Stimulation-induced increases in cerebral blood flow and local capillary vasoconstriction depend on conducted vascular responses

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Functional neuroimaging, such as fMRI, is based on coupling neuronal activity and accompanying changes in cerebral blood flow (CBF) and metabolism. However, the relationship between CBF and events at the level of the penetrating arterioles and capillaries is not well established. Recent findings suggest an active role of capillaries in CBF control, and pericytes on capillaries may be major regulators of CBF and initiators of functional imaging signals. Here, using two-photon microscopy of brains in living mice, we demonstrate that stimulation-evoked increases in synaptic activity in the mouse somatosensory cortex evokes capillary dilation starting mostly at the first- or second-order capillary, propagating upstream and downstream at 5–20 μm/s. Therefore, our data support an active role of pericytes in cerebrovascular control. The gliotransmitter ATP applied to first- and second-order capillaries by micropipette puffing induced dilation, followed by constriction, which also propagated at 5–20 μm/s. ATP-induced capillary constriction was blocked by purinergic P2 receptors. Thus, conducted vascular responses in capillaries may be a previously unidentified modulator of cerebrovascular function and functional neuroimaging signals.

Conducted vascular responses | pericytes | neurovascular coupling | purinergic signaling | cerebral capillaries

Brain function emerges from signaling in and between neurons and astrocytes, causing fluctuations in the cerebral metabolic rate of oxygen and cerebral blood flow (CBF). Normal brain function depends on a preserved supply of glucose and oxygen, which is mediated by neurovascular coupling, the robust coupling between brain activity and CBF. Neurovascular coupling depends on the functional properties of the association of brain microvessels, astrocytes, pericytes, and neurons, which together constitute the neurovascular unit (1). Brain arterioles are traditionally thought to control CBF and brain capillaries to serve in the exchange of substances between the blood and brain. This view of CBF dynamics was revolutionized recently by the discovery that both arterioles and capillaries take part in substance exchange (2) and cerebrovascular resistance (3, 4). Specifically, modified smooth muscle cells called pericytes are attached to capillaries and can regulate CBF at the capillary level (3, 5, 6). However, this regulation is not completely understood. Retinal pericytes are constricted by ATP and dilated by neurotransmitters in vitro (5), and they constrict in vivo following stroke (7). In response to light stimulation, retinal capillaries dilate and regulate blood flow independent of arterioles (8). Furthermore, glial Ca2+ signaling regulates capillary, but not arteriole, blood flow in both the retina and the cerebral cortex (8, 9). Nevertheless, capillary pericytes have been suggested not to be contractile, and that the regulation of CBF in the CNS is only mediated by smooth muscle cells in penetrating arterioles (p.a.s) and capillaries, but not by pericytes on capillaries (10–12). This controversy may be more apparent than real because it depends on how a pericyte and capillary are defined rather than the role of brain capillaries in cerebrovascular control.

We have chosen to analyze the change in brain capillaries based on the branching order from the p.a. (13). Using this unbiased methodology, our study may contribute to understanding the contribution of capillaries and pericytes to cerebrovascular control and the interplay between capillaries and arterioles.

All capillaries have pericyte coverage (14), and pericytes are almost completely covered by astrocyte end-feet (15), which raises the possibility that soluble signal molecules released into the microenvironment by astrocytes are sensed by specialized surface receptors on pericytes. ATP is the main transmitter by which astrocytes communicate with neighboring astrocytes (16), as well as an important paracrine transmitter in signaling to neurons (17) and possibly pericytes (18). Therefore, an important part of this study was an examination of the effect of ATP on brain capillary pericytes in vivo.

The current study used in vivo two-photon microscopy of a transgenic mouse model with fluorescent pericytes. The activity-dependent increase in synaptic activity was examined to determine whether capillaries of all branching orders are dilated or constricted, or only capillaries close to the p.a., and whether capillaries exhibit conducted vascular responses (CVRs) similar to pial arterioles.

Our study supports the notion that pericytes play active roles in neurovascular coupling. Furthermore, the results suggest that both arterioles and capillaries contribute to cerebrovascular control during physiological stimulation, and that spatially restricted CVRs may contribute to regulating the flow in brain capillaries and the spatiotemporal characteristics of functional neuroimaging signals.

Significance

Pericytes are located at the outside wall of capillaries. However, whether and how pericytes are involved in the regulation of blood flow in brain capillaries is still debated. We report that capillary vascular responses are mostly initiated and peak at near-arteriole capillaries. These vascular responses are conducted along capillaries at a speed of 5–20 μm/s. Conducted vascular responses in brain capillaries appear to involve pericytes, the mural cells of microvessels, and may be a novel modulator of vascular function in the brain.

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Fig. 1. Functional vessel dilation in the mouse barrel cortex. (A) A two-photon image of the barrel cortex of a NG2-DsRed mouse at ∼150 μm depth. The p.a. branch out a capillary horizontally (first order). Further branches are defined as second- and third-order capillaries. Pericytes are labeled with a red fluorophore (NG2-DsRed) and the vessel lumen with FITC-dextran (green). ROIs are placed across the vessel to allow measurement of the vessel diameter (colored bars). (Scale bar: 10 μm.) (B) Vessel diameters at different orders of capillaries. p.a., 15.09 ± 4.15 μm; 1st cap (first-order capillaries), 7.18 ± 1.93 μm; 2nd cap (second-order capillaries), 6.25 ± 2.43 μm; 3rd cap (third-order capillaries), 7.63 ± 2.47 μm. The p.a. diameter is significantly larger than all orders of capillaries. *** P < 0.001, one-way ANOVA with post hoc test. (C) Example trace of fluorescent intensity over time at the blue ROI in A is shown as the gray image, and the two red curves indicate the vessel wall (Upper). The distance of the two red curves is calculated as the time course of vessel diameter (Lower). (D) The normalized diameter change over time at different orders of capillaries in response to whisker-pad stimulation. The short vertical bar is where the curve reaches 50% peak, which is defined as response onset. (E) Distribution of the locations where the functional dilation initiated (n = 29 locations). (F) Multiple ROIs at the p.a. and first-, second-, and third-order capillaries are marked as red, blue, green, and yellow, respectively. (Scale bar: 10 μm.) (G) In this mouse experiment, the half-maximal dilation latency of each ROI is plotted with corresponding colors on the left along the geographic distance from the p.a. Dashed lines show the linear fit of the conducted dilation. (H) The maximal dilation amplitude is plotted with corresponding colors on the left along the geographic distance from the p.a. (I) Eighteen out of 29 imaged vasculatures exhibited conducted functional dilation, with an upstream conductive speed of 12.65 ± 0.96 μm/s and downstream conductive speed of 12.83 ± 0.64 μm/s. No significant difference was found between upstream and downstream conductive speeds. n.s., not significant; P > 0.05, unpaired t test. (J) Time to 50% maximal dilation was significantly longer in the third-order capillaries than the p.a. and first-order capillaries. The second-order capillaries dilated significantly slower than the first-order capillaries. * P < 0.05, one-way ANOVA with post hoc test. (K) Maximal dilation amplitude in different order capillaries. First- and second-order capillaries exhibited significantly larger responses than other locations. * P < 0.05, one-way ANOVA with post hoc test. All error bars represent SEM.
Results

CVRs initiate at Capillaries or p.a.s. We used in vivo two-photon microscopy to image the vasculature in the whisker-barrel cortex of anesthetized mice expressing DsRed in pericytes under control of the NG2 promoter. FITC-dextran was used to label the blood plasma (shown as green in Fig. L4). The p.a.s were identified unequivocally in vivo by tracing their connections back to the pial arterioles and by the clear continuous rings of smooth muscle around them. Only p.a.s with a longitudinal axis perpendicular to the x–y plane were used for data analysis. Capillaries were identified as microvessels branching off from the p.a. with a longitudinal axis parallel to the x–y plane. This geometric arrangement was necessary for reliable measurement of changes in the arteriolar and capillary diameter. Pericytes were identified as NG2-positive mural cells on capillaries branching off from the arteriole. Pericyte cell bodies were spatially separated from the p.a. and each other, and individual pericytes were identified by processes extending longitudinally along capillaries (Fig. L4).

Based on z-stacks of the cortex, we segmented the blood vessels by branching order, 0 being the p.a., 1 being the first-order capillary branching off the arteriole, and so on (Fig. L4). In the resting state, the diameters of the p.a. and first-, second-, and third-order capillaries were 15.09 ± 0.10 μm, 7.18 ± 0.04 μm, 6.25 ± 0.10 μm, and 6.75 ± 0.28 μm, respectively. The p.a. was significantly wider than the capillaries, but the capillary diameter was similar among the first three orders of capillaries (Fig. 1B).

Reportedly, first-order capillaries dilate first in response to somatosensory stimulation and the time to vasodilation in first-order capillaries commonly precedes dilation in the p.a. (3). In the present study, the time resolution did not allow us to assess differences in the time of onset of the stimulation-induced vasodilation, but as a proxy we used the latency from stimulus onset to 50% maximal dilation (Fig. 1D and Movie S1). Out of 29 preparations, stimulation-evoked dilation was achieved first in first-order capillaries in 55% of experiments, whereas dilation was achieved first in the p.a. in 21% and in second- or third-order capillaries in 24% of experiments (Fig. 1E). Next, we evaluated whether a pattern exists in the development of capillary dilation, that is, whether dilation occurs first at a particular point and whether the reaction spreads according to the branching order of the capillaries. For this purpose, multiple regions of interest (ROIs) rectangles with the long side perpendicular to the vessel wall were drawn as indicated by the color coding in Fig. 1F. The half-maximal latency and maximal vascular dilation for each vessel wall were drawn as indicated by the color coding in Fig. 1.

To probe the mechanism of ATP-puffing-induced dilation and constriction demonstrated linear or near-linear conduction in the upstream and downstream direction (Fig. 2 H and I). The conductive speed of dilation to upstream and downstream vessels was 11.47 ± 3.37 μm/s and 14.78 ± 3.85 μm/s, respectively, whereas the conductive speed of constriction to upstream and downstream vessels was 6.54 ± 1.05 μm/s and 6.55 ± 1.22 μm/s, that is, slower than for dilation (Fig. 3F). Hyperstack imaging during ATP puffing confirmed conductive responses for both ATP-induced dilation and constriction in five of five experiments (Supporting Information, Fig. S3, and Movie S4). The faster conductive speed for downstream dilation suggests that ATP-induced conducted vascular dilation and constriction are modulated by different mechanisms.

CVRs Initiated by Local ATP Injection. Purinergic signaling may affect the neurovascular unit in pathological states, such as during cerebral ischemia when ATP is released in high concentrations (19–22). ATP constricts retinal pericytes and capillaries in vitro, which is of interest because brief periods of ischemia lead to the no-reflow phenomenon and a reduction in the caliber of small vessels (7, 23). We examined the effect of purinergic receptor activation on pericytes and capillaries in vivo by local injection of ATP into the barrel cortex of NG2-DsRed mice. Guided by the two-photon microscope, a glass micropipette was inserted into the cortex and advanced to close proximity of the p.a. and the first few orders of capillaries. A mixture of 10 μM Alexa 594 (red color in the glass micropipette) and 1 mM ATP was puffed from the micropipette by air pressure (Fig. 2 A and B). ATP puffing evoked capillary dilation, followed by constriction (Fig. 2B and Movie S2). Fifteen rectangular ROIs were studied at different-order capillaries (Fig. 2C) and the normalized diameter change was plotted over time for each ROI (Fig. 2D). Amplitudes of dilation or constriction were defined as positive or negative amplitudes at maximal vascular response. The latency of dilations and constrictions were reported as the time to half positive or negative maximum after puffing onset (Fig. 2E). The four variables were plotted as a function of the geographic distance along the vasculature from the p.a. (Fig. 2F–I). The same color coding was used for the squares representing ROIs. The branching point of the first- to second-order capillary exhibited the strongest and earliest dilation and constriction, whereas the third-order capillary had a very small change in diameter (Fig. 2 F and G). A significantly higher amplitude of both vasodilation and vasoconstriction was found at the first- and second-order capillaries, whereas the diameters of higher-order capillaries were almost unaltered (n = 7; Fig. 3A and B). The latencies of vasodilation and vasoconstriction at third-order and higher capillaries were significantly longer than at lower-order capillaries (Fig. 3 C and D). No significant difference was found for the mean distance from the pipette tip to the different-order capillaries, which excludes an influence of distance to pipette tips on the conducted responses (Fig. 3E). Furthermore, traces from individual mice indicated no correlation between pipette distance and the response properties (i.e., latency and amplitude) (Fig. S2).

ATP-puffing-induced dilation and constriction demonstrated linear or near-linear conduction in the upstream and downstream direction (Fig. 2 H and I). The conductive speed of dilation to upstream and downstream vessels was 11.47 ± 3.37 μm/s and 14.78 ± 3.85 μm/s, respectively, whereas the conductive speed of constriction to upstream and downstream vessels was 6.54 ± 1.05 μm/s and 6.55 ± 1.22 μm/s, that is, slower than for dilation (Fig. 3F). Hyperstack imaging during ATP puffing confirmed conductive responses for both ATP-induced dilation and constriction in five of five experiments (Supporting Information, Fig. S3, and Movie S4). The faster conductive speed for downstream dilation suggests that ATP-induced conducted vascular dilation and constriction are modulated by different mechanisms.
ATP-induced constriction observed in the absence of inhibitor at first- and second-order capillaries, and to a lesser extent at the p.a. and third-order and higher capillaries (Fig. 3), was due to P2 purergic receptor activation.

Activation of both P2X and P2Y Receptors Leads to Similar Vessel Responses. In vitro studies of arteries and arterioles have shown that the activation of P2Y receptors on smooth muscle cells leads to vessel constriction, whereas the activation of P2X receptors on arteriolar endothelial cells (ECs) leads to vessel dilation (24–27). To test whether the same mechanisms contribute to brain capillary control in vivo, we investigated the vessel responses elicited by both P2X and P2Y receptor agonists. P2X receptor agonist αβATP (αβ-methylene-ATP) and P2Y receptor agonist UTP were administered (1 mM each) by puffing in close proximity to the p.a. and the first few order capillaries. As adenosine hydrolyzed from ATP is a potent vasodilator (28), a more stable ATP analog, ATPγS, was used at a concentration of 1 mM for micro-pipette puffing experiments. Finally, control experiments were performed by puffing 10 μM Alexa 594 only to rule out the effect of puffing itself.

We compared the effect of the compounds on the first-order capillary responses because these capillaries had the most robust and profound responses upon ATP puffing (Fig. 3; see also Fig. 2).
ATP-puffing-induced dilation and constriction. Compounds found no significant difference (Fig. 5E and C). Capillaries with visible pericyte cell bodies exhibited more constriction than capillaries devoid of pericyte cell bodies (Fig. 6C).

Next, we evaluated whether purinergic receptors were involved in the ischemia-induced capillary constriction by pericytes. For this purpose, we superfused the exposed mouse cortex with 0.5 mM PPADS for at least 2 h before cardiac arrest. This mitigated constriction of the p.a. and first- and second-order capillaries (Fig. 6B and C). These results are consistent with prior studies of brain slices and postmortem studies suggesting that pericytes constrict in ischemia (3, 7) and that preconditioning the animals with PPADS helps in the recovery from experimental stroke (29, 30). We conclude that pericytes at the first several orders of capillaries constrict severely after ischemia in vivo and that blocking purinergic receptors mitigates the constriction of both arterioles and capillaries.

Discussion
Understanding neurovascular signaling in response to neuronal or astrocytic activity is crucial to understanding how brain processes are supplied with energy and functional neuroimaging signals are generated. Our results demonstrate that first- and second-order capillaries initiate functional dilation more often than the p.a. and higher-order capillaries. In addition, local and direct administration of ATP induces vessel dilation, followed by constriction at the first several orders of capillaries. Functional dilation and ATP-puffing-induced dilation and constriction are initiated mostly at the first- or second-order capillaries, and CVRs develop both upstream and downstream. However, the velocity of conducted vasodilation is faster than for conducted vasoconstriction. Furthermore, minutes after cerebral ischemia, pericytes at near-arteriolar sites constrict via a P2 receptor-dependent mechanism.

The contribution of pericytes to CBF regulation has been controversial (13). Some in vivo studies suggest a role of pericytes in the regulation of capillary blood flow (3, 6), whereas others have indicated that flow control was detectable only in arterioles, but not in capillaries, and that vascular smooth muscle cells, but not pericytes, contribute to the regulation of CBF responses (10–12). However, most of those studies have suggested that pericytes close to the p.a. are contractile during normal brain activity, and pericytes on first- and second-order capillaries have hybrid features of both smooth muscle cells and capillary pericytes (31). In our studies, pericytes were identified by two-photon microscopy as red-fluorescent cells on the capillary wall in NG2-DsRed mice. Nevertheless, we describe the changes in capillary function according to the branching orders from the p.a. and involvement of pericytes in this context. Our results strengthen the importance of using a defined vessel geometry with respect to the cortical surface to reliably assess small changes in the diameters of capillaries.

Similar to other studies (3, 10), our data suggest a key role of capillaries close to the p.a. in local blood flow control. As a powerful tool for studying CBF (32), the two-photon imaging microscope was
used to focus on one horizontal plane at a depth of 100–200 μm to obtain good image quality. This horizontal plane most commonly included one p.a. and the associated first-, second-, or third-order capillaries. Our results showed that capillary dilation as a response to increased synaptic activity (i.e., the neurovascular coupling response) is initiated in most cases in first-order capillaries. This is consistent with earlier results indicating active relaxation of pericytes before relaxation of arteriolar smooth muscle cells (3), and with recent studies showing that smooth muscle actin is present in pericytes at near-arteriole capillaries (10, 33). In addition, pericytes at near-arteriole capillaries may have denser smooth muscle actin (12, 34), as the first- and second-order capillaries react with the earliest and most profound dilation.

ATP puffing induced dilation and constriction in the first few orders of capillaries. ATP puffing onto higher than second-order capillaries induced almost no changes in diameter. The observations indicate that pericytes have different sensitivities to purinergic stimulation, in accordance with previous studies (5). Dilatation induced by ATP puffing has been suggested to be mediated by the activation of P2X receptors on arteriolar ECs (24). However, a recent study proposed that ATP may also act on astrocytic P2X1 receptors to evoke the release of PGE2, which relaxes pericytes (9). Other studies have shown that the ATP analog ATPγS has its own pharmacological profile; for example, it may in fact be hydrolyzed to adenosine (35), which may be another explanation for ATP-puffing-induced vasodilation.

ATP-puffing-induced vessel constriction was profoundly attenuated by the purinergic type 2 receptor antagonist PPADS, indicating the involvement of purinergic type 2 receptors. Similar to our study, ATP puffing in brain slices was previously shown to increase cytosolic Ca2+ in glial cells, followed by adjacent vasoconstriction, which was abolished by preincubation with P2Y1 receptor blocker (36). Our in vitro studies with pericytes in monoculture (Supporting Information, Fig. S5, and Movie S5) confirmed that ATP constricted capillary pericytes in a manner dependent on P2 receptor activation and increased cytosolic Ca2+. This study demonstrates that intracellular Ca2+ increases in pericytes may be the mechanism underlying pericyte contraction in response to ATP. ATP applied to cerebral arterioles in vitro produced a biphasic vessel response, constriction followed by dilation (24), which is the opposite of what we observed in capillaries. The data suggest that the effect of the purinergic signaling cascade in capillaries is different from the effect in arterioles (9).

In arteries and arterioles, CVRs are primarily characterized by fast (1–3 mm/s) and far-reaching electrical conduction along well-coupled endothelium and into smooth muscle (37, 38). Upon G-coupled receptor stimulation, a secondary slow and spatially limited Ca2+ wave spreads along the endothelium (~100 μm/s), giving rise to nitric oxide and prostaglandin production (Fig. 7A and B). Inhibition of the electrical component has demonstrated that the speed of the slow, diffusion-based CVR is ~20 μm/s, similar to the slow speed of propagated vasodilation observed in the present study (39). Furthermore, hyperpolarizing pulses propagate along the ECs in capillaries, with a conductive speed 100 times faster than the diffusion-mediated CVR (37). In contrast, the vascular relaxation times are the same for the two types of conducted responses. It is possible that the final common path of both types of vascular responses may involve axon boutons dumping potassium concurrent with rapid spiking (40), but this will need to be addressed in more detail in future studies.

Although the underlying mechanism remains unclear, a diffusion-based conduction of vasomotor responses emanating from first- and second-order capillaries can be envisioned, for example paracrine signaling along astrocytic end-feet or intracellular diffusion across gap junctions connecting ECs and/or pericytes (Fig. 7C). However,
During ischemia, cerebral pericytes constrict and stop the blood flow in capillaries within a few minutes. The constriction is dependent propagated vasodilation (25, 26). The compounds are 1 mM ATP, 1 mM P2X receptor agonist (αβATP), 1 mM ATP + S, and 10 µM Alexa 594 as control. n/a, not available; *P < 0.05, ***P < 0.001, one-way ANOVA with post hoc test. Note that the latency of the control experiment is marked as not available for both dilation (C) and constriction (D). This is due to the small responses upon control puffing and the suboptimal measurements of latency. All error bars represent SEM. n.s., not significant.

documentation of gap junction coupling between the ECs of first- and second-order capillaries in mouse cortex is lacking. In addition, in contrast to arterial endothelium, capillary ECs do not harbor SKCa/IKCa channels (37), which are thought to underlie the initiation of fast electrical CVRs (41). This may also explain why microapplication of ATP to pial arteries and p.a.s in vitro has been observed to result in constriction followed by endothelium-dependent propagated vasodilation (25, 26).

During ischemia, cerebral pericytes constrict and stop the blood flow in capillaries within a few minutes. The constriction is pronounced at the p.a. and first- and second-order capillaries, whereas the diameters of higher capillaries remained constant. This supports and modifies the notion that pericytes contribute to the long-lasting decrease in capillary blood flow after cerebral ischemia (3, 7, 42, 43). The most ischemia-sensitive region of the vasculature is first-order capillaries at the near-arteriole site. Ischemia leads to severe constriction of capillaries at the near-arteriole site and preconditioning of P2 receptor blockers mitigates constriction of capillaries. (A) Image stacks (1-µm step size, average intensity projection) of the vasculature, including the p.a. and first few orders of capillaries. Five minutes after ischemia by cardiac arrest (CA), severe constriction was observed at the p.a. and first- and second-order capillaries, but third-order and higher capillaries were moderately constricted. Dashed lines indicate the vessel contours of first-order capillaries before cardiac arrest. (Scale bars: 20 µm.) (B) Preconditioning with 0.5 mM PPADS for 2 h rescued severe constriction of the p.a. and first-order capillary 5 min after CA. Dashed lines indicate the vessel contours of first-order capillaries before CA. (Scale bars: 20 µm.) (C) The most severe constrictions at the first- and second-order capillaries colocalized with pericytes. The third-order and higher capillaries exhibited moderate constriction. Preconditioning with PPADS mitigated vasconstriction at the p.a. and first- and second-order capillaries. For the p.a., an unpaired t test was used. n/a, not available; ***P < 0.001. For the other order capillaries, one-way ANOVA with post hoc test was used (*P < 0.05). All error bars represent SEM.

![Fig. 5. Vessel responses of first-order capillaries to puffing with ATP, P2X, P2Y receptor agonists, ATP+ S, and red dye. (A) Comparison of different puffing compounds with amplitude of dilation, (B) amplitude of constriction, (C) latency of dilatation, (D) latency of constriction, and (E) conductive speed. The compounds are 1 mM ATP, 1 mM P2X receptor agonist (αβATP), 1 mM P2Y receptor agonist (UTP), 1 mM ATP+S, and 10 µM Alexa 594 as control. n/a, not available; *P < 0.05, ***P < 0.001, one-way ANOVA with post hoc test. Note that the latency of the control experiment is marked as not available for both dilation (C) and constriction (D). This is due to the small responses upon control puffing and the suboptimal measurements of latency. All error bars represent SEM. n.s., not significant.](image-url)
Materials and Methods

Animal Handling. All procedures involving animals were approved by the Danish National Ethics Committee according to the guidelines set forth in the ARRIVE (Animal Research: Reporting in Vivo Experiments) guidelines. Forty NG2-DsRed mice [Tg(Cspg4-DsRed.T1)1Akik/J; Jackson Laboratory] of both sexes were used at 4–7 mo of age. The mice were anesthetized by i.p. injection of a mixture of ketamine (30 mg/kg) of ketamine every 20 min. Upon completion of all surgical procedures and pharmacological interventions.

Whisker-Pad Stimulation. The mouse sensory barrel cortex was activated by stimulation of the contralateral ramus infratrochlearis of the trigeminal nerve using a set of custom-made bipolar electrodes inserted percutaneously. The cathode was positioned relative to the hiatus infraorbitalis (IO), and the anode was inserted into the masticatory muscles (45). Thalamocortical IO stimulation was performed at an intensity of 20 μA and a frequency of 20 Hz.

Micropipette Puffing. The glass micropipettes for puffing were produced by a micropipette puller (P-97; Sutter Instrument) with a resistance of 3–3.5 MΩ. The micropipette was loaded with a mixture of 10 μM Alexa 594 and active substances to visualize the pipette under the two-photon microscope. Guided by the two-photon microscopy, the pipette was inserted into the cortex and the vascular response was monitored using a two-photon microscope (Fig. 2A).

Possible mechanisms of ATP-puffing-induced dilation and constriction. (A) The pial artery and p.a.s consist of endothelium surrounded by smooth muscle cells (light red). As capillaries branch off the p.a., smooth muscle is replaced by pericytes (light blue) with heterogeneous morphologies across first-, second-, and higher-order capillaries (i.e., going from the p.a. to the venous side). (B) Fast and long-range conduction along arterioles and arteries via electrical conduction and the local Ca2+ wave. (C) Observed slow and long-range conduction of vasomotor responses emanating mostly from first- and second-order capillaries seem to involve signaling by diffusion. Both paracrine signaling along astrocyte end-feet and intracellular diffusion along putative gap junctions can be envisioned.
stimulation/puffing. The response latency was defined as the latency of half-maximal amplitude.

Drug Application. Upon completion of all surgical procedures, FITC-dextran (FD20005; Sigma-Aldrich) was injected i.v. through the femoral vein catheter to label blood serum and visualize the vasculature under the two-photon microscope (green color). In the micropipette ATP puffing study, the puffing substance was a mixture of 10 μM Alexa Fluor 594 (A-10438; Life Technologies; red color) and 1 mM ATP (A9187; Sigma-Aldrich) dissolved in aCSF. The same method was used for the ATP5-S (A1388; Sigma-Aldrich), UTP (U6875; Sigma-Aldrich), and αβ-methylene-ATP (M6517; Sigma-Aldrich) studies. In the preconditioned ATP puffing study using P2 receptor antagonist PPADS (P178; Sigma-Aldrich), aCSF containing 0.5 mM PPADS was used to superfuse the exposed cortex immediately after the dura mater was removed and to prepare both agarose and bathing fluid for the cranial window during imaging. ATP puffing experiments occurred after at least 2 h of PPADS exposure.

The same procedure for PPADS application and exposure was used in the subset of experiments in which cardiac arrest and cerebral ischemia was induced by i.v. application of 0.05 mL (200 mg/mL) pentobarbital.

Statistical Analysis. Responses are presented as mean ± SEM. P values are from one-way ANOVA with Tukey–Kramer post hoc test or unpaired Student’s t tests, as appropriate. P ≤ 0.05 was considered significant. All statistical analyses were performed using MATLAB.

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