel wall and allowing RBCs to pass more rapidly, as proposed by [14].

To date, there is unanimity that in response to neuronal activity pericytes on the first 4 capillary branch orders increase CBF, and it is documented that 1st and 2nd order capillaries dilate before arterioles, but it is unclear to what extent pericytes at different positions in the vascular bed contribute to NVC. This is mostly because we lack specific cellular markers to examine their function, a gap in our knowledge we will revisit below.

3. Messengers signalling to the cerebral vasculature

As outlined in the Introduction, numerous messengers released by neuronal activity may contribute to NVC [5]. These include enzyme-derived agents such as NO and arachidonic acid (AA) metabolites (vasodilatory PgE2 and EETs, and vasoconstricting 20-HETE), ATP which acts on astrocyte P2X receptors to release AA derivatives [29]•• (see Figure 1B), ATP-derived adenosine which can elicit vasodilation via mural cell receptors and K^+ which may hyperpolarise mural or endothelial cells [9,10]. Finally, local reductions in O_2 could increase blood flow by making RBCs deform more easily as they pass through capillaries [11] (see Figure 1C). How might all these messengers interact?

Nitric oxide

A meta-analysis suggested that, of all these messenger systems, blockage of neuronal NO synthase (nNOS) may cause the largest reduction in NVC [6]••. NO dilates by raising the cGMP level, and by blocking synthesis of 20-HETE [15] which blocks BK_{Ca} channels and thus depolarises contractile cells causing vasoconstriction [30]. NO appears to act on arterioles in cerebral cortex [29]••, but in cerebellum dilates capillaries via pericytes by inhibiting 20-HETE production [15], suggesting brain region-dependent differences in the molecular organisation of NVC.

AA metabolites

PgE2 and EET formation from AA and release is stimulated by a neuronal activity-evoked rise in astrocytic $[Ca^{2+}]_i$, and - at least in brain slices - arteriolar dilation does not depend on astrocytic $[Ca^{2+}]_i$ changes or PgE2 release [8,29,31]. However, neurons may also release AA which is taken up by astrocytes and converted into PgE2 and EET release in the absence of astrocytic $[Ca^{2+}]_i$ changes [32], and furthermore it has been suggested that an increase in arteriole pressure or flow evokes a rise in astrocyte $[Ca^{2+}]_i$ rather than vice versa [33]. The PgE2 released from astrocytes is a major mediator of NVC at capillaries [15,29], apparently acting via pericyte EP4 receptors (EP4R) [15,29,34], to relax pericytes and thus dilate capillaries. Curiously, while EP4R block decreases pericyte-mediated hyperaemia [15,29], neither immunohistochemistry nor transcriptomic work report high levels of EP4R expression by brain pericytes, perhaps suggesting an unknown intermediate signalling step between PgE2 and pericyte relaxation.

ATP and adenosine

Following synaptic activity, ATP is released into the extracellular space. In addition to ATP and its metabolites' actions on neuronal excitability [35], ATP and adenosine have also vasomodulatory effects. ATP raises astrocytic $[Ca^{2+}]_i$ by activating $P2X_1$ receptors and this elicits a phospholipase D-dependent release of PgE2 which dilates pericytes [29]••. ATP is degraded rapidly to ADP and adenosine by extracellular endonucleotidases. Blocking adenosine receptors reduced stimulation-evoked blood flow [36] and adenosine may dilate both arterioles and capillaries [37,38]. Adenosine acts via the A2A receptor, a G_sPCR,

[38,39], and neuronal activity-induced increases in adenosine level correlated with and preceded [O_2] increases [39]. This raises the question of whether ATP and adenosine are equally important for functional hyperaemia. Blocking the ATP-astrocyte pathway inhibited capillary dilation by ~70% [29]•• and promoting the conversion of ATP to adenosine by overexpressing endonucleotidase resulted in reduced functional hyperaemia [40], suggesting that the ATP-astrocyte-PgE2-pericyte-relaxing pathway is more important than the actions of adenosine.

Potassium

In parallel with enzyme-derived messenger systems, neuronal activity raises $[K^+]_o$ (see Figure 1C). Conventionally, $[K^+]_o$ elevations depolarise cells, but for inward rectifier K^+ (Kir) channels, the conductance is increased by a rise of $[K^+]_o$ [41], resulting in an increased outward current and cell hyperpolarisation (and activation of the Na/K-ATPase by external K^+ may also contribute an outward current, at least transiently [42]). In arteriolar smooth muscle, this is suggested to close voltage-gated Ca^{2+} channels, lowering $[Ca^{2+}]_i$ and causing relaxation [9]. Brain pericytes also express the inward rectifier channel subunits Kir2.1 and Kir2.2 [43,44], and patch-clamping showed Kir2-mediated currents in retinal pericytes [45,46]. Thus, brain pericytes may also respond directly to rises in $[K^+]_o$ with hyperpolarisation, as suggested by a preprint [47] and our own unpublished data, and pericyte voltage changes can spread rapidly (through gap junctions with the endothelial cells) to other pericytes and possibly to arteriolar SMCs further along the capillary [14,48].

Interestingly, capillary endothelial cells (cECs) also express Kir2.1 and Kir2.2 and a neuronal activity-induced rise in $[K^+]_o$ hyperpolarises cECs [10]••. This hyperpolarisation propagates upstream to the PA and spreads to arteriolar SMCs through myoendothelial gap junctions, relaxing the SMCs. Genetic ablation of Kir2.1 from ECs suggested that this mechanism confers ~50% of stimulation-evoked hyperaemia, although this knock-out may have unanticipated effects on the pericytes and SMCs overlying the ECs. This EC to arteriolar SMC signalling is unlikely to be the first event in NVC, however, since pericyte-mediated capillary dilation precedes arteriolar dilation [15,17,18,20,24]. Indeed, pericytes are

also electrically coupled to ECs via myoendothelial gap junctions [49–51] and may hence relax in response to cEC hyperpolarisation before upstream SMC relaxation. Longden et al. (2017) reported that raising $[K^+]_o$ around capillaries had no effect on capillary diameter but our own unpublished experiments on brain slices demonstrate a dilation at pericyte locations, which is also echoed by more recent work from the Nelson lab [19]••.

Retinal pericytes have a different resting membrane potential (V_M) according to their position in the vascular tree: pericytes on capillaries near arterioles are more hyperpolarised (V_M ~-45 mV) than pericytes deeper in the capillary bed (V_M ~-35 mV) [46]. If brain pericytes exhibit a similar V_M stratification, when $[K^+]_o$ rises the resulting difference in outward current and degree of activation of voltage-gated Ca^{2+} channels may result in pericytes at different locations evoking $[Ca^{2+}]_i$ (and capillary diameter) changes of different magnitude and kinetics, as observed by [20]• and also seen in our own unpublished data.

In conclusion, neuronal-evoked elevations in $[K^+]_o$ are likely to contribute to dilations of capillaries and arterioles. Surprisingly, we lack information on how much $[K^+]_o$ rises near endothelial cells, pericytes and arteriolar smooth muscle cells, and on its spatio-temporal dynamics following neuronal activity, for which we propose concrete experiments below.

Oxygen

Neuronal activity can locally reduce O_2 concentration, which increases RBC deformability [11]. This would allow RBCs to deform to pass through capillaries more readily, thus decreasing vascular resistance and increasing blood flow. Although interesting, this effect cannot explain NVC for both qualitative and quantitative reasons. First, when neurons are active, although the local $[O_2]$ can initially fall [52], the increase in blood flow produced by NVC brings in excess extra O_2 so that the local $[O_2]$ rises above its original value even while a maintained increase in blood flow is produced [53]. As the $[O_2]$ is higher, the increase in blood flow hence cannot be being driven by low $[O_2]$ affecting RBCs. Second, the effect of the O_2-evoked increase of RBC deformability on flow is quantitatively very small. When cortical neurons were activated by hindlimb stimulation they lowered cortical $[O_2]$ by 1.9 mm Hg (Fig. 5F of [11]). The same decrease of $[O_2]$, when applied to RBCs in PBS flowing

through a 5 μm diameter glass tube produced only a 1.3% increase of RBC speed (Fig. 4D of [11]), which is less than 9% of the RBC speed increase seen in the cortex during neuronal activation (15%, Fig. 5C of [11]). Although perfusion through a glass microtube may not well recapitulate the environment of flow through a capillary owing to lack of the glycocalyx which lines the capillaries, it appears that the O_2 effect on deformability can only explain less than 10% of NVC. RBCs may nevertheless contribute to NVC by releasing, in response to low [O_2], ATP, which activates NO production in ECs [54].

4. Interactions of enzyme-generated messengers and K^+ in NVC

Are the various mechanisms of NVC discussed above simply additive, or do they interact in a synergistic or occlusive manner? The meta-analysis of Hosford and Gourine showed that inhibiting the production of enzymatically-derived messengers leads to at most a 65% reduction of NVC, while blocking K^+ channels reduces NVC by a highly variable amount with a mean suppression of 40% [6]••. Interpreting the effects of applying these inhibitors is complicated by the fact that, as described below, enzyme-derived messengers affect the Kir channels that can contribute to NVC, while blockers of K^+ channels (even if they only affect the target cell, which is often not the case) will affect not only changes of membrane current evoked by $[K^+]_o$ rises but also the cell's resting potential (thus potentially altering Ca^{2+} influx through voltage-gated Ca^{2+} channels).

When examining K^+-evoked hyperpolarisation in ECs, Harraz et al. demonstrated that activation of G_qPCRs (which activate phospholipase C to convert the lipid PIP_2 to Ca^{2+}-releasing IP_3) leads to a fall of PIP_2 level which inhibits Kir2 channels (see Figure 2) [55,56]. As a result, the hyperpolarisation generated when $[K^+]_o$ rises, and its propagation from capillaries to arterioles, are inhibited. Vasodilating PgE2 (generated in the ATP-astrocyte-PgE_2-pericyte pathway for regulating blood flow) acts on G_q-coupled EP1 receptors expressed in the ECs (www.mousebrain.org/genesearch). Thus, we would expect the activation of NVC via PgE2 release to suppress NVC mediated by Kir2 channels on ECs. Conversely block of the PgE2 pathway would allow a larger K^+-mediated response to spread

to arteriolar SMCs and perhaps pericytes, resulting in an underestimate of the contribution of PgE2 to NVC.

Since pericytes and arteriolar SMCs also express some EP1 receptors (www.mousebrain.org/genesearch) one might expect that PgE2 would also suppress Kir2 channels in these cells, and thus also inhibit NVC evoked by a $[K^+]_o$ rise around these contractile cells. Surprisingly, however, Kir channels in contractile cells appear to be much less sensitive to a fall in PIP_2 level than are EC Kir2 channels [57].

5. Coordination of NVC at the capillary and arteriolar level

Mishra *et al.* proposed [29]•• that NVC at the capillary level was largely mediated by PgE2, and at the arteriolar level was mediated largely by NO, although K^+ may contribute in both locations. The faster dilation of capillaries than of arterioles [15] might then reflect differences in the kinetics of generation of these messengers (the relative speed of capillary dilation mediated by PgE2 and by K^+ is unknown), or the fact that capillaries are in general closer to active neurons than are arterioles [58] so the messengers need to diffuse less distance to activate capillary NVC. If there was extensive propagation of vasodilating voltage signals along ECs between the capillaries and arterioles [10]•• then one would presumably expect some mixed pharmacology at both locations when testing blockers of the PgE_2 and NO pathways, but capillary to arteriole endothelial connections may often be disrupted in the brain slices largely used in the experiments of Mishra *et al.* [29]••.

An interesting question is whether propagation of hyperpolarisation along capillary ECs has an intrinsic directionality to it (e.g. upstream to arterioles). A mechanism for this has recently been suggested in the retina [59], based on gap junction coupling between pericytes and ECs facilitated by NO which opens connexin-43 containing gap junctions [60,61]. A preference for hyperpolarising upstream ECs was proposed to reflect accumulation of caveolae (cell membrane invaginations) containing caveolin-1 and eNOS at the upstream end of the ECs [62]. However, it is unclear how such a mechanism alone would generate directional selectivity, as the upstream end of one EC is the downstream end

of the next one; conceivably a gradient of gap junctional strength along the endothelium also contributes.

Supporting the notion that caveolin-1 is important in NVC, deleting caveolin-1 from arteriolar ECs roughly halved both the 15% increase in somatosensory cortex arteriole diameter and the ~2.7-fold increase in capillary blood flow evoked by whisker stimulation [63]•. This was attributed to altered NVC at the arteriole level mediated by EC caveolae, conceivably modulating a propagated voltage change in ECs arriving from capillaries. Despite the association of caveolin-1 with eNOS (see above), this effect proved to be independent of eNOS, although eNOS did contribute to NVC (perhaps activated by ATP release from astrocytes [64]). However, quantitative considerations argue against such a (previously unknown) mechanism acting solely at the arteriole level. The 15% increase of arteriolar diameter in control conditions [63]• would evoke a 1.75-fold increase in flow (by simplistic use of Poiseuille's law) if arterioles were the only significant resistance in the cortical vasculature. However, arterioles comprise only around $1/7^{th}$ of the total intracortical vascular resistance [13]•• so, to explain the 2.7-fold increase in downstream capillary blood flow [63]•, most of the increase in blood flow evoked by neuronal activity must be attributed to relaxation of pericytes on downstream capillaries. This suggests that the change of NVC seen when deleting caveolin-1 is not occurring at the arteriole SMC adjacent to the ECs where the caveolae are being suppressed, but somehow instead occurs at downstream capillary pericytes.

Some limitations on vasodilatory signal spread may be imposed by thin processes ("nanotubes") reported to link pericytes on different capillary trees [65]•. Following pericyte-mediated capillary dilation, the pericytes connected by nanotubes to a dilating pericyte are reported to constrict their underlying capillary, perhaps generating a focus of capillary dilation surrounded by other constricted capillaries. It has also been proposed that such a focus in blood supply can be achieved by pericytes located at branch points through preferentially dilating only one of the downstream daughter vessels [19]••. This phenomenon

could be instructed by a hyperpolarising signal backpropagating through the endothelium, hence ensuring the supply of RBC to locations with enhanced energy need.

6. Future directions and conclusions

Given the uncertainty about the properties of pericytes and ECs at different positions in the capillary bed, and how they interact with each other and with arteriolar SMCs, we suggest the following key avenues for tackling the largest gaps in our knowledge (see Figure 3).

Defining mural cell differences in the brain vascular bed using spatial transcriptomics

Although it has been suggested that all pericytes are essentially identical and lack contractile proteins [44], the current consensus, based on imaging pericyte contraction and relaxation, is that pericytes on at least the first 4 branch orders of capillaries are contractile [14,15,17,18,20,22] and possibly those on higher order vessels as well [28], but also that pericytes differ in their properties along the capillary bed. Spatial transcriptomic techniques, such as seqFISH+ [66], will allow researchers to define the mRNA expression profile of mural (and endothelial) cells as a function of their location in the vascular bed (see Figure 3A), and hence facilitate the creation of population-specific conditional CreER-driver mouse lines.

Simultaneous read-out of pericyte and endothelial cell activity

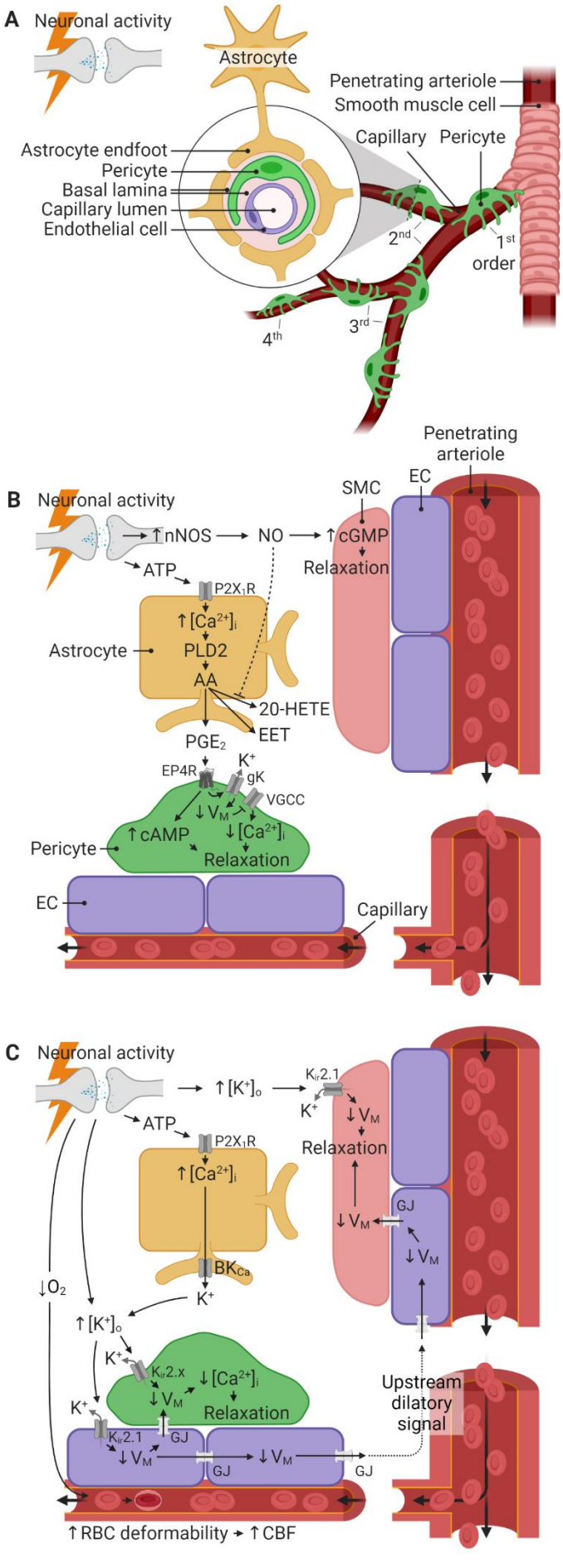
Monitoring and manipulating cells simultaneously will be key to establishing the relative contribution to NVC of SMCs, pericytes and endothelial cells at different locations in the vascular bed, and assimilation of technical advances from circuit neuroscience laboratories will facilitate progress (see Figure 3B). Use of the existing rich optogenetic and DREADD toolbox is still in its infancy in the brain blood flow field, but optogenetic modulation of pericytes is emerging as a promising tool [28,67,68]. Simultaneous two-colour Ca^{2+} imaging in pericytes and cECs paired with neuronal activation, puff application of candidate signalling molecules (e.g. K^+ and PgE2), and cell (population) specific manipulation will also allow testing of whether and how pericytes and cECs interact to accomplish NVC.

Defining the locus and spread of neuronal activity-mediated $[K^+]_o$ rise

Imaging the origin, kinetics and spread of K^+ is hampered by a lack of available tools. Conventionally, K^+-sensitive electrodes are used to estimate changes in $[K^+]_o$ [69], but they provide poor temporal resolution, and could damage the tissue. Extracellular use of standard K^+-sensitive indicators suffers from poor selectivity against Na^+. While indicators sensitive enough to report neuronal activity-derived changes in $[K^+]_o$ have been developed [70], a better solution would be a genetically expressed indicator similar to the glutamate-sensitive fluorescence reporter (GluSnFR) family [71] (see Figure 3C).

Concluding remarks

Capillaries comprise the largest vascular resistance within the brain, and the brain's main energy supply. Pericytes are the only contractile cell type modulating capillaries but, along with capillary endothelial cells, they are understudied. This review emphasises the importance of understanding control of capillary haemodynamics, which underpins not only an adequate brain energy supply but also non-invasive BOLD fMRI imaging.


A
Neuronal activity
Astrocyte
Penetrating arteriole
Smooth muscle cell
Capillary
Pericyte
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Basal lamina
Capillary lumen
Endothelial cell
2nd
1st order
3rd
4th
B
Neuronal activity
↑nNOS
NO
↑cGMP
SMC
EC
Penetrating arteriole
Relaxation
ATP
P2X1R
↑[Ca2+]i
Astrocyte
PLD2
AA
20-HETE
EET
PGE2
EP4R
K+
gK
VGCC
↓VM
↑cAMP
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P2X1R
↑[Ca2+]i
GJ
↓VM
BKCa
↓O2
K+
↑[K+]o
K+
Kir2.x
↓[Ca2+]i
↓VM
Relaxation
K+
Kir2.1
GJ
↓VM
↓VM
GJ
GJ
Upstream dilatory signal
↑RBC deformability → ↑CBF

Figure 1: Current view of neurovascular coupling. (**A**) Introduction to the vascular tree and neurovascular unit. Capillaries branch off from penetrating arterioles and are classified according to their branch order. Smooth muscle cells and pericytes are contractile cells regulating the diameter of arterioles and capillaries, respectively. Inset: Endothelial cells (purple) make up the vessel wall, pericytes (green) are tightly wrapped around the endothelial cells. Both share a basal lamina. Astrocyte endfeet are in close apposition to the vessel. (**B**) Enzyme-derived messengers mediating NVC. Postsynaptic activation of nNOS in interneurons leads to NO production, which relaxes SMCs by raising cGMP levels, and dilates arterioles. NO acts on capillaries by blocking 20-HETE production (arrow with bar at end). Neuronal activity-derived ATP binds to astrocyte $P2X_1$ receptors raising $[Ca^{2+}]_i$ to elicit PgE2 and EET formation from AA via PLD2. PgE2 acts on pericyte EP4R. This raises [cAMP], activates a hyperpolarising K^+ efflux and thus inhibits voltage-gated Ca^{2+} channels (VGCCs) which all result in pericyte relaxation and capillary dilation. (**C**) Contribution of inorganic K^+ ions and O_2 molecules to NVC. Neuronal activity raises $[K^+]_o$ during neuronal repolarisation and by evoking K^+ release from astrocytes through Ca^{2+}-dependent K^+ channels (BK_{Ca}). Increased $[K^+]_o$ hyperpolarises SMCs by evoking a K^+ efflux through Kir2.1 channels, which leads to closure of VGCCs, and a $[Ca^{2+}]_i$ reduction, SMC relaxation and arteriolar dilation. Pericytes may also respond to $[K^+]_o$ elevations with hyperpolarisation and relaxation, causing capillary dilation. A $[K^+]_o$ rise hyperpolarises cECs via Kir2.1 channels. This hyperpolarisation propagates upstream to the penetrating arteriole via myoendothelial gap junctions and relaxes SMCs. Endothelial cells are also gap junctionally coupled with pericytes and may hyperpolarise these as well, dilating capillaries. Neuronal activity can introduce a local dip in $[O_2]$ which increases RBC deformability, increasing cerebral blood flow somewhat.

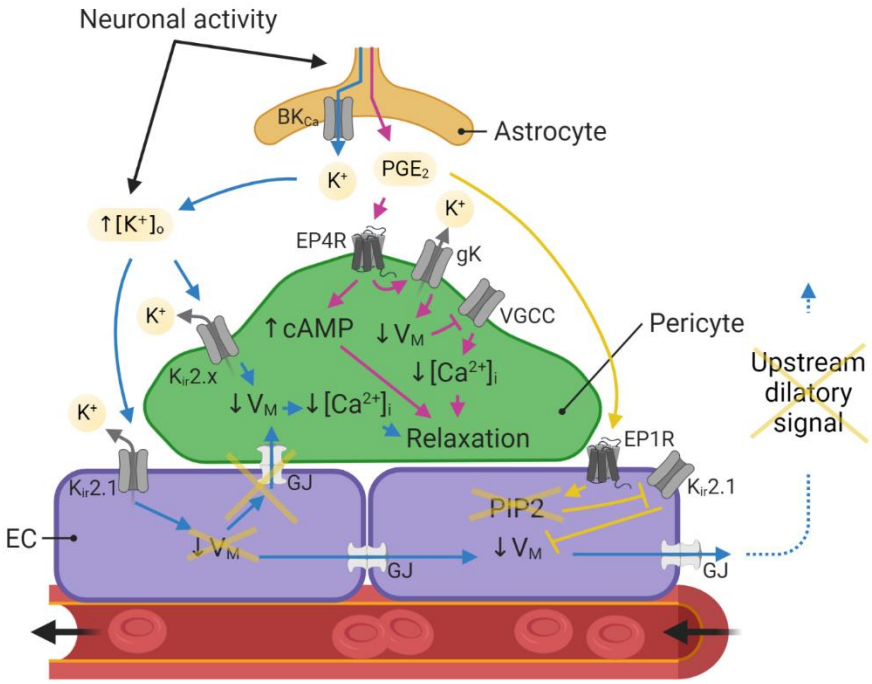

Figure 2: Interaction of PgE_2 and K^+ in NVC. PgE_2 dilates pericytes via EP4 receptors, as in Fig. 1 (purple arrows). Neuronal activity-evoked $[K^+]_o$ rise may produce local pericyte-mediated capillary dilation by Kir2 based hyperpolarisation of cECs and subsequent gap junction coupling-based hyperpolarisation of pericytes, or direct pericyte hyperpolarisation via pericyte Kir2 channels (blue arrows). Upstream arteriolar dilation may in part be achieved by the backpropagation of an endothelial hyperpolarising signal relaxing SMCs via myoendothelial gap junctions. In cECs, PgE_2 binds to G_q-coupled EP1Rs, leading to PIP_2 depletion which inhibits Kir2 channels and thus prevents the backpropagation of the hyperpolarising signal (yellow arrows and crosses). For illustration purposes gap junction coupling of pericytes and cECs, and PIP_2 depletion are each only shown in one cEC.

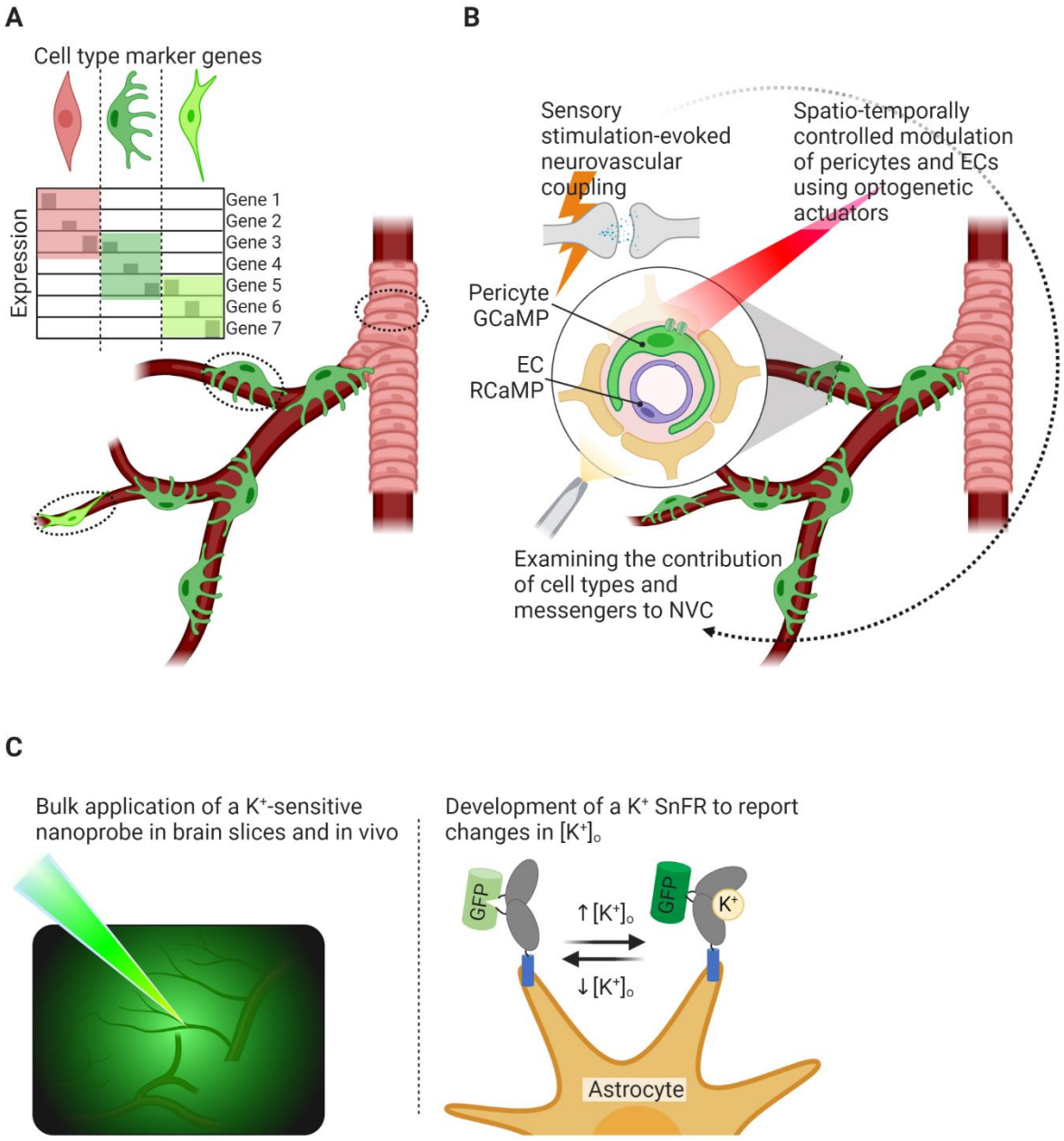

Figure 3: Future experimental directions. (**A**) Defining differences in contractile cells around the vessel wall in the vascular bed based on gene expression profiles using spatial transcriptomics. (**B**) Simultaneous monitoring of pericyte and endothelial cell activity paired with spatio-temporally controlled manipulation (e.g. using optogenetic actuators) and puff-application of substances modulating signalling pathways following neuronal activity, should facilitate dissection of the cells and messengers mediating NVC. (**C**) Application of novel extracellular K^+ indicators and the development of genetically-expressed indicators

comparable to the glutamate-sensitive fluorescence reporter (GluSnFR) may facilitate identifying the origin and magnitude of the rise in K^+ concentration and its spread.

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References

1. Attwell D, Laughlin SB: **An energy budget for signaling in the grey matter of the brain.** J Cereb Blood Flow Metab 2001, 21:1133–1145.

2. Iadecola C: **The Neurovascular Unit Coming of Age: A Journey through Neurovascular Coupling in Health and Disease.** Neuron 2017, 96:17–42.

3. Koehler RC, Gebremedhin D, Harder DR: **Role of astrocytes in cerebrovascular regulation.** J Appl Physiol 2006, 100:307–317.

4. Garthwaite J, Charles S, Chess-Williams R: **Endothelial-derived relaxing factor release on activation of NMDA receptors suggests role as intraceullar messenger in the brain.** Nature 1988, 53:1689–1699.

5. Attwell D, Buchan AM, Charpak S, Lauritzen M, MacVicar BA, Newman EA: **Glial and neuronal control of brain blood flow.** Nature 2010, 468:232–243.

6. •• Hosford PS, Gourine A V.: **What is the key mediator of the neurovascular coupling response?** Neurosci Biobehav Rev 2019, 96:174–181.

Remarkable meta-analysis of the NVC literature highlighting our lack of understanding of NVC by demonstrating that none of the currently investigated signalling pathways account for more than 70% of the control of blood flow.

7. Paulson O, Newman EA: **Does the release of potassium from astrocyte endfeet regulate cerebral blood flow.** Science 1987, 237:1979–1982.

8. Metea MR, Kofuji P, Newman EA: **Neurovascular coupling is not mediated by potassium siphoning from glial cells.** J Neurosci 2007, 27:2468–2471.

9. Filosa JA, Bonev AD, Straub S V., Meredith AL, Wilkerson MK, Aldrich RW, Nelson MT: **Local potassium signaling couples neuronal activity to vasodilation in the brain.** Nat Neurosci 2006, 9:1397–1403.

10. •• Longden TA, Dabertrand F, Koide M, Gonzales AL, Tykocki NR, Brayden JE, Hill-Eubanks D, Nelson MT: **Capillary K+-sensing initiates retrograde hyperpolarization to increase local cerebral blood flow.** Nat Neurosci 2017, 20:717–726.

Developed the concept that capillary endothelial cells act as a sensory web to detect changes in neuronal activity using Kir2 channels. The resulting hyperpolarisation is translated into upstream arteriolar dilation mediated by SMCs.

11. Wei HS, Kang H, Rasheed IYD, Zhou S, Lou N, Gershteyn A, McConnell ED, Wang Y, Richardson KE, Palmer AF, et al.: **Erythrocytes Are Oxygen-Sensing Regulators of the Cerebral Microcirculation.** Neuron 2016, 91:851–862.

12. Krogh A: **The supply of oxygen to the tissues and the regulation of the capillary circulation.** 1919

13. •• Gould IG, Tsai P, Kleinfeld D, Linninger A: **The capillary bed offers the largest hemodynamic resistance to the cortical blood supply.** J Cereb Blood Flow Metab 2017, 37:52–68.

Identified that most vascular resistance in the brain is located in the capillaries, and not in arterioles, supporting the importance of capillary pericytes for regulating cerebral blood flow.

14. Peppiatt CM, Howarth C, Mobbs P, Attwell D: **Bidirectional control of CNS capillary diameter by pericytes.** Nature 2006, 443:700–704.

15. Hall CN, Reynell C, Gesslein B, Hamilton NB, Mishra A, Sutherland BA, Oâ Farrell FM, Buchan AM, Lauritzen M, Attwell D: **Capillary pericytes regulate cerebral blood flow in health and disease.** Nature 2014, 508:55–60.

16. Nortley R, Korte N, Izquierdo P, Hirunpattarasilp C, Mishra A, Jaunmuktane Z, Kyrargyri V, Pfeiffer T, Khennouf L, Madry C, et al.: **Amyloid b oligomers constrict human capillaries in Alzheimer's disease via signaling to pericytes.** Science 2019, 365, eaav9518.

17. Grubb S, Cai C, Hald BO, Khennouf L, Murmu RP, Jensen AGK, Fordsmann J, Zambach S, Lauritzen M: **Precapillary sphincters maintain perfusion in the cerebral cortex.** Nat Commun 2020, 11, 395.

18. Kisler K, Nelson AR, Rege S V., Ramanathan A, Wang Y, Ahuja A, Lazic D, Tsai PS, Zhao Z, Zhou Y, et al.: **Pericyte degeneration leads to neurovascular uncoupling and limits oxygen supply to brain.** Nat Neurosci 2017, 20:406–416.

19. •• Gonzales AL, Klug NR, Moshkforoush A, Lee JC, Lee FK, Shui B, Tsoukias NM, Kotlikoff MI, Hill-eubanks D, Nelson MT: **Contractile pericytes determine the direction of blood flow at capillary junctions.** Proc Natl Acad Sci USA 2020, doi:10.1073/pnas.1922755117.

Demonstrated that junctional pericytes determine the directional distribution of red blood cells. The underlying pericytes relaxation is initiated by $[K^+]_o$ mediated backpropagating hyperpolarisation through the endothelium.

20. • Rungta RL, Chaigneau E, Osmanski BF, Charpak S: **Vascular Compartmentalization of Functional Hyperemia from the Synapse to the Pia.** Neuron 2018, 99:362-375.e4.

First characterization of the contribution of the different vascular compartments to NVC in the olfactory bulb.

21. Hill RA, Tong L, Yuan P, Murikinati S, Gupta S, Grutzendler J: **Regional Blood Flow in the Normal and Ischemic Brain Is Controlled by Arteriolar Smooth Muscle Cell Contractility and Not by Capillary Pericytes.** Neuron 2015, 87:95–110.

22. Attwell D, Mishra A, Hall CN, O'Farrell FM, Dalkara T: **What is a pericyte?** J Cereb Blood Flow Metab 2016, 36:451–455.

23. Damisah EC, Hill RA, Tong L, Murray KN, Grutzendler J: **A fluoro-Nissl dye identifies pericytes as distinct vascular mural cells during in vivo brain imaging.** Nat Neurosci 2017, 20:1023–1032.

24. Khennouf L, Gesslein B, Brazhe A, Octeau JC, Kutuzov N, Khakh BS, Lauritzen M: **Active role of capillary pericytes during stimulation-induced activity and spreading depolarization.** Brain 2018, 141:2032–2046.

25. Alarcon-Martinez L, Yilmaz-Ozcan S, Yemisci M, Schallek J, Kılıç K, Can A, Di Polo A, Dalkara T: **Capillary pericytes express α-smooth muscle actin, which requires prevention of filamentous-actin depolymerization for detection.** Elife 2018, 7:1–17.

26. Somlyo AP, Somlyo A V: **Signal transduction and regulation in smooth muscle.** Nature 1994, 372:231–236.

27. Fukata Y, Kaibuchi K, Amano M, Kaibuchi K: **Rho-Rho-kinase pathway in smooth muscle contraction and cytoskeletal reorganization of non-muscle cells.** Trends Pharmacol Sci 2001, 22:32–39.

28. Hartmann D, Berthiaume A-A, Grant R, Harrill S, Noonan T, Costello J, Tieu T, McDowell K, Faino A, Kelly A, et al.: **Brain capillary pericytes exert a substantial but slow influence on blood flow.** bioRxiv 2020, doi:10.1101/2020.03.26.008763.

29. •• Mishra A, Reynolds JP, Chen Y, Gourine A V., Rusakov DA, Attwell D: **Astrocytes mediate neurovascular signaling to capillary pericytes but not to arterioles.** Nat Neurosci 2016, 19:1619–1627.

Discovered that NVC at the capillary level is mostly mediated by astrocyte-derived PgE2 acting on pericytes, and that NVC at the arteriolar level is mostly mediated by NO.

30. Gebremedhin D, Lange AR, Lowry TF, Taheri MR, Birks EK, Hudetz AG, Narayanan J, Falck JR, Okamoto H, Roman RJ, et al.: **Production of 20-HETE and its role in autoregulation of cerebral blood flow.** Circ Res 2000, 87:60–65.

31. Howarth C, Sutherland B, Choi HB, Martin C, Lind BL, Khennouf L, Ledue JM, Pakan JMP, Ko RWY, Ellis-Davies G, et al.: **A critical role for astrocytes in hypercapnic vasodilation in brain.** J Neurosci 2017, 37:2403–2414.

32. Nippert AR, Biesecker KR, Newman EA: **Mechanisms Mediating Functional Hyperemia in the Brain.** Neuroscientist 2018, 24:73–83.

33. Kim KJ, Iddings JA, Stern JE, Blanco VM, Croom D, Kirov SA, Filosa JA: **Astrocyte contributions to flow/pressure-evoked parenchymal arteriole vasoconstriction.** J Neurosci 2015, 35:8245–8257.

34. Perrot CY, Herrera JL, Fournier-Goss AE, Komatsu M: **Prostaglandin E2 breaks down pericyte–endothelial cell interaction via EP1 and EP4-dependent downregulation of pericyte N-cadherin, connexin-43, and R-Ras.** Sci Rep 2020, 10:1–20.

35. Badimon A, Ayata P, Strasburger HJ, Chen X, Ikegami A, Graves SM, Ishikawa M, Chan AT, Wang Y-C, Loh YE, et al.: **Negative feedback control of neuronal activity by microglia.** Nature 2020, 585:417-423.

36. Dirnagl U, Niwa K, Lindauer U, Villringer A: **Coupling of cerebral blood flow to neuronal activation: Role of adenosine and nitric oxide.** Am J Physiol - Hear Circ Physiol 1994, 267.

37. Ko KR, Ngai AC, Winn HR: **Role of adenosine in regulation of regional cerebral blood flow in sensory cortex.** Am J Physiol - Hear Circ Physiol 1990, 259.

38. Matsugi T, Chen Q, Anderson DR: **Adenosine-induced relaxation of cultured bovine retinal pericytes.** Investig Ophthalmol Vis Sci 1997, 38:2695–2701.

39. Wang Y, Venton BJ: **Correlation of transient adenosine release and oxygen changes in the caudate-putamen.** J Neurochem 2017, 140:13–23.

40. Wells JA, Christie IN, Hosford PS, Huckstepp RTR, Angelova PR, Vihko P, Cork SC, Abramov AY, Teschemacher AG, Kasparov S, et al.: **A critical role for purinergic signalling in the mechanisms underlying generation of BOLD fMRI responses.** J Neurosci 2015, 35:5284–5292.

41. Hagiwara S, Miyazaki S, Rosenthal NP: **Potassium current and the effect of cesium on this current during anomalous rectification of the egg cell membrane of a starfish.** J Gen Physiol 1976, 67:621–638.

42. Longden TA, Nelson MT: **Vascular inward rectifier K+ channels as external K+ sensors in the control of cerebral blood flow.** Microcirculation 2015, 22:183–196.

43. Zeisel A, Hochgerner H, Lönnerberg P, Johnsson A, Memic F, van der Zwan J, Häring M, Braun E, Borm LE, La Manno G, et al.: **Molecular Architecture of the Mouse Nervous System.** Cell 2018, 174:999-1014.e22.

44. Vanlandewijck M, He L, Mäe MA, Andrae J, Ando K, Del Gaudio F, Nahar K, Lebouvier T, Laviña B, Gouveia L, et al.: **A molecular atlas of cell types and zonation in the brain vasculature.** Nature 2018, 554:475–480.

45. Quignard JF, Harley EA, Duhault J, Vanhoutte PM, Félétou M: **K+ channels in cultured bovine retinal pericytes: Effects of β-adrenergic stimulation.** J Cardiovasc Pharmacol 2003, 42:379–388.

46. Matsushita K, Puro DG: **Topographical heterogeneity of KIR currents in pericyte-containing microvessels of the rat retina: Effect of diabetes.** J Physiol 2006, 573:483–495.

47. Glück C, Ferrari KD, Keller A, Saab AS, Stobart JL, Weber B: **Distinct signatures of calcium activity in brain pericytes.** bioRxiv 2020, doi:10.1101/2020.07.16.207076.

48. Wu D, Minami M, Kawamura H, Puro D: **Electrotonic transmission within pericyte-containing retinal microvessels.** Microcirculation 2006, 13:353–363.

49. Cuevas P, Gutierrez-Diaz JA, Reimers D, Dujovny M, Diaz FG, Ausman JI: **Pericyte endothelial gap junctions in human cerebral capillaries.** Anat Embryol (Berl) 1984, 170:155–159.

50. Armulik A, Abramsson A, Betsholtz C: **Endothelial/pericyte interactions.** Circ Res 2005, 97:512–523.

51. Armulik A, Genové G, Betsholtz C: Pericytes: **Developmental, Physiological, and Pathological Perspectives, Problems, and Promises.** Dev Cell 2011, 21:193–215.

52. Enager P, Piilgaard H, Offenhauser N, Kocharyan A, Fernandes P, Hamel E, Lauritzen M: **Pathway-specific variations in neurovascular and neurometabolic coupling in rat primary somatosensory cortex.** J Cereb Blood Flow Metab 2009, 29:976–986.

53. Jessen SB, Brazhe A, Lind BL, Mathiesen C, Thomsen K, Jensen K, Lauritzen M: **GABAA receptor-mediated bidirectional control of synaptic activity, intracellular Ca2+, Cerebral Blood flow, and oxygen consumption in mouse somatosensory cortex in vivo.** Cereb Cortex 2015, 25:2594–2609.

54. Richardson KJ, Kuck L, Simmonds MJ: **Beyond oxygen transport: Active role of erythrocytes in the regulation of blood flow.** Am J Physiol Circ Physiol 2020, doi:10.1152/ajpheart.00441.2020.

55. Harraz OF, Longden TA, Eubanks DH, Nelson MT: **PIP2 depletion promotes TRPV4 channel activity in mouse brain capillary endothelial cells.** Elife 2018, 7:1–24.

56. Harraz OF, Longden TA, Dabertrand F, Hill-Eubanks D, Nelson MT: **Endothelial GqPCR activity controls capillary electrical signaling and brain blood flow through PIP2 depletion.** Proc Natl Acad Sci U S A 2018, 115:E3569–E3577.

57. Sancho M, Fabris S, Hald BO, Brett SE, Sandow SL, Poepping TL, Welsh DG: **Membrane Lipid-KIR2.x Channel Interactions Enable Hemodynamic Sensing in Cerebral Arteries.** Arterioscler Thromb Vasc Biol 2019, 39:1072–1087.

58. Lovick TA, Brown LA, Key BJ: **Neurovascular relationships in hippocampal slices: Physiological and anatomical studies of mechanisms underlying flow-metabolism coupling in intraparenchymal microvessels.** Neuroscience 1999, 92:47–60.

59. Kovacs-Oller T, Ivanova E, Bianchimano P, Sagdullaev BT: **The pericyte connectome: spatial precision of neurovascular coupling is driven by selective connectivity maps of pericytes and endothelial cells and is disrupted in diabetes.** Cell Discovery 2020, 6:39.

60. Looft-Wilson RC, Billaud M, Johnstone SR, Straub AC, Isakson BE: **Interaction between nitric oxide signaling and gap junctions: Effects on vascular function.** Biochim Biophys Acta - Biomembr 2012, 1818:1895–1902.

61. Straub AC, Lohman AW, Billaud M, Johnstone SR, Dwyer ST, Lee MY, Bortz PS, Best AK, Columbus L, Gaston B, et al.: **Endothelial cell expression of haemoglobin α regulates nitric oxide signalling.** Nature 2012, 491:473–477.

62. Harding IC, Mitra R, Mensah SA, Herman IM, Ebong EE: **Pro-atherosclerotic disturbed flow disrupts caveolin-1 expression, localization, and function via glycocalyx degradation.** J Transl Med 2018, 16:1–20.

63. • Chow BW, Nuñez V, Kaplan L, Granger AJ, Bistrong K, Zucker HL, Kumar P, Sabatini BL, Gu C: **Caveolae in CNS arterioles mediate neurovascular coupling.** Nature 2020, 579:106–110.

Proposed a novel mechanism underpinning NVC based on caveolae in ECs.

64. Toth P, Tarantini S, Davila A, Noa Valcarcel-Ares M, Tucsek Z, Varamini B, Ballabh P, Sonntag WE, Baur JA, Csiszar A, et al.: **Purinergic glio-endothelial coupling during neuronal activity: Role of P2Y1 receptors and eNOS in functional hyperemia in the mouse somatosensory cortex.** Am J Physiol - Hear Circ Physiol 2015, 309:H1837–H1845.

65. • Alarcon-Martinez L, Villafranca-Baughman D, Quintero H, Kacerovsky JB, Dotigny F, Murai KK, Prat A, Drapeau P, Di Polo A: **Interpericyte tunnelling nanotubes regulate neurovascular coupling.** Nature 2020, 585:91–95.

Showed that tunnelling nanotubes connect retinal pericytes and may generate a focus of pericyte-mediated capillary dilation, by instructing connected pericytes on nearby capillaries to constrict and hence reduce surrounding blood flow.

66. Eng CHL, Lawson M, Zhu Q, Dries R, Koulena N, Takei Y, Yun J, Cronin C, Karp C, Yuan GC, et al.: **Transcriptome-scale super-resolved imaging in tissues by RNA seqFISH+.** Nature 2019, 568:235–239.

67. Nelson AR, Sagare MA, Wang Y, Kisler K, Zhao Z, Zlokovic B V.: **Channelrhodopsin Excitation Contracts Brain Pericytes and Reduces Blood Flow in the Aging Mouse Brain in vivo.** Front Aging Neurosci 2020, 12:1–11.

68. Tong L, Yuan P, Chen M, Chen F, Bewersdorf J, Zhou ZJ, Grutzendler J: **Single cell in vivo brain optogenetic stimulation by two-photon excitation fluorescence transfer.** bioRxiv 2020, https://doi.org/10.1101/2020.06.29.179077

69. Adámek S, Vyskočil F**: Potassium-selective microelectrode revealed difference in threshold potassium concentration for cortical spreading depression in female and male rat brain.** Brain Res 2011, 1370:215–219.

70. Wellbourne-Wood J, Rimmele TS, Chatton J-Y: **Imaging extracellular potassium dynamics in brain tissue using a potassium-sensitive nanosensor.** Neurophotonics 2017, 4:015002.

71. Marvin JS, Borghuis BG, Tian L, Cichon J, Harnett MT, Akerboom J, Gordus A, Renninger SL, Chen T-W, Bargmann CI, et al.: **An optimized fluorescent probe for visualizing glutamate neurotransmission.** Nat Methods 2013, doi:10.1038/nmeth.2333.