Astrocytes mediate neurovascular signaling to capillary pericytes but not to arterioles

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Active neurons increase their energy supply by dilating nearby arterioles and capillaries. This neurovascular coupling underlies blood oxygen level–dependent functional imaging signals, but its mechanism is controversial. Canonically, neurons release glutamate to activate metabotropic glutamate receptor 5 (mGluR5) on astrocytes, evoking Ca\textsuperscript{2+} release from internal stores, activating phospholipase A2 and generating vasodilatory arachidonic acid derivatives. However, adult astrocytes lack mGluR5, and knockout of the inositol 1,4,5-trisphosphate receptors that release Ca\textsuperscript{2+} from stores does not affect neurovascular coupling.

We now show that buffering astrocyte Ca\textsuperscript{2+} inhibits neuronally evoked capillary dilation, that astrocyte [Ca\textsuperscript{2+}], is raised not by release from stores but by entry through ATP-gated channels, and that Ca\textsuperscript{2+} generates arachidonic acid via phospholipase D2 and diacylglycerol lipase rather than phospholipase A2. In contrast, dilation of arterioles depends on NMDA receptor activation and Ca\textsuperscript{2+}-dependent NO generation by interneurons. These results reveal that different signaling cascades regulate cerebral blood flow at the capillary and arteriole levels.

Functional hyperemia is the phenomenon by which active brain regions induce a local increase in blood flow to match their energy demands, via a process termed neurovascular coupling. Traditionally, regulation of cerebral blood flow was thought to occur at the level of arterioles\textsuperscript{1–3}. However, capillaries in the brain are also wrapped by contractile cells called pericytes\textsuperscript{4}, which can respond to neuronal activity and control blood flow at a more local level than arterioles\textsuperscript{5,6}. Although neurovascular coupling is the basis of blood oxygen level–dependent (BOLD) functional magnetic resonance imaging (fMRI), an increasingly routine method used to measure brain activity in the clinic and in cognitive studies, our understanding of the underlying signaling mechanisms is still incomplete.

Neurovascular coupling can partly occur as a result of direct signaling from neurons to the vasculature\textsuperscript{7}. However, over the last decade, a role for astrocytes, which have endfoot processes near blood vessels, has also been demonstrated: neurons signal to astrocytes, which in turn release vasoactive substances onto vessels\textsuperscript{8}. Initial data suggesting astrocyte-mediated neurovascular signaling came from experiments in which stimulating neurons led to a rise in astrocyte [Ca\textsuperscript{2+}], as well as arteriole dilation\textsuperscript{1}. It was further shown that raising astrocyte [Ca\textsuperscript{2+}] evokes vascular dilations and constrictions\textsuperscript{2,3,9}, which were produced by metabolites of arachidonic acid (AA) generated by phospholipase A2 (PLA2). Dilation occurs via the formation of prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) and epoxyeicosatrienoic acids (EETs), while production of 20-hydroxyeicosatetraenoic acid (20-HETE) results in constriction (see ref. 8 for review).

Although these findings were obtained in several laboratories, controversies still abound. Astrocyte Ca\textsuperscript{2+} signals may be too small or slow\textsuperscript{10}, or too infrequent\textsuperscript{11}, to have a causative role in neurovascular coupling. However, these conclusions were based on measuring Ca\textsuperscript{2+} signals within astrocyte cell bodies, rather than in the fine processes\textsuperscript{12} of astrocytes near synapses that are presumably the first responders to neuronal activity. Indeed, recent in vivo studies have reported rapid, physiological stimulation-evoked [Ca\textsuperscript{2+}], rises in astrocyte somata and endfeet in the somatosensory cortex\textsuperscript{13}, and in the processes but not the somata of olfactory bulb astrocytes\textsuperscript{14}.

A second controversy concerns how elevations of astrocyte Ca\textsuperscript{2+} concentration are generated. While these were originally thought to reflect activation of mGluR5 and downstream inositol 1,4,5-trisphosphate (IP\textsubscript{3})-dependent release of Ca\textsuperscript{2+} from internal stores\textsuperscript{1–3}, recent studies show that mGluR5 expression is downregulated in astrocytes from adult animals\textsuperscript{15}. Furthermore, animals lacking IP\textsubscript{3}R2, the primary IP\textsubscript{3} receptor in astrocytes, display unaltered neurovascular coupling\textsuperscript{16}. Nevertheless, there are other mechanisms that could raise astrocyte [Ca\textsuperscript{2+}], including Ca\textsuperscript{2+}-permeable AMPA receptors\textsuperscript{17}, NMDA receptors\textsuperscript{18}, ATP receptors\textsuperscript{19} and TRPA1 channels\textsuperscript{20}, and thus mediate astrocyte-to-vessel signaling.

Finally, it is unclear whether neurovascular coupling at the capillary level is mediated by the same pathways as that at the arteriole level. Although dilation of capillaries and arterioles both rely on relaxation of actomyosin, in pericytes and in arteriolar smooth muscle respectively, it seems plausible that local (capillary) and spatially broader (arteriolar) control of blood flow might be driven by different signaling pathways.

Here we investigated the role of astrocytes in mediating neurovascular coupling in the cerebral cortex, in brain slices and in vivo. We provide, to our knowledge, the first direct demonstration that a rise in astrocyte [Ca\textsuperscript{2+}], is necessary for neuronally evoked capillary dilation to occur. We show that the source of Ca\textsuperscript{2+} involved in this process and its molecular effects are different from what was previously believed.

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We also show that arteriole dilation is not mediated by astrocyte Ca²⁺ signaling, but instead depends on NMDA receptor–mediated nitric oxide release (Supplementary Fig. 1).

RESULTS

Neuronal activity evokes capillary dilation

We assessed whether cerebral cortical capillaries can respond to electrically stimulated synaptic activity in cortical slices from P21 (adolescent) rats. Stimulation-evoked fiber volley and field excitatory postsynaptic currents (fEPSCs) were recorded in all experiments to confirm activation of the neuropil near the capillary being studied (Supplementary Fig. 2). Vascular responses are known to be modulated by the oxygen concentration in the tissue: physiological [O₂]₂⁺ containing 30 mM of the faster Ca²⁺ chelator BAPTA (1,2-bis(2-amino-5-phosphonopentoxy)ethane-N,N',N'-tetraacetic acid) or one containing 30 mM of the faster Ca²⁺ chelator BAPTA (1,2-bis(2-amino-5-phosphonopentoxy)ethane-N,N',N'-tetraacetic acid). Spread of the pipette solution between astrocytes via gap junctions was visualized using the dye Alexa Fluor 488 (40 µM). After 10–15 min of dialysis, the astrocyte network, including endfeet along the vessel of interest, was filled with Alexa Fluor 488 (Fig. 2a). Stimulating neuronal activity when astrocytes were dialed with the control (EGTA) internal solution evoked robust capillary dilations (16.2 ± 1.7% ± 20% ± 20%; n = 12; Fig. 2b,c). However, when astrocytes were dialed with BAPTA to buffer rises in [Ca²⁺], stimulation-evoked dilation was reduced by 64% to 5.9 ± 2.0% (n = 17; P = 0.0007; Fig. 2b,c). The free [Ca²⁺] in both the EGTA and BAPTA internal solutions was set to ∼24 nM, so the effects observed are not attributable to differences in baseline free [Ca²⁺], implying that a transient rise in astrocyte [Ca²⁺], in response to neuronal stimulation is, at least in part, necessary for neurovascular signaling to capillaries.

In some experiments, we imaged vessels as the internal solution was switched from either the EGTA or BAPTA internal solution. In the EGTA solution, the capillaries dilated with stimulation, and the width of the dye spread from the patch-clamped astrocyte into the endfoot processes, to the pericytes) studied. In box and whisker plots, central line shows the median, central dot shows the mean, the edges of the box define the upper and lower quartile values, and whiskers show the minimum–maximum range. P values are from appropriate t-tests, as described in the Statistics section of the Online Methods.

Capillary dilation is mediated by Ca²⁺-dependent astrocyte signaling

Next we investigated whether astrocyte Ca²⁺ signaling mediates neuron-to-capillary signaling. To address this, astrocytes near the vessel of interest were whole-cell patch-clamped and dialyzed with either a control internal solution containing 1 mM EGTA (ethylene glycol-bis(2-aminoethylether)-N,N',N"-N',N"-tetraacetic acid) or one containing 30 mM of the faster Ca²⁺ chelator BAPTA (1,2-bis(2-aminoethoxy)ethane-N,N',N"-N',N"-tetraacetic acid). Spread of the

Figure 1 Neuronal activity evokes capillary dilation. (a) A cortical capillary response to 200 nM U46619 and superimposed neuronal stimulation (Stim). Lines show lumen diameters plotted in b. (b) U46619-evoked constriction and stimulation-evoked dilation at regions indicated in a (in this and subsequent example traces, a large response is shown for illustrative purposes). (c) TTX (500 nM) blocks stimulation-evoked capillary dilation. (d) 10 µM NBQX blocks stimulation-evoked dilation. (e) d-AP5 (25 µM) did not reduce stimulation-evoked dilation. (f-h) Mean data showing the block of capillary dilation by TTX (f) and NBQX (g) but not by d-AP5 (h). Numbers below plots are capillary regions (putative pericytes) studied. In box and whisker plots, central line shows the median, central dot shows the mean, the edges of the box define the upper and lower quartile values, and whiskers show the minimum–maximum range. P values are from appropriate t-tests, as described in the Statistics section of the Online Methods.

P2X1 receptors raise astrocyte [Ca²⁺]

Many mechanisms leading to a [Ca²⁺] rise in astrocytes have been suggested. Group I metabotropic glutamate receptors (mGluR1 and mGluR5) are activated by the glutamate analog quinoxaline-7-sulfonamide, 10 µM, raising astrocyte [Ca²⁺] in both the EGTA and BAPTA internal solutions was set to ~24 nM, so the effects observed are not attributable to differences in baseline free [Ca²⁺], implying that a transient rise in astrocyte [Ca²⁺], in response to neuronal stimulation is, at least in part, necessary for neurovascular signaling to capillaries.

In some experiments, we imaged vessels as the internal solution spread from the patch-clamped astrocyte into the endfoot processes, to observe whether buffering astrocyte Ca²⁺ rises had any effect on baseline capillary diameter. There was no significant change in the baseline diameter of the vessels when using either the control internal solution (2.0 ± 1.8% constriction in 4 capillaries, not significantly different from zero, P = 0.3) or the BAPTA internal (5.4 ± 3.2% dilation in 8 capillaries; not significantly different from zero, P = 0.1; diameter change in BAPTA was not significantly different from that in EGTA, P = 0.2).
and the Alexa Fluor 488 fill of the astrocyte network after whole-cell patch-clamp dialysis for 1 min (middle) and 10 min (right) after breaking into the cell. Whole-cell patch-clamped cell is indicated by arrowhead and endfoot by arrow. (b,c) Example traces (b) and mean data (c) demonstrating that stimulation-evoked capillary dilation is intact when the astrocyte network is dialyzed with a control internal solution containing 1 mM EGTA but significantly reduced when filled with 30 mM BAPTA, a fast Ca\(^{2+}\) chelator. (d-f) An inhibitor of group I and II mGluRs, (S)-MCPG (1 mM; d), the P2Y1 blocker MRS2179 (25 μM; e) and the TRPA1 blocker A967079 (10 μM; f) do not block stimulation-evoked capillary dilation. (g) The P2X1 blocker NF449 (100 nM) significantly reduces stimulation-evoked capillary dilation. (h) Puff-application of the P2X1 agonist α,β-methylene-ATP (α,β-meATP, 100 μM) to the neuropil downstream of the vessel induces capillary dilation. (i–l) Quantification of the effect of (S)-MCPG (l), MRS2179 (j), A967079 (k) and NF449 (l) on capillary dilation. (m) Mean response of capillaries in experiments like those in h, puffing external solution (control) or α,β-methylene-ATP. Change in diameter in control experiments was measured as a 30-s average centered around the largest response seen between 30 and 120 s after puff of α,β-methylene-ATP. Box and whisker plots as defined in Figure 1. P values are from appropriate t-tests, as defined in the Statistics section of the Online Methods.

mGluR5\(^2\) and the ATP receptor P2Y1 (ref. 24) are both G-protein-coupled receptors associated with G\(_{q}\) signaling that induces Ca\(^{2+}\) release from internal stores by generating IP\(_3\). In particular, mGluR5 has long been thought to be the primary cause of astrocyte [Ca\(^{2+}\)]\(_i\) rises\(^3\). Recently, group II mGluRs were also shown to raise [Ca\(^{2+}\)]\(_i\), in astrocytes via an as yet undefined mechanism\(^2\). Furthermore, the Ca\(^{2+}\)-permeable heteromeric ATP receptor P2X1/5 (ref. 19) and transient receptor potential A1 (TRPA1) channels\(^20\) are also possible routes for Ca\(^{2+}\) influx into astrocytes. We tested the role of each of these receptors in mediating capillary-level neurovascular coupling. Stimulation-evoked capillary dilation was not reduced by bath application of a nonspecific blocker of both group I and II mGluRs, (S)-MCPG ((S)-α-methyl-4-carboxyphenylglycine, 1 mM, P = 0.1; Fig. 2d,i), the P2Y1 receptor blocker MRS2179 (25 μM, P = 0.8; Fig. 2e,j) or the TRPA1 blocker A967079 (10 μM, P = 0.3; Fig. 2f,k). However, the P2X1 blockers NF449 (100 nM; Supplementary Table 1) and NF023 (5 mM) reduced the stimulation-evoked capillary dilation by 58% (P = 0.0007; Fig. 2g,l) and 94% (P = 0.001; Supplementary Fig. 4d), respectively. Similar results were obtained using shorter stimulation durations than the 1 min used above (NF449 reduced dilation by 94% (P = 0.005) and 60% (P = 0.01) for 200 ms and 5 s stimulation, respectively; Supplementary Fig. 4a-c), and in P45 mice (66% reduction with NF449, P = 0.00008 using 5 s stimulation; Supplementary Fig. 4e).

If P2X1 receptors raise astrocyte [Ca\(^{2+}\)]\(_i\), to evoke the dilation, then we should also be able to evoke dilation simply by activating this receptor. Indeed, a 5-s puff application of α,β-methylene-ATP (100 μM; Online Methods), an agonist for P2X1 (ref. 26), onto the neuropil induced a 10.7 ± 2.3% dilation of cortical capillaries, whereas application of vehicle did not (0.4 ± 1.3% constriction, significantly different, P = 0.0007; Fig. 2h,m). These data suggest that capillary-level neurovascular coupling occurs largely through a P2X1 receptor-dependent pathway in astrocytes.

To study stimulation-evoked changes in [Ca\(^{2+}\)]\(_i\) in astrocyte endfeet, we whole-cell clamped astrocytes to selectively fill them with the membrane-impermeant Ca\(^{2+}\) indicator dye Fluo-4 (50 μM), along with a reference dye (Alexa Fluor 594, 40 μM) to allow ratiometric imaging, as ratio R = (Fluo-4 intensity)/Alexa Fluor 594 intensity). We observed spontaneous local Ca\(^{2+}\) transients in astrocyte cell bodies and processes, as previously reported\(^27\). Stimulating neuronal activity evoked an increase in [Ca\(^{2+}\)]\(_i\) in astrocytes (AR/R = 41.4 ± 11.7% in endfeet, n = 9, P = 0.003 compared to zero; ΔR/R = 58.4 ± 20.2% in processes, n = 23, P = 0.006; ΔR/R = 60.2 ± 24.3% in somata, n = 13, P = 0.02; Fig. 3a–c). The amplitude of this signal was reduced by 81% and 85% in endfeet and processes, respectively, by the P2X1 receptor blocker NF449 (ΔR/R = 7.9 ± 3.1% in endfeet, n = 6, P = 0.02 compared to control data; AR/R = 8.5 ± 3.4% in processes, n = 14, P = 0.02; Fig. 3b,c). NF449 did not significantly block the calcium rise in somata (Fig. 3c). These data are consistent with P2X1 receptor–mediated signaling in astrocytes mediating the
Arachidonic acid metabolites mediating capillary dilation

Astrocyte-mediated neurovascular coupling is thought to occur via the synthesis of AA by Ca\(^{2+}\)-activated phospholipase A2 followed by metabolism into vasoactive substances such as prostaglandins (by the activity of cyclooxygenase (COX)) or EETs (by the activity of epoxygenases). We tested the contribution of these enzymes using specific inhibitors. The COX1 inhibitor SC-560 (1 μM) reduced the stimulation-evoked capillary dilation by 64% (P = 0.0002; Fig. 4a,d), but the COX2 inhibitor NS-398 (10 μM; Fig. 4b,e) and the epoxygenase inhibitor PPOH (2-(2-propenyl)-benzenehexanoic acid, 25 μM; Fig. 4c,f) had no effect on the dilation. COX1 generates several prostaglandin derivatives, including the vasodilators PGE\(_2\) and PGI\(_2\). Blocking the EP4 receptor for PGE\(_2\) with L-161,982 (1 μM) reduced the stimulation-evoked dilation by 72% (P = 0.004; Fig. 4g,j). In contrast, blocking the prostaglandin I\(_2\) (IP) receptor for PGI\(_2\) with CAY10441 (1 μM) did not affect the dilation (Fig. 4h,k). Nitric oxide has also been shown to contribute to arteriolar vasodilation in the cortex\(^{20,31}\); however, when the NO synthase (NOS) inhibitor \(N^\text{G}\)-nitro-l-arginine (l-NAME; 100 μM) was applied, stimulation-evoked capillary dilation was not reduced (Fig. 4i,l), implying that NO does not contribute to capillary dilation in the cortex. Thus, PGE\(_2\) generated by COX1 and acting on EP4 receptors is the primary vasoactive signal mediating capillary dilation.

We used immunohistochemistry to investigate the cellular location of the enzymes metabolizing AA. We found COX1 labeling in processes along arterioles and capillaries that colabeled for aquaporin 4 (AQP4), a protein highly expressed in astrocyte endfeet (Supplementary Fig. 5a). In contrast, COX2 did not colocalize with the astrocyte structural protein glial fibrillary acidic protein (GFAP), but was expressed diffusely in the neuropil with higher expression in neuronal somata (Supplementary Fig. 5b). Antibodies against the CYP2C11 isofrom of epoxygenase, reported to be the predominant epoxygenase in the rodent brain\(^{22}\), labeled AQ4P-expressing endfeet along larger vessels, but not along capillaries (Supplementary Fig. 5c). Furthermore, antibodies against PGE\(_2\) synthase (PGES) also labeled GFAP-expressing astrocyte cell bodies, processes and endfeet (Supplementary Fig. 5d). These findings are consistent with the functional data in Figure 4 suggesting that PGE\(_2\) is the main vasoactive metabolite of AA that signals to capillaries and that it is synthesized within astrocytes.
PLD2, not PLA2, initiates neurovascular coupling at the capillary level

The Ca^{2+}-dependent enzyme PLA2 is thought to synthesize the AA that is converted into PGE_{2} (refs. 2,8,33). We found that PLA2 immunolabeling colocalized with GFAP on astrocyte endfeet along capillaries (Supplementary Fig. 6), suggesting a possible role for this enzyme. Surprisingly, however, stimulation-evoked capillary dilation was unchanged when PLA2 was blocked with its inhibitor MAFP (10 µM, Fig. 5a,d). Thus, despite being in the correct cellular compartment, PLA2 is not the primary enzyme involved in the AA synthesis required for signaling to capillaries. We therefore sought other possible AA sources.

The enzymes phospholipase C (PLC) and PLD can produce AA via a multistep process. PLC breaks down phosphatidylinositol-4,5-bisphosphate to DAG (diacylglycerol) and IP_{3}, while PLD acts on membrane phospholipids to synthesize phosphatidic acid, which is then converted to DAG by phosphatidate phosphatase. DAG produced by PLC or PLD in this way can be further metabolized by DAG lipase (DAGL) to produce AA. A specific blocker of PLC, U73122 (10 µM), did not reduce stimulation-evoked capillary dilation (Fig. 5b,e). In contrast, the PLD-specific blocker FIPI (4-fluoro-N-(2-(4-(5-fluoro-1H-indol-1-yl)pyperidin-1-yl)ethyl)benzamide, 1 µM) reduced dilation by 64% (P = 0.0005; Fig. 5c,f), suggesting that PLD plays a major role in synthesizing AA. Two isoforms of PLD are expressed in mammals, PLD1 and PLD2, both of which are regulated by Ca^{2+}. A specific inhibitor of PLD1, VU0155069 (500 nM), had no effect on stimulation-evoked dilation (Fig. 5g,j); however, a blocker of PLD2, CAY10594 (1 µM), completely abolished the capillary dilation (P = 0.0002; Fig. 5h,k), suggesting that AA synthesis is PLD2 dependent. If so, then the downstream enzyme DAGL, which is required for AA production from DAG, should also be involved. Indeed, the DAGL inhibitor RHC80267 (50 µM) also blocked the stimulation-evoked capillary dilation (reduced by 81%, P = 0.0004; Fig. 5i,l), suggesting the PLD2–DAGL pathway as the mechanism for AA synthesis.

We considered whether PLD2 might, instead of being in the pathway for AA synthesis, be activated downstream of the PGE_{2} receptor EP4 in pericytes to cause the capillary dilation. However, there was no difference between PGE_{2}-induced dilation of cortical capillaries in the absence (8.1 ± 1.7%) and presence (8.5 ± 2.1%) of the PLD blocker FIPI (Supplementary Fig. 7), supporting our conclusion that the PLD2–DAGL pathway is upstream of AA synthesis.

Consistent with these results, immunolabeling for PLD1 showed that it was expressed diffusely in the neuropil and strongly in endothelial cells lining arteries (Supplementary Fig. 8a), but it was not in astrocyte endfeet, pericytes or capillary endothelial cells (Supplementary Fig. 8a,b). In contrast, PLD2 immunolabeling occurred in GFAP-positive astrocyte endfeet on capillaries (Supplementary Fig. 8c) and cell bodies (Supplementary Fig. 8d), but not in pericytes or endothelial cells, consistent with a role in astrocyte-mediated neurovascular signaling to capillary pericytes.

Arteriole dilation is mediated by a different signaling pathway

To compare the data above on capillary dilation with the dilation of arterioles evoked by neuronal activity, we carried out similar experiments imaging arterioles. U46619 preconstricted arterioles by 11.8 ± 0.8% (n = 36 arterioles from 26 animals, Supplementary Fig. 9). The neuronal-stimulation-evoked dilation of arterioles (7.0 ± 0.7%, n = 36; mean of all control data in Fig. 6) was smaller than that evoked in capillaries (12.1 ± 1.9%; Supplementary Fig. 4b; both with 5 s stimulation). Remarkably, in contrast to the capillary dilation, the arteriole dilation was unaffected by dialyzing the astrocyte network with BAPlA (Fig. 6a,b) or by blocking P2X1 receptors, PLD2 or PLA2 (Fig. 6c–e,h,i), indicating that a completely different signaling pathway, independent of astrocyte [Ca^{2+}], changes, mediates arteriole dilation. However, again unlike the capillary dilation (Figs. 1h and 4l), arteriole dilation was strongly inhibited by blocking NMDA receptors (P = 0.008, Fig. 6f,j) or NO synthase (P = 10^{-5}, Fig. 6g,k).

Neurovascular signaling in vivo

To determine whether the ATP-dependent astrocyte signaling mechanism also mediates neurovascular coupling at the capillary level in vivo, we used two-photon excitation fluorescence microscopy to image cortical capillaries and arterioles in vivo in anesthetized rats, with intravascular FITC-dextran to visualize the blood vessels (Fig. 7a,b and Online Methods). We evoked neuronal activity in the somatosensory cortex by electrically stimulating the forepaw. The diameter of all imaged capillaries was 6.7 ± 0.2 µm (n = 102) and that of arterioles was 13.4 ± 0.5 µm (n = 77). Forepaw stimulation evoked dilation in 22% of capillaries and 24% of arterioles (Fig. 7c–e).

Figure 5 PLD2, not PLA2, initiates neurovascular coupling at the capillary level. (a) The PLA2 inhibitor MAFP (10 µM) does not block stimulation-evoked capillary dilation. (b) The PLC blocker U73122 (10 µM) does not reduce dilation. (c) The PLD blocker FIPI (1 µM) inhibits dilation. (d–f) Mean data showing the effects of MAFP (d), U73122 (e) and FIPI (f) on capillary dilation. (g) Blocking PLD1 with VU0155069 (500 nM) has no effect on the dilation. (h,i) The PLD2 blocker CAY10594 (1 µM; h) and the DAGL blocker RHC80267 (50 µM; i) significantly reduce the capillary dilation. (j–l) Mean data showing the effect of VU0155069 (j), CAY10594 (k) and RHC80267 (l) on capillary dilation. Box and whisker plots as defined in Figure 1. P values are from appropriate t-tests, as defined in the Statistics section of the Online Methods.
In those vessels, capillary dilation averaged 29.3 ± 5.8% and arteriolar dilations averaged 10.9 ± 2.2% (Fig. 7f).

Local infusion of the P2X1 blocker NF449 (5 μM) into the cortical region did not significantly affect the percentage of capillaries dilating (reduced to 13%, P = 0.3, Fig. 7e). However, the capillary dilations that did occur were reduced by 68% in size to 9.5 ± 1.8% (P = 0.006, Fig. 7d,f). In contrast, NF449 did not significantly alter either the percentage of dilating arterioles (18%, P = 0.6) or the magnitude of the arteriole dilations (9.7 ± 1.7%, P = 0.7; Fig. 7c,e,f), just as in slices (Fig. 6h).

DISCUSSION

Neurovascular coupling plays a crucial role in controlling cerebral blood flow, both physiologically and in disease. Capillary dilation...
generates a large portion of the blood flow increase evoked by neuronal activity and thus is expected to contribute substantially to the BOLD signal\(^6\). In ischemia, pericytes constrict capillaries, contributing to the lack of reperfusion of the microvasculature that occurs after a thrombus is removed from an upstream artery\(^6,36\). Consequently, characterizing the signaling mechanisms that regulate capillary and arteriole dilation is important for understanding the blood flow response to physiological activity and to disease. In this study, we have demonstrated that neurovascular coupling at the capillary and arteriole levels differ mechanistically (Supplementary Fig. 1). Neurovascular coupling at the capillary level is largely dependent on astrocyte \(\text{Ca}^{2+}\) signaling, and our data reveal a new mechanism by which astrocytes alter their \(\text{Ca}^{2+}\), in response to neuronal activity: influx of \(\text{Ca}^{2+}\) via the ionotropic ATP receptor P2X1. Thus astrocyte \(\text{Ca}^{2+}\) can be raised without a need for mGluR5 (ref. \(^{37}\)) and IP\(_1\)/R2-mediated signaling, which have recently been suggested to be unnecessary for neurovascular coupling in adult animals\(^15,16,38\), and astrocyte \(\text{Ca}^{2+}\) transients observed in IP\(_1\)/R2 knockout mice\(^39\) may partly reflect activation of P2X1 receptors. Our data are consistent with work showing that overexpression of an ectonucleotidase (to promote rapid ATP degradation) strongly reduces BOLD fMRI signals triggered by somatosensory stimulation in rats\(^40\). We also show that, although signaling to capillaries is mediated by AA metabolites, the synthesis of AA depends not upon PLA2, as generally assumed\(^15\), but rather on PLD2 and DAGL activity. Furthermore, neuronal activity induced capillary dilation is unaffected by block of NO synthase (thus differing from the pericyte-mediated dilation we previously characterized in cerebellum\(^6\), presumably because of the difference in brain region, as the cerebellum expresses more NOS, and/or because dilation was evoked by glutamate superfusion in the latter study but by endogenous glutamate release here). In contrast, neurovascular signaling to cortical arterioles is not mediated by P2X1, astrocyte \(\text{Ca}^{2+}\), PLD2 or PLA2 but, unlike cortical capillary dilation, requires the activation of NMDA receptors and production of NO.

Our data suggest that neuronal activity results in postsynaptic neurons (Fig. 1) releasing ATP, which acts on astrocyte ATP receptors containing P2X1 subunits to produce a \(\text{Ca}^{2+}\) rise (Figs. 2 and 3). This activates PLD2 (Fig. 5 and Supplementary Fig. 8), resulting in AA synthesis via DAGL and downstream metabolism by COX1 into vasodilatory PGE\(_2\), which is released onto capillary pericytes to induce dilation via the EP4 receptor (Fig. 4 and Supplementary Fig. 5). P2X1 receptors are expressed in cortical astrocytes along with P2X5 receptors\(^19\), which can together form heterotrimers and contribute to \(\text{Ca}^{2+}\) influx. The astrocyte \(\text{Ca}^{2+}\) influx causing capillary dilation (Figs. 2, 3 and 7 and Supplementary Fig. 4) may be via P2X1 homotrimers or P2X1/5 heterotrimers\(^19\), as both are blocked by NF449 and activated by \(\alpha,\beta\)-methylene-ATP (puffed onto the neuropil; Fig. 2h and Supplementary Fig. 10). Although it was previously reported that ATP is released\(^40\) from presynaptic terminals\(^41\), we show that NBQX blocks the capillary dilation, implying that postsynaptic glutamate receptor activation is needed for ATP release, at least in the cortex. The mechanism of this ATP release has yet to be investigated.

In contrast to capillary dilation, arteriole dilation does not depend on P2X1 receptors, PLA2, PLD2 or astrocyte calcium signaling, but is dependent on NMDA receptor activation and NO synthesis (Figs. 6 and 7), as previously suggested\(^30,31\). It has been suggested that activation of NMDA receptors, by \(\text{Ca}^{2+}\)-dependent release of \(\delta\)-serine from astrocytes, generates NO from endothelial NOS in endothelial cells and thus helps to dilate penetrating arterioles\(^31\). Our data showing that buffering astrocyte \(\text{Ca}^{2+}\), does not inhibit arteriole dilation (Fig. 6a,b) argue against a role for astrocyte \(\text{Ca}^{2+}\) in triggering arteriole dilation in our experiments, and furthermore transcriptome data suggest there are no NMDA receptors on endothelial cells\(^42\). We therefore assume that the NO is generated by neuronal NOS, which is \(\text{Ca}^{2+}\)-dependent and predominantly located in interneurons in the cortex\(^33,34\).

It has previously been suggested that, in the cortex, NO release is needed to maintain neurovascular coupling but not to mediate it\(^45\). This is similar to the idea that, in cortex\(^33\) and in cerebellum\(^6\), NO serves to suppress the generation of the vasoconstrictor 20-HETE from AA while another AA derivative, PGE\(_2\), dilates the arterioles. We have shown that blocking AA production by inhibiting PLA2 or PLD2 has no effect on the arteriole dilation evoked by neuronal activity (Fig. 6d,e,i), leading us to favor the idea that NO may directly mediate arteriole dilation in our preparation. However, we cannot rule out the possibility that AA is generated by PLC and DAG lipase, and that downstream generation of PGE\(_2\) (or EETs; see below) dilates the arterioles provided that NO suppresses 20-HETE formation.

The lack of dependence of arteriole dilation on astrocyte \(\text{Ca}^{2+}\), changes contrasts with earlier work\(^1-3\) in which mGluR agonists or Ca\(^{2+}\) uncaging evoked arteriole dilation by raising astrocyte \(\text{Ca}^{2+}\),\(^2\). We assume this relates to the different kinds of stimuli applied: release of endogenous neurotransmitter glutamate from synapses in this paper versus the generation of a more spatially extensive \(\text{Ca}^{2+}\), rise by bath superfusion of an mGluR agonist or essentially simultaneous \(\text{Ca}^{2+}\) uncaging in several astrocytes. Consequently, we cannot exclude the possibility that with other stimulation procedures (or in other brain regions) mGluR-driven astrocyte \(\text{Ca}^{2+}\) signaling may contribute to arteriole dilation, although this mechanism may decrease in importance with age\(^15\).

Overall, our data indicate that two cellually and chemically distinct \(\text{Ca}^{2+}\)-dependent pathways mediate neurovascular coupling in response to neuronal-activity-evoked glutamate release (Supplementary Fig. 1): ATP-evoked signaling in astrocytes generates PGE\(_2\), which dilates capillaries via pericytes, and glutamate-evoked NO release from interneurons dilates arterioles. Previous data\(^8\) suggest that the astrocyte-capillary pathway is faster, and quantitatively more important for increasing blood flow, than the interneuron-arteriole pathway, so our current data imply that purinergic signaling contributes substantially to generating BOLD signals.

We observed a similar block of capillary dilation after astrocyte \(\text{Ca}^{2+}\) buffering (64% reduction: Fig. 2c) and inhibition of P2X1 receptors (58% reduction: Fig. 2i). The incomplete nature of this block (compared to that produced by inhibiting PLD2: Fig. 5k) may reflect an insufficient concentration of BAPTA or NF449 being applied. Alternatively, another mechanism might generate the remaining fraction of the response. We have ruled out direct signaling from neurons to pericytes via NMDA receptor mediated NO release\(^30\) (Fig. 1e,h and 4i,l), but other signals or \(\text{Ca}^{2+}\)-independent (for example, cyclic nucleotide) mechanisms in astrocytes could be involved.

Previous reports have suggested that astrocyte-mediated neurovascular signaling involves AA metabolites, and our data demonstrate that this is true for signaling to capillaries. Conceivably, EETs may contribute to neurovascular signaling to arterioles under some conditions\(^9,46,47\), as we detected epoxyenase expression in astrocyte endfeet along larger vessels (but not in endfeet along capillaries (Supplementary Fig. 5c), and EETs do not contribute to capillary dilation\(^6\); Fig. 4c,f). Although AA production has been attributed to the activity of \(\text{Ca}^{2+}\)-dependent PLA2 (refs. \(^{2,8}\)), which is expressed in astrocytes\(^53\) (Supplementary Fig. 6), we found that PLA2 was not involved in neurovascular coupling. Instead, another \(\text{Ca}^{2+}\)-dependent phospholipase, PLD2, was responsible for AA production (Fig. 5h,k).
Calcium-sensitive cytosolic phospholipase A2 (PLA2)

Capillary pericytes regulate cerebral blood flow in health and disease.

Astrocytes are endogenous regulators of basal transmission at somatosensory cortex-evoked astrocyte Ca2+ signaling.

In conclusion, neuronal activity results in capillary dilation in a manner that is largely dependent on Ca2+-dependent signaling in astrocytes. The astrocyte [Ca2+]i rise causing dilation depends on ionotropic P2X1 receptor activation, but not on G-protein-coupled receptors or TRPA1 channels. AA synthesis via a PLD2–DAGL pathway and downstream metabolism into prostaglandin E2 by COX1 are necessary for capillary dilation, with the required synthetic enzymes being expressed in astrocytes. In contrast, neurally evoked arteriolar dilation depends at least partly on the activation of neuronal NMDA receptors and subsequent synthesis and release of NO. These data demonstrate that astrocytes are important contributors to neurovascular coupling at the capillary level and suggest that capillaries, which contain most of the resistance to blood flow in the brain parenchyma, could be specifically targeted therapeutically to increase blood flow in pathological conditions.

Note added in proof: consistent with this paper, Biesecker et al.1 have just reported that, in the retina, glial Ca2+ signaling regulates capillary but not arteriolar diameter.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Animals. Sprague-Dawley rats of both sexes were used in all experiments. Animal procedures were carried out in accordance with the guidelines of the UK Animals (Scientific Procedures) Act 1986 and European Directive 2010/63/EU under the oversight of the UK government Home Office. Each experiment was conducted on brain slices from at least three animals (at least one of each sex).

Brain slice preparation. As described previously\(^\text{22}\), 300-µm-thick coronal cortical slices were prepared from P21 rats on a vibrotome in ice-cold oxygenated (95% O\(_2\), 5% CO\(_2\)) solution containing (in mM) 93 N-methyl-d-glucamine chloride, 2.5 KCl, 30 NaHCO\(_3\), 10 MgCl\(_2\), 1.2 NaHPO\(_4\), 25 glucose, 0.5 CaCl\(_2\), 20 HEPES, 5 sodium ascorbate, 3 sodium pyruvate and 1 kynurenic acid. The slices were incubated at 34 °C in the same solution for 15–20 min, then transferred to a similar solution with the NMDG chloride, MgCl\(_2\), and CaCl\(_2\) replaced by (in mM) 93 NaCl, 1 MgCl\(_2\) and 2 CaCl\(_2\) and incubated at room temperature (21–23 °C) until used in experiments.

Imaging of capillaries in brain slices. Slices were perfused with bicarbonate buffered aCSF containing (in mM) 124 NaCl, 2.5 KCl, 26 NaHCO\(_3\), 1 MgCl\(_2\), 2 CaCl\(_2\), 1 NaHPO\(_4\), 10 glucose, 1 sodium ascorbate, heated to 31–35 °C and gassed with 95% O\(_2\), 5% CO\(_2\) and 75% N\(_2\). Capillaries were imaged using differential interference contrast (DIC) microscopy at 20–50 μm depth within layers III–VI of cortical slices containing the motor and prefrontal cortices, using a 40x water immersion objective, a Coolsnap HQ2 CCD camera, and ImagePro Plus acquisition software. Images were acquired every 5 s, with an exposure time of 100 ms. The pixel size was 160 nm. The thromboxane A\(_2\) analog U46619 (200 nM) was used to preconstrict capillaries in all experiments where the vessel was vascularized before the experiment started. The solution contained (in mM) 125 potassium glutonate, 2 NaCl, 0.1 CaCl\(_2\), 10 HEPES, 1 tetrapotassium EGTA, 2 MgCl\(_2\), 2 Na\(_2\)-ATP, 0.5 Na\(_2\)-GTP, 10 disodium phosphate and 0.04 Alexa Fluor 488, with pH set to 7.1 using KOH. The internal solution containing BAPTA (tetrapotassium salt) was similar but with the CaCl\(_2\) and EGTA replaced with (in mM) 3 CaCl\(_2\) and 30 BAPTA (so that the free [Ca\(^2+\)]) was –24.5 nM in both the control and the BAPTA internal solutions, calculated using MaxChelator (http://maxchela tor.stanford.edu/webmaxc/webmaxcE.htm) with the temperature set to 35 °C and ionic strength set to 145 mM and with osmolarity differences compensated by lowering the potassium gluconate concentration. Osmolarity measurements showed that tetrophotophotophosphatase BAPTA does not dissociate completely so that corresponding less compensation of osmolarity was needed. The solution contained (mM) 67 potassium glutonate, 2 NaCl, 3 CaCl\(_2\), 10 HEPES, 30 tetrathiol BAPTA, 2 MgCl\(_2\), 2 Na\(_2\)-ATP, 0.5 Na\(_2\)-GTP, 10 disodium phosphate and 0.04 Alexa Fluor 488, with pH set to 7.1 using KOH. Patch-clamped cells were confirmed to be astrocytes morphologically by their arborization pattern revealed by dye filling and electrically by their large negative resting membrane potential (−91.6 ± 0.6 mV, n = 33), low input resistance (4.6 ± 0.6 MΩ) and passive V–I relationship.

Calcium imaging of astrocytes. All neurally evoked astrocyte (Ca\(^2+\)) imaging was carried out by confocal microscopy using a Zeiss LSM700 microscope. The internal solution used for Ca\(^2+\) imaging was similar to the control internal, but with added 50 μM Fluo-4 to sense Ca\(^2+\) and 40 μM Alexa 594 as a reference dye to allow ratiometric imaging, and with CaCl\(_2\) increased to 0.105 mM. The intensities of Fluo-4 and Alexa 594 were measured using GECiQuant\(^\text{23}\) in a region of interest (ROI), and a background intensity measured far from the filled astrocyte at each time point was subtracted from the value in the ROI at the same time point. Data were quantified as fractional changes in the ratio of background-subtracted Fluo-4 fluorescence to Alexa 594 fluorescence. Images were acquired at 0.4 Hz.

Immunohistochemistry. Coronal cortical slices 200 μm thick were incubated in aCSF bubbled with 95% O\(_2\) and 5% CO\(_2\) containing 1 μM isoleucine B\(_2\) conjugated to Alexa 488 (which binds to α-d-galactose residues in the vascular basement membrane\(^\text{23}\)) for 30 min to label vessels, then washed with aCSF and fixed in 4% paraformaldehyde for 1 h. Slices were then washed three times in phosphate-buffered saline (PBS) and incubated in a blocking solution (0.05% Triton X-100,
10% horse serum and 1% bovine serum albumin prepared in PBS) for 1 h at room temperature. Slices were incubated with the primary antibody for 48–72 h at 4 °C with agitation, washed three times with PBS, incubated with the secondary antibody overnight at 4 °C with agitation and washed again three times with PBS. The primary and secondary antibody solutions were prepared in PBS containing 0.005% Triton X-100, 1% horse serum and 0.1% bovine serum albumin. Controls to test for nonspecific labeling of the secondary antibody were carried out in exactly the same manner, except that slices were incubated in just the vehicle (PBS containing 0.005% Triton X-100, 1% horse serum and 0.1% bovine serum albumin) during the primary antibody incubation step. The slices were then mounted with VectaShield hard set mounting medium containing DAPI to label nuclei. Immunohistochernistry experiments were conducted on three separate occasions on tissue from three different animals. Primary antibodies used were chicken GFAP (Abcam, ab4674, 1:1,000), rabbit AQ49 (Santa Cruz, sc-20812, 1:500) or goat AQ49 (Santa Cruz, sc-9888, 1:500), goat COX1 (Abcam, ab22720, 1:1,000), goat COX2 (Abcam, ab23672, 1:1,000), goat PGES (Santa Cruz, sc-12268, 1:500), rabbit PDE1 (Santa Cruz, sc-25512, 1:300), rabbit PDE2 (Santa Cruz, sc-25513, 1:200), mouse PLA2 (Santa Cruz, sc-454, 1:300) and rabbit CYP2C11 (Abcam, ab3571, 1:300). Secondary antibodies used were donkey anti-chicken Alexa 644 (Molecular Probes, 1:1,000), donkey anti-chicken Cy3 (Jackson Laboratories, 1:1,000), donkey anti-rabbit Alexa 647 (Molecular Probes, 1:1,000), donkey anti-goat Alexa 568 (Molecular Probes, 1:1,000), donkey anti-rabbit Alexa 555 (Molecular Probes, 1:1,000) and donkey anti-mouse Cy3 (Jackson Immunoresearch Laboratories, 1:500). Immunolabeled slices were imaged using a Zeiss LSM700 confocal microscope.

In vivo experiments: preparation. Experiments were conducted in male Sprague-Dawley rats (4 to 6 weeks of age). Animals were initially anesthetized using isoflurane (5% induction, 2% maintenance). The femoral artery was cannulated for continuous blood pressure recording, as well as periodic blood gas monitoring. Following cannulation, a mixture of urethane (a single dose of 0.75 g/kg) and alpha-chloralose (an initial dose of 50 mg/kg supplemented with 10 mg/kg/h) was administered intravenously while discontinuing isoflurane. Adequate anesthesia was ensured by maintaining a stable arterial blood pressure and the absence of a withdrawal response to a paw pinch. The trachea was cannulated and the animal was mechanically ventilated during imaging sessions (1:2 oxygen/nitrogen ratio at 80 strokes min⁻¹ and a tidal volume of 1 ml/100 g body weight). Body temperature was maintained at 37 ± 0.5 °C. The PO₂, PCO₂, and pH of the arterial blood were periodically measured using a RAPiDlab 348EX blood gas analyzer (Siemens), and the ventilation parameters were adjusted to keep these variables within their physiological ranges (PO₂ > 80 mm Hg, PCO₂ 30–45 mm Hg and pH 7.35–7.45). Mean arterial blood pressure was stable at 90–110 mm Hg throughout the experiment. The animal was secured in a stereotaxic frame and a craniothoracic frame of approximately 4 mm diameter was carried out over the right primary somatosensory cortex, immediately caudal to the coronal suture and approximately 2–6 mm laterally from the midline. In vivo photon microscopy of the intraluminal dye fluorescein isothiocyanate–dextran (FITC-dextran, MW 2,000 kDa, 50 mg/kg, 1 ml iv.) was carried out using a Newport-Spectraphysics Tiscarry MaiTai laser pulsing at 80 MHz, and an Olympus VF1000 with XLPPlan N 25× water immersion objective (NA 1.05). Acquisitions were carried out using a wavelength of 800 nm and the mean laser power under the objective was kept at 20–30 mW. Penetrating arterioles were identified by chromatically separated Alexa 633 hydrazide fluorescent labeling of the smooth muscle elastin⁵⁵. Recordings were made at a depth between 50 and 250 µm from the cortical surface. xy time series (at 1–4 Hz with a pixel dwell time of 2 µs and pixel size of 0.248–0.496 µm) were taken of regions of interest to record vessel dilations in response to somatosensory stimulation. Unilateral forepaw stimulation (0.3 ms pulse width, 3 Hz, 0.2–2 mA, 20 s) was applied using an electrical stimulator triggered by a 1401 interface (Cambridge Electronic Design).

In vivo experiments: analysis. The presence of red blood cells (RBCs) leads to apparent holes in the images of the capillary and (to a lesser extent) the arteriole. This image noise was reduced by smoothing images in the time series with a rolling window (in time) maximum intensity projection not exceeding 2 s duration, as previously described⁶. The resulting time series was then median filtered (radius 1 pixel) using ImageJ. A 3-pixel-wide line segment was drawn perpendicular to the vessel in ImageJ and the fluorescence profile across the vessel was extracted. Relative diameter was determined by fitting a function to the fluorescence profile in Matlab (using the sum of two Gaussian functions) and calculating the full width at quarter-maximum of the peak fluorescence intensity. The peak dilation and the time to 10% of the peak dilation were calculated by fitting the data with a function of the form: (max dilation)(time)/(time² + R²), with max dilation, n and K arbitrary constants.

Choice of drug concentrations used. To ensure that enzyme and receptor inhibitors were used at concentrations and with incubation times that would produce effective block, we compiled a dossier of the relevant parameters from the previous literature (Supplementary Table 1)⁵⁵–⁶⁶.

Statistics. Data are shown as box and whisker plots in which the central line shows the median, the central dot shows the mean, the edges of the box define the upper and lower quartile values, and the outer whiskers show the minimum–maximum range of the data, or are shown as mean ± s.e.m. N numbers below plots or on bars are of capillaries or regions or arterioles. The variability of responses observed at different regions (presumed regions of active pericyte control) along the same capillary was higher than that between different capillaries, slices or animals. Therefore, capillary regions that constituted to 200 nm U46619 were used as the observational units. Normality of data was checked using the Kolmogorov-Smirnoff test and the equality of variance confirmed using the F-statistic. All data were compared to interleaved controls using a homoscedastic, two-tailed Student’s t-test (equal variance), except in Figures 3c, 4 and 7f and Supplementary Figure 4d, where the variances of the data were unequal, and therefore a heteroscedastic, two-sided Student’s t-test was used; in Figure 3c (soma data with NF449), where the data were not normally distributed and so a Mann-Whitney test was used; and in Figure 7e, where we used Fisher’s exact test to compare percentages. Data in Supplementary Figure 2 were compared using a one-way ANOVA and post hoc t-tests. Data were corrected for multiple comparisons using a procedure equivalent to the Holm-Bonferroni method (for N comparisons, the most significant P value is multiplied by N, the second most significant by N − 1, the third most significant by N − 2, etc.; corrected P values were considered significant if they were less than 0.05). An estimate of the sample size needed for a typical experiment is as follows: for a control response of 100%, a typical response s.d. of 40%, a response in a drug of 30% (70% inhibition), a power of 80% and P < 0.05, seven vessels are needed (http://www.biomath.info/power/ttest.htm) in each of the control and drug groups. The exact numbers depend on the drug effect size and standard error of the data. A Supplementary Methods Checklist is available.

Materials. TTX, NBQX, d-AP5, (S)-MCPG, MR2179 and A967079 were obtained from Tocris Biosciences; U46619, NF449, L-161982, U73122, CAY10441, PPOH, MAFP and FIP1 from Cayman Chemicals; NS-398 and SC-560 from Calbiochem-Merck Millipore; RHC-80267 from Enzo Life Science; U105069 and CAY1094 from Santa Cruz; t-NNa, t-ß-methylene-ATP, NF023 and PGE1 from Sigma; Alexa Fluor 488, Alexa Fluor 594, Fluo-4 pentapotassium salt, BPAT tetrapotassium salt and Alexa Fluor 488– or FITC-conjugated IB4 from Life Technologies; and PBS and Alexa Fluor 633 hydrazide from Thermo Scientific. All other salts and reagents were purchased from Sigma.

Data availability. The data that support the findings of this study are available from the corresponding author upon request.
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Corrigendum: Astrocytes mediate neurovascular signaling to capillary pericytes but not to arterioles

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In the version of this article initially published, the abstract referred to diacylglycerol kinase; this should have been diacylglycerol lipase. The error has been corrected in the HTML and PDF versions of the article.