

A Critical Role for the Vascular Endothelium in Functional Neurovascular Coupling in the Brain

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Background—The functional modulation of blood flow in the brain is critical for brain health and is the basis of contrast in functional magnetic resonance imaging. There is evident coupling between increases in neuronal activity and increases in local blood flow; however, many aspects of this neurovascular coupling remain unexplained by current models. Based on the rapid dilation of distant pial arteries during cortical functional hyperemia, we hypothesized that endothelial signaling may play a key role in the long-range propagation of vasodilation during functional hyperemia in the brain. Although well characterized in the peripheral vasculature, endothelial involvement in functional neurovascular coupling has not been demonstrated.

Methods and Results—We combined in vivo exposed-cortex multispectral optical intrinsic signal imaging (MS-OISI) with a novel in vivo implementation of the light-dye technique to record the cortical hemodynamic response to somatosensory stimulus in rats before and after spatially selective endothelial disruption. We demonstrate that discrete interruption of endothelial signaling halts propagation of stimulus-evoked vasodilation in pial arteries, and that wide-field endothelial disruption in pial arteries significantly attenuates the hemodynamic response to stimulus, particularly the early, rapid increase and peak in hyperemia.

Conclusions—Involvement of endothelial pathways in functional neurovascular coupling provides new explanations for the spatial and temporal features of the hemodynamic response to stimulus and could explain previous results that were interpreted as evidence for astrocyte-mediated control of functional hyperemia. Our results unify many aspects of blood flow regulation in the brain and body and prompt new investigation of direct links between systemic cardiovascular disease and neural deficits. (*J Am Heart Assoc.* 2014;3:e000787 doi: 10.1161/JAHA.114.000787)

Key Words: endothelial hyperpolarization • functional magnetic resonance imaging • neurovascular coupling • optical imaging • vascular endothelial function • vascular reactivity

Increases in local blood flow almost always accompany stimulus-evoked neuronal activity in the cerebral cortex. These actively mediated increases in blood flow cause an inflow of oxygenated blood that exceeds oxygen consumption. The resulting decrease in the local concentration of (paramagnetic) deoxygenated hemoglobin is the basis of contrast in functional magnetic resonance imaging (fMRI).¹ Identifying the mechanisms driving neuronally coupled blood flow modulations in the brain is thus central to interpretation of fMRI data in both the healthy and diseased brain.^{2,3} Moreover,

understanding neurovascular coupling is also fundamental to understanding brain physiology and the way in which the brain manages its energy demands. Evidence is building for a connection between impaired neurovascular coupling and neurodegeneration.^{4–6} A more complete understanding of the mechanisms of neurovascular coupling could potentially lead to new diagnostic and therapeutic approaches to a range of neurological conditions.

It has become widely accepted that the control of functional hyperemia in the brain employs a specialized mechanism compared to the rest of the body.^{7,8} However, despite a range of studies exploring the potential role of astrocytes, pericytes, neurons, and interneurons in neurovascular coupling, a cohesive and consistent model has yet to emerge.^{6,7,9–13} The prevailing assumption is that astrocytes, sensing neuronal activity through metabotropic glutamate receptors,¹⁴ release arachidonic acid derivatives onto the smooth muscle cells of penetrating arterioles.⁹ However, recent studies have begun to directly challenge the involvement of astrocytes in neurovascular coupling.^{11,15,16} In particular, it has been difficult to reconcile the physical distribution, connectivity, and timing of intracellular calcium

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increases of astrocytes with the spatiotemporal properties of vasodilation during functional hyperemia.^{15,17,18}

The role of the vascular endothelium in blood flow modulation is well characterized in the peripheral vasculature,^{8,19–22} particularly the ability of the endothelium to propagate vasodilation. Earlier studies using pharmacological stimuli, such as acetylcholine (ACh), in both the *in vivo* brain^{23,24} and in isolated vessels^{25,26} have demonstrated the presence of endothelial reactivity and propagation of vasodilation in the cortical vasculature. However, the assumed role of astrocytes in functional coupling in the brain has led to few to consider the potentially important role of the vascular endothelium in functional neurovascular coupling.^{7,27,28} Our previous studies benchmarking the timing, pattern, and spatial range of arterial vasodilation during functional hyperemia¹⁸ found a close match to the properties of endothelial signaling pathways,²² leading us to hypothesize that the endothelium might play a critical role during functional hyperemia. As described further in the Discussion, establishing the involvement of the vascular endothelium in the process of neurovascular coupling is important, because it can provide explanations for many previous results that have been interpreted as evidence for, and against, other neurovascular mechanisms^{7,9,15,29} and could have wider implications for understanding neurovascular impairment. Re-evaluating earlier works, we find that no previous studies have specifically assessed whether endothelial mechanisms contribute to normal, cortical functional hyperemia in response to normal, physiological stimulation in the *in vivo* brain.

In this study, we disrupt the vascular endothelium of cerebral arteries *in vivo* and demonstrate that this disruption halts retrograde propagation of vasodilation during stimulus-evoked functional hyperemia. We further show that wide-field disruption of the endothelium of superficial arteries can profoundly alter functional hemodynamics in the cortex. These findings suggest that the vascular endothelium is a critical component of the functional hemodynamic response to somatosensory stimulus in the living brain.

Methods

Our overall experimental approach was to selectively disrupt the vascular endothelium of cortical arterioles in the intact, living rat brain and assess the effects of this disruption on the cortical hemodynamic response to sensory stimulation. All animal procedures were reviewed and approved by the Columbia University Institutional Animal Care and Use Committee.

Animal Preparation and Optical Recording of Stimulus-Evoked Hemodynamics

The cortical hemodynamic response to electrical hindpaw stimulation was recorded using multispectral optical intrinsic

signal imaging (MS-OISI), an established technique used for assessment of functional hemodynamics in exposed-cortex *in vivo* models.^{1,18,30} Preparatory surgery was performed under isoflurane anesthesia (2% to 3% inhalation in 3:1 air/oxygen mix) and included placement of a tracheotomy for ventilation (TOPO, Kent Scientific, Torrington, CT) and left femoral artery and vein cannulation for continuous blood pressure monitoring (BP1; World Precision Instruments Inc., Sarasota, FL) and drug/dye delivery. A ≈ 3 mm \times 5 mm region of the rat somatosensory cortex was then exposed by craniotomy, the fourth ventricle was opened to relieve intracranial pressure, and the dura overlying the exposed region was then removed. Dental acrylic (Acraweld; Henry Schein, Dublin OH) was used to seal a glass coverslip with a drop of 1.5% agarose in artificial cerebrospinal fluid (aCSF) over the exposed region, creating a window for imaging while minimizing brain motion and contamination (aCSF: 125 mmol/L of NaCl, 5 mmol/L of KCl, 4.1 mmol/L of CaCl₂, 10 mmol/L of glucose, 2.8 mmol/L of MgCl, and 10 mmol/L of HEPES). For imaging, animals were removed from isoflurane and anesthetized by intravenous alpha-chloralose (40 mg/kg⁻¹/h⁻¹). To rule out anesthetic-dependent effects, intraperitoneal urethane anesthesia (1.5 mg/g) was used for both surgery and imaging in a subset of controls. The animal's core body temperature was measured and maintained throughout at 37°C with a rectal thermometer attached to a homeothermic heating pad (Stoelting Co., Wood Dale, IL). In animals where electrophysiology was performed, a thin silicone sheet (gloss/gloss; Specialty Manufacturing, Inc., Saginaw, MI) was used instead of a glass coverslip to allow insertion of a recording electrode through the window. Stimulation electrodes were placed on the right hindpaw and connected to an electrical stimulation isolation unit (A365; World Precision Instruments Inc.) delivering 0.3-ms pulses at 3 Hz with 1.0 ± 0.1 mA amplitude under computer control.

MS-OISI employs a camera to capture high-speed images of the cortex.³⁰ Image sequences were acquired using our home-built MS-OISI system consisting of a Dalsa 1M60 camera and synchronized, filtered light-emitting diodes (LEDs) for strobed illumination.³⁰ Data were acquired using red (630 nm centered, M625L2; Thorlabs, Inc., Newton, NJ) and green (530 nm centered, M530L2; Thorlabs) LEDs with a 534/43 nm filter in front of the green LED (NT67-031; Edmund Optics Inc., Barrington, NJ). Dual-wavelength data were acquired at 74 frames per second (fps; equivalent to 37 fps for each LED) with a 5-ms exposure time and 512 \times 512 pixel resolution. Each imaging “run” lasted 40 seconds and consisted of 6 seconds of prestimulation, 12 or 2 seconds of stimulation, and 22 or 32 seconds of poststimulation, respectively. Runs were typically acquired in sets of 5 sequential repetitions. Sensory-evoked hemodynamic responses were recorded before and after light-dye disruption. A 496 nm long-pass emission filter (FF01-496/LP-25; Semrock, Inc., Rochester, NY) was placed in

front of the CCD camera to allow fluorescent imaging of the dextran-conjugated fluorescein isothiocyanate (FITC-dx) during blue light illumination. Negligible absorption of green LED light was found for FITC-dx at in vivo concentrations.

In Vivo Light-Dye Disruption of the Cortical Vascular Endothelium and Controls

Used widely in studies of endothelial propagation of vasodilation in the peripheral vasculature, light-dye treatment involves filling a vessel with a fluorescent dye, in our case FITC-dx, and illuminating with bright light at the dye's excitation wavelength. The reactive oxygen species generated by this illumination induce highly localized disruption of the vessel's endothelial cell (EC) membranes, interrupting their ability to conduct signals while leaving the rest of the vessel and smooth muscle cells intact and functioning.^{22,24,31,32} Here, we implemented the light-dye method in 2 ways: first, using a fine focused line of laser light to transect signaling within the endothelium of a pial artery that had been observed to dilate in response to stimulation in pre-light-dye recordings. In a second cohort, a larger wide-field area of the pial surface was illuminated to attempt to disrupt the endothelium of all pial arteries previously observed to dilate in response to stimulus.

Parameters for in vivo light-dye treatment were carefully determined in preliminary experiments. For the data shown, following acquisition of pre-light-dye stimulation runs, rats were slowly injected with 0.5 mL of 10% FITC-dx (molecular weight, 70 kDa; 46945; Sigma-Aldrich, St. Louis, MO) in saline through the femoral vein catheter (≈ 1.2 mL/h). After equilibration and acquisition of control "dye-only" stimulus runs, the endothelium was disrupted by exciting the dye for a period of 6 to 7 minutes with either (1) 488 nm light from a single-mode, fiber-coupled, solid-state, continuous-wave laser (laser 85BCD-030-115 [Melles Griot Light Sources Group, Barlsbad, CA] and fiber P3-460A-FC-10 [Thorlabs]) focused using a 50-mm focal-length cylindrical lens (LJ1695RM-A; Thorlabs) to generate a light line 50 to 100 microns wide and 2.5 mm long before tissue scattering (total power, ≈ 2 to 3 mW) or (2) 470 nm-centered blue light from a 400- μ m optical fiber coupled to a high-power LED (M470F1; Thorlabs) focused to a ≈ 1 -mm-diameter spot (before scattering) with ≈ 6 mW/mm² for wide-field treatment. Analysis of the likely penetration depth of these 2 illumination configurations is included in Data S1. In each rat, control runs during somatosensory stimulation were acquired before injection of FITC-dx with blue light illumination of the cortex only, as well as after FITC-dx injection prior to combined blue light illumination.

Further control measurements were performed in each light-dye-treated rat after completion of stimulus-evoked data acquisition to ensure that only the vascular endothelium had

been disrupted.^{22,24,32,33} These controls included imaging (without stimulation) during cortical application of ACh (0.1 mmol/L), to test for the presence (or lack) of endothelium-dependent vasodilation in untreated and treated regions, and during cortical application of sodium nitroprusside (SNP; 0.1 μ mol/L) to test that arterial smooth muscle cells were still responsive in all regions. In each case, the cranial window was removed and an acrylic well was built around the exposed region of the cortex and then filled with either ACh or SNP. A field of view containing both light-dye-treated and nontreated vessels was then imaged for 1 minute after application of ACh or SNP. After each imaging run, the brain was flushed with aCSF and allowed to return to baseline for 15 minutes. Control and treatment area dilations were assessed in arteries of similar resting diameter. Only vessels that exhibited intact smooth muscle-dependent dilation to SNP, but not endothelial-dependent dilation to ACh, were included in analysis. In additional experiments, ACh (1 to 2 μ mol/L) was used to isolate the effects of arterial predilation on functional hyperemia to rule out the possible influence of light-dye-induced dilation.

Electrophysiology measurements were performed to ensure that wide-field light-dye treatment did not alter the neuronal response to somatosensory stimulation. A tungsten microelectrode (0.005-mm diameter, 0.5 M Ω ; A-M Systems, Sequim, WA) mounted on a micromanipulator was stereotaxically inserted into the cortex to a depth of ≈ 400 to 500 μ m in the center of the responding region identified through initial MS-OIS imaging. Electrode signals were recorded using Spike2 software (Cambridge Electronic Design Limited, Cambridge, UK). Local field potential (LFP) signals were filtered (0.1 to 100 Hz) and processed using MATLAB (The MathWorks, Inc., Natick, MA).

Data Processing

The dynamic dilation and constriction of vessels on the pial surface can be resolved from raw MS-OIS images, whereas light absorption by blood in vessels, including deeper capillary beds, provides a measure of parenchymal hyperemia (corresponding to a superficially weighted sum of contributions from deeper layers³⁴; see Data S2). By synchronously illuminating the cortex with different interlaced wavelengths of light, the oxygenation-dependent absorption of light by blood permits mapping of the relative changes in oxy-, deoxy- and total hemoglobin (Δ [HbO], Δ [HbR], and Δ [HbT])^{1,18,30} (calculated here from 630 and 530 nm reflectance data using the modified Beer Lambert law with wavelength-dependent path-length factors calculated using Monte Carlo simulations^{1,35}). Data were analyzed by averaging responses over repeated identical stimulation runs. Data were temporally filtered at 5 Hz and affine coregistered where necessary. Time

courses were averaged over pixel regions indicated. Vessel diameters were calculated as the full width of the vessel profile on raw 530 nm reflectance data. Vessels examined for diameter measurements were roughly the same resting size (same degree of branching). Functional response maps represent stimulus-evoked changes over the full field of view.

In the data presented here, we primarily focus on ΔHbT as a measure of physical vascular actuation and hyperemia, rather than HbO and HbR dynamics, which combine the effects of vessel volume and oxygenation changes and are influenced by both blood flow changes and oxygen consumption.

Statistical Analysis

Raw data consists of time series of images acquired over multiple repetitions of the stimulation paradigm or, in some cases, pharmacological trials. Parameters are extracted from different locations within the image time series, for multiple repetitions of stimulation, and under different conditions (such as pre- and post-light-dye treatment). Unless otherwise specified, 5 individual stimulus runs were averaged to

construct a single data point. This was necessary, for example, in vessel diameter calculations, to ensure sufficient image signal to noise for accurate parameter calculation. Where multiple assessments within a single rat are used, standard errors (SEs) were corrected to account for intraclass correlation. All statistical comparisons are 2-sided *t* tests using Student *t* distribution. Inspection of residuals (eg, through quantile comparisons) indicate that residuals are approximately normally distributed and thus that Student *t* distribution is appropriate. Randomization was not possible for pre- and post-light-dye studies, because the light-dye effect is irreversible, although controls were performed to ensure that light exposure, dye exposure, and time alone were insufficient to generate the effects observed.

Results

Properties of Cortical Functional Hyperemia With Intact Vascular Endothelium

Figure 1a illustrates the vascular organization of the cerebral cortex, with deeper capillary beds fed by perpendicularly

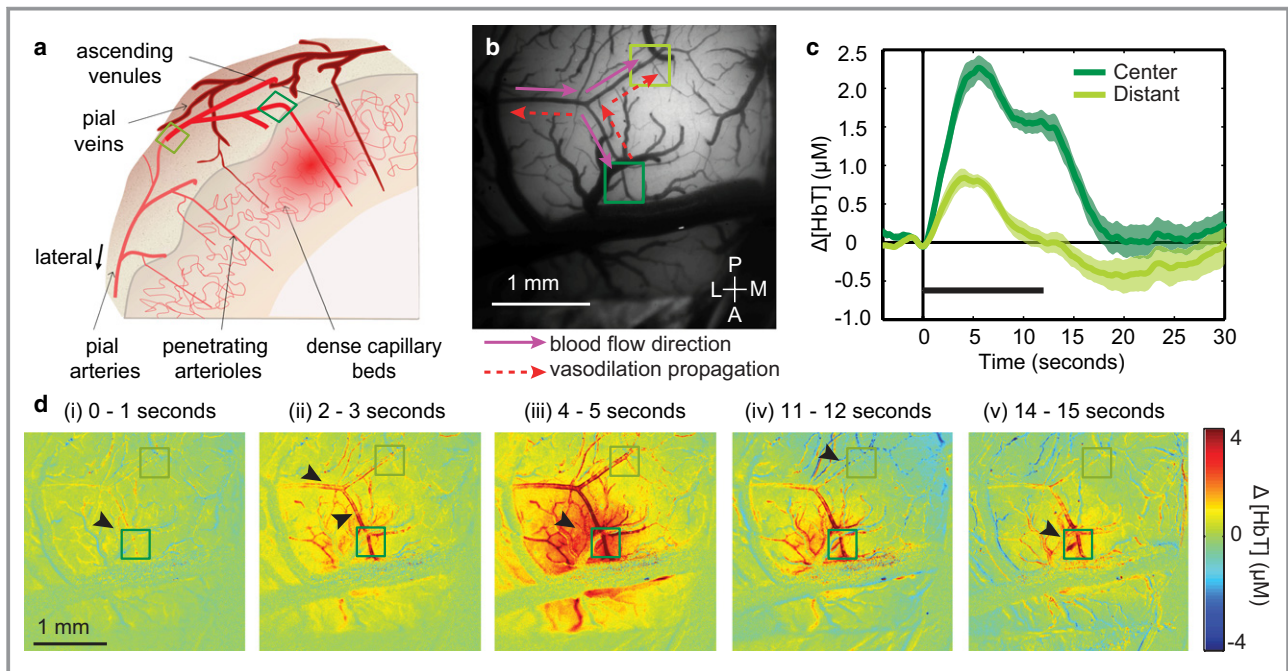


Figure 1. Normal spatiotemporal evolution of the hemodynamic response to somatosensory stimulation. a, Vascular organization of the cerebral cortex. Envisaged capillary hyperemia and retrograde dilation of penetrating arterioles and pial arteries feeding the active region is overlaid in red. b, Grayscale image of the exposed somatosensory cortical surface under 534 nm-centered illumination with arrows indicating direction of blood flow (purple) and propagated vasodilation (red dash). c, Time courses of change in total hemoglobin ($\Delta[\text{HbT}]$) extracted from regions of interest indicated in (a), (b) and (d) (average and SEM of $N=10$ repeated stimulus runs, same rat as in [b] and [d], and in Figure 3a through 3d), and in Figure 3a through 3d). d (i through v), Time series of functional maps of $\Delta[\text{HbT}]$ after onset (at $t=0$) of 12-second, 3-Hz electrical hindpaw stimulation (same rat and field of view as in [b], averaged over times indicated, average of $N=5$ repeated stimulus runs). ΔHbT contrast shows both parenchymal hyperemia and the dilation of specific pial arteries. Arrowheads point to features described in the text. A indicates anterior; L, lateral; M, medial; P, posterior.

oriented penetrating arterioles that branch from superficial pial arteries. Figure 1b shows a grayscale image of the exposed somatosensory cortex, with arrows along one of the main feeding arteries indicating both the direction of blood flow and anticipated direction of propagated vasodilation. Figure 1d through 1dv shows an MS-OISI $\Delta[\text{HbT}]$ time series of the same field of view during a 12-second, 3-Hz electrical hindpaw stimulus. This sequence demonstrates the typical

spatiotemporal evolution of the cortical hemodynamic response to stimulation: (i) Slight capillary hyperemia is visible as enhanced ΔHbT contrast in the parenchyma before pial arteries are seen to dilate (ii).^{1,18,36,37} Arterial dilation appears as increased ΔHbT contrast resulting from increased vessel volume and can be visualized as an increase in the physical diameter of the vessel (see also Figure 2g). Parenchymal hyperemia continues to rise slowly (iii); however,

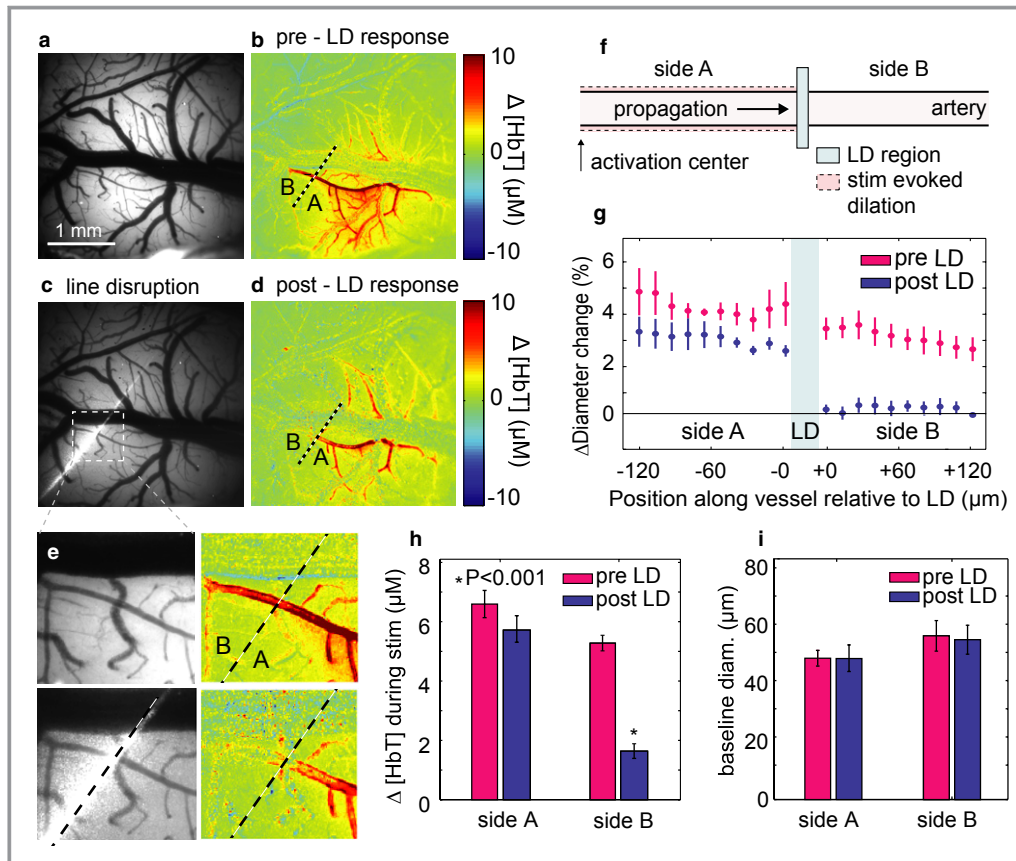


Figure 2. Light-line disruption to endothelial propagation of vasodilation. a, Grayscale image of the exposed cortical surface (534 nm-centered illumination). b, Pre-light-dye (LD) total hemoglobin (ΔHbT) functional response map (averaged over 4 to 6 seconds after stimulus onset, stimulus duration=12 seconds, average of $N=5$ runs from 1 rat). c, Image of 488 nm laser focused into a “light-line” positioned to transect a responding arteriole. d, Post-LD hemodynamic response equivalent to (b), average of $N=5$ runs from 1 rat. The dilatatory response no longer reaches side B. e, Zoomed-in views of the region indicated in (c) for each case shown in (a) through (d). f, Schematic showing LD treatment geometry. g, Percent change in vessel diameter at the response peak (4 to 6 seconds) relative to baseline on either side of the LD-treated vessel segment before (pink) and after (blue) LD treatment (average from $n=3$ rats, pre-LD runs=5 per rat, post-LD runs=5 per rat). Error bars: SEM. Position along the vessel is measured relative to the edges of the treated segment. The treated vessel segment was 83 ± 15 μm long. h, Average ΔHbT 4 to 6 seconds after stimulus onset selected from side A and side B before (pink) and after (blue) LD treatment ($n=4$ rats, runs=20 per rat per condition). Side B shows significantly attenuated ΔHbT responses after LD treatment ($P<0.001$; Student t test). Error bars show SEs corrected for intraclass correlation. One rat included in ΔHbT analysis was excluded from diameter calculations owing to vessel overlap. i, Baseline vessel diameters on either side of the illuminated segment were unaffected by the LD treatment ($n=3$ rats, runs=15 per rat per condition: $P>0.7$, side A, $P>0.9$, side B; Student t test). Error bars show SEs corrected for intraclass correlation. Results from an additional light-line rat with acetylcholine (ACh) control are shown in Figure 4.

dilation in more-distant pial arteries begins to return to baseline after an initial peak at ~4 to 6 seconds (iv).^{36,38} Continued stimulation maintains higher perfusion of a localized region of parenchyma in the center of the responding region, with minimal continued dilation of the more-distant pial arteries (v).^{36,38–40} This difference is clear from ΔHbT time courses extracted from a “distant” pial artery versus the “center” of the responding region (Figure 1c). The “central” response exhibits an early peak and a later plateau phase,^{36,38–40} whereas the “distant” component shows only an early peak. Models of neurovascular coupling to date have failed to explain these spatiotemporal patterns and have not presented a mechanism that can explain the long-range, rapid dilation of pial arteries both up- and downstream from the active region (see arterial flow vs. propagation directions indicated in Figure 1b). In earlier work, we characterized the onset propagation speed of this distant, retrograde pial artery dilation as exceeding 2 mm/s.¹⁸ These observations led us to hypothesize that initial pial artery dilation involves rapidly propagated vasodilation within the vascular endothelium.²²

Interruption of Retrograde Propagation of Vasodilation

To determine whether dilation of distant pial arteries is dependent on endothelial propagation of vasodilation, the light-dye method was implemented using a thin, focused line of 488 nm of laser light. This line was positioned and oriented to transect pial artery branches that had been observed to dilate in response to stimulation preceding light-dye treatment.

Figures 2a and 2b show a grayscale image of the exposed cortex and a corresponding ΔHbT functional map of the pre-light-dye cortical response to a 12-second hindpaw stimulus in 1 rat. A similar pattern of selective pial artery dilation can be seen as in Figure 1d. Figure 2c shows the location of the applied “light-line,” intended to specifically disrupt endothelial signaling in a targeted pial artery. Figure 2d shows that following this light-line light-dye treatment, stimulus-evoked dilation of the artery segment distal to the light-dye treatment line (with respect to the center of the responding region) was strongly attenuated (side B). Arterial dilation on the proximal side (side A) was unaffected. Figure 2e shows a higher magnification view of the region indicated, for each case. This effect was consistent across all rats ($n=4$; $P<0.001$), both in terms of arterial diameter change (Figure 2g) and ΔHbT (Figure 2h).

It is important to note that the dilatory response was eradicated in the distal segment of vessel that was not itself illuminated by the light-dye laser, such that baseline diameters of each examined segment were the same before and after light-dye treatment (Figure 2i). Additional control measurements shown in Figure 4 confirm that dilation to

application of ACh was observed on both sides of the light line. We conclude that selective, discrete disruption of the endothelium prevented transmission of stimulus-evoked vasodilatory signaling from the proximal (side A) to the distal segment (side B). We also note that this result clearly demonstrates that stimulus-evoked retrograde dilation of pial arteries is not the result of flow-mediated coupling.⁴¹

Wide-Field Disruption of the Vascular Endothelium

The light-line results described above provide strong evidence of an endothelial pathway for retrograde propagation of vasodilation along pial arteries during functional hyperemia. However, this result does not demonstrate the relative importance of this pathway in generating functional hyperemia as a whole. To address this, wide-field light-dye treatment was implemented using more distributed illumination from a LED centered at 470 nm. Because blue light is heavily scattered in brain tissue, we expect the main action of this illumination to be impairment of endothelial signaling within the superficial pial arteries (see Data S1 for depth analysis). The results from these measurements are summarized in Figure 3.

As expected, wide-field light-dye treatment significantly attenuated stimulus-evoked dilation of pial arteries (Figure 3a through 3d). Plotting the time courses of changes in ΔHbT occurring in the center of the responding region before and after light-dye treatment, we observe that wide-field light-dye disruption also has a strong effect on the overall ΔHbT time course (Figure 3e). Specifically, the fast increase and initial peak of the ΔHbT response is strongly attenuated, leaving behind only a gradual rise in $\Delta[\text{HbT}]$. This residual response was found to localize to the parenchyma, rarely recruiting dilation of pial arteries. Equivalent results for a 2-second stimulus are compared in Figure S3. Corresponding HbO and HbR dynamics are shown in Figure S4.

Comparing these results to the spatiotemporal patterns shown in Figure 1, we note the similarity between the initial peak of the “distant” artery response in Figure 1c and the missing “peak” component of the ΔHbT response removed by wide-field light-dye treatment in Figure 3e. We also note the similarity between the spatial map of the pre-light-dye ΔHbT response at 14 to 16 seconds in Figure 1dv and the post-light-dye response (Figure 3d and 3e, same rat). This suggests that endothelium-dependent, long-range propagated vasodilation of pial arteries has a more significant role in the early phase and initial peak of the cortical hemodynamic response to stimulus than in later phases. We estimate that attenuation resulting from scattering and absorption in the intact brain would reduce the irradiance of wide-field 470 nm illumination to below the threshold for the light-dye effect within 100 to 200 microns of the pial surface (Data S1). This suggests that

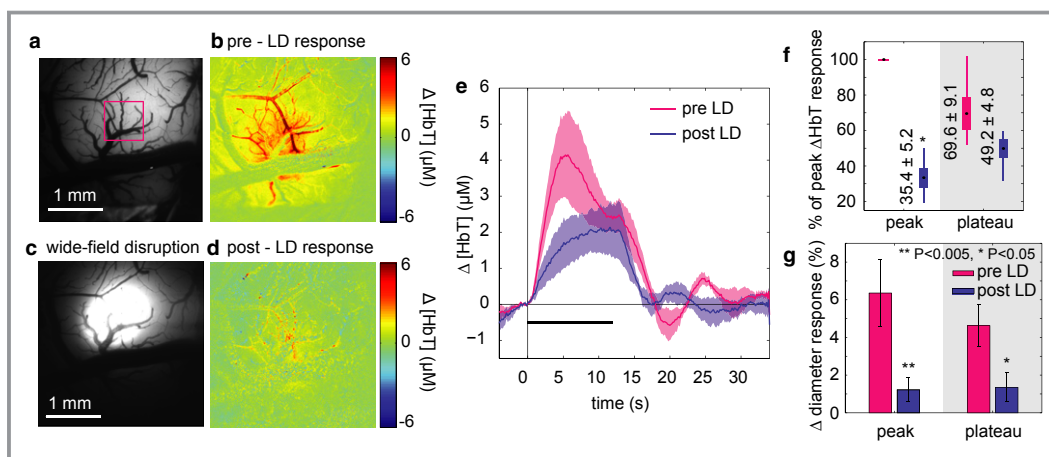


Figure 3. The hemodynamic response before and after wide-field light-dye treatment. a, grayscale image of the exposed cortical surface (534 nm-centered reflectance) of a representative rat (same rat as shown in Figure 1). b, Pre-light-dye treatment functional map showing total hemoglobin ($\Delta[\text{HbT}]$) “peak” response relative to baseline averaged over 4 to 6 seconds after stimulus onset (average of $N=5$ runs, 1 rat). c, Image showing the blue light spot used for wide-field light-dye treatment (≈ 1 mm in diameter). d, Post-light-dye $\Delta[\text{HbT}]$ functional map (same rat, time-period average and color scale as [c]) (average of $N=5$ runs, 1 rat). e, Pre- and post-light-dye $\Delta[\text{HbT}]$ time-course response to 12 seconds of stimulation averaged over $n=7$ rats, runs=5 per rat per condition, responses averaged per rat. Error bounds show SEM over rats. Responses to 2-second stimulus are compared in Figure S3. Equivalent traces for oxy- and deoxy-hemoglobin concentration changes are shown in Figure S3. The region of interest was the same for all conditions and was centered over the responding region and included both arteries and parenchyma. f, $\Delta[\text{HbT}]$ amplitudes response averaged over the “peak” (4 to 6 seconds) and “plateau” (10 to 12 seconds) shown as a percentage of the pre-light-dye “peak” response (data from e). Black dots: mean, boxes: SEM, whiskers: range of data. Post-LD peak amplitude is significantly attenuated relative to pre-LD ($P<0.05$; Student t test). g, Percent stimulus-evoked change in diameter of responding pial arteries pre- and post-light-dye for “peak” and “plateau” ($n=5$ rats, 5 runs per rat per condition). Diameters calculated from averages of 5 runs=5 measurements per condition. Error bars show SEM. P values from Student t test.

our wide-field light-dye treatment most strongly affected the pial arteries, and therefore long-range signaling, leaving the endothelium of deeper segments of pial arterioles and capillaries intact.

Controls

To ensure that light-dye treatment was indeed *only* disrupting the vascular endothelium, and not smooth muscle cells, we performed established pharmacological control measurements at the end of each wide-field light-dye experiment.^{22,24,33,42} Endothelium-dependent dilator ACh and endothelium-independent dilator SNP were each applied to the exposed cortex in turn, and MS-OIS1 data were acquired in the absence of stimulation. As expected, pial arteries in the untreated regions dilated to ACh application, whereas pial arteries within the treated area failed to dilate ($P<0.005$; Student t test), indicating endothelial disruption. The same treated arteries *did* dilate in response to SNP application, verifying that smooth muscle cells were intact and reactive after light-dye treatment (Figure 4a). Neither intravenous delivery of FITC-dx nor blue light exposure alone affected the amplitude of the peak

response to stimulus or the baseline diameter of pial arteries (Figure 4b). ACh applied after light-line light-dye experiments caused *both* proximal and distal segments to dilate (Figure 4c through 4e), even though stimulus-evoked dilation of the same distal segment was significantly attenuated after light-line disruption. These results confirm that the distal segment was itself undamaged, and still physically able to dilate, indicating that the light line selectively interrupted the path taken by stimulus-evoked endothelial signaling. The data shown in Figure 4c through 4e were acquired in an animal under urethane anesthesia to rule out the influence of anesthesia on our findings. The slightly smaller pre-light-dye percentage stimulus-evoked arterial diameter increase seen here, compared to Figure 2, is likely a result of the larger baseline diameter of the chosen pial artery.

To assess whether wide-field light-dye treatment altered neuronal activity, microelectrode recordings of LFP were performed in 2 rats during 12 seconds of electrical hindpaw stimulation, before and after wide-field light-dye treatment. Traces in Figure 4f show identical patterns of larger amplitudes at stimulus onset, with a decay to lower amplitudes for the remainder of the stimulus. Individual LFPs also did not exhibit

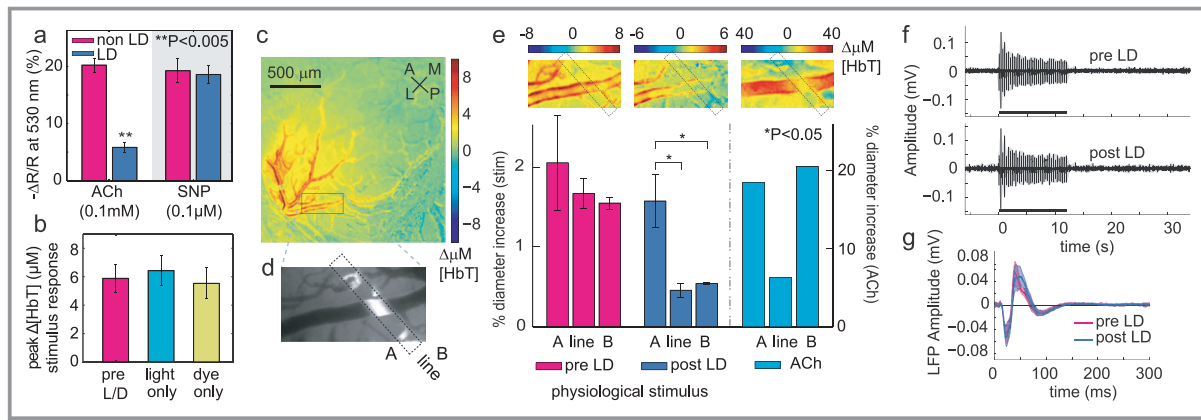


Figure 4. Smooth muscle cell function and neural activity were unaltered by light-dye treatment. **a**, Maximum $\Delta[\text{HbT}]$ change induced by topical cortical application of acetylcholine (ACh) (0.1 mmol/L) and sodium nitroprusside (SNP) (0.1 $\mu\text{mol/L}$) after wide-field light-dye (LD) treatment. LD values were extracted from within the treated blue light illumination area, whereas non-light-dye values were extracted from unilluminated, peripheral regions. Vasodilation to ACh in light-dye-treated regions was significantly attenuated (** $P < 0.005$; Student t test; $n = 3$ rats, 2 trials per rat per condition). The temporal dynamics of dilation to SNP in light-dye-treated and untreated regions were also unaffected (see Figure S6). **b**, Controls for 3 conditions: pre-light-dye response, response after exposure to blue light spot only (no dextran-conjugated fluorescein isothiocyanate (FITC-dx)), and response after FITC-dx dye intravenous injection only (no blue light). Peak $\Delta[\text{HbT}]$ response was averaged over the hindpaw response region between 4 and 6 seconds after stimulus onset ($n = 4$ rats, average of 15 runs per rat for each condition, responses were averaged over 5 runs in each rat = 12 measurements per condition; error bars show SEM) ($P > 0.6$ and $P > 0.7$, respectively; Student t test). **c** through **f**, Light-line ACh control. **c**, Peak $\Delta[\text{HbT}]$ map in response to 12-second stimulation averaged over 4 to 6 seconds after stimulus onset (**d**) shows the subregion during light-line illumination of a selected pial artery. **e**, $\Delta[\text{HbT}]$ images of same region of interest under 3 different conditions: (1) the peak response to stimulation before light-line treatment ($N = 5$ runs); (2) the peak response to stimulation after light-line treatment ($N = 5$ runs); and (3) The response of the same region (after light-line treatment) to topical application of ACh (1 trial). Bar graphs show calculated percentage change in diameter on either side of the light line for these 3 conditions in the same rat (stimulus results show an average of 10 repeated runs; error bars show SEM) * $P < 0.05$; Student t test. **f**, Electrophysiological recordings of neural activity during 12 seconds of hindpaw stimulation before and after wide-field LD treatment. Black time course represents the average over $n = 2$ rats, with runs = 5 per rat per condition. Gray envelope indicates SEM. **g**, Averaged individual local field potential (LFP) spikes from the time courses in (**f**). Shaded region shows SEM.

significant differences in amplitude or shape throughout the stimulus period (Figure 4g), confirming that wide-field light-dye treatment did not itself alter neuronal activity.³⁸

Our analysis of arterial diameters shown in Figure 3g did reveal one potential confound in our wide-field light-dye experiments: Pial arteries with baseline diameters between 30 and 50 μm exhibited a $\approx 19\%$ ($7.8 \pm 1.2 \mu\text{m}$) increase in their baseline diameters after wide-field light-dye treatment. The predilating effect of light-dye treatment has been described previously^{31,33,42} and does not imply that the vessel is unable to dilate further (as confirmed in every case by our SNP control). However, to verify that this predilation was not itself causing alterations to the hemodynamic response time course, we performed further *in vivo* control measurements in which we recorded responses to somatosensory stimulation during gradually increasing levels of predilation induced by a single topical application of 1 to 2 [$\mu\text{mol/L}$] ACh (Data S5). Arteries that were predilated to equivalent levels, as induced by wide-field light-dye treatment, were found to still respond normally to somatosensory stimulation, with

responses exhibiting a normal peak and plateau pattern and similar peak ΔHbT amplitudes as pre-light-dye response amplitudes, even for predilations exceeding 40%. This control demonstrates that moderate predilation alone does not affect the ability of pial arteries to dilate further in response to somatosensory stimulation. This result also shows that any increase in blood flow resulting from an increase in baseline diameters does not affect driving mechanisms sufficiently to alter the peak-plateau shape or amplitude of the response to stimulation.³⁸ We note also that our light-line results (Figures 2 and 4c through 4e) showed that discrete disruption of a small section of pial artery strongly attenuated dilation in distal arterial segments that were neither illuminated nor predilated. These distal segments were also responsive to ACh application, confirming that changes observed after light-dye treatment were not the result of physical damage to the unresponsive vessel segment. The small extent of the treated vessel segment in our light-line study also rules out the possible influence of post-light-dye increases in baseline blood flow.

Additional analysis was performed to assess the possible influence of blood pressure and other systemic parameters. Our recordings of intra-arterial blood pressure indicated that a measurable decrease (up to 14%) in systemic blood pressure occurred in most rats immediately after FITC-dx injection. However, these changes in systemic blood pressure did not correlate with observed changes in arterial reactivity to stimulation, as evidenced by our “light-only” and “FITC-dx-only” measurements (Figure 4b). Similarly, whereas analysis also revealed a small (<2%) increase in systemic blood pressure in response to stimulation,⁴³ the temporal shape of these blood pressure responses were not significantly different for pre- and post-light-dye data. Light-line control experiments shown in Figure 4d and 4e were intentionally performed under intraperitoneal urethane anesthesia, rather than intravenous alphachloralose, to rule out the possible influence of anesthetic choice on our findings. In an equivalent cohort of animals, blood gases were found to be within normal ranges (PaCO₂ between 35 and 45 mm Hg, PaO₂ between 100 and 180 mm Hg, and pH between 7.3 and 7.45). In all cases, we conclude that the highly localized changes in the cortical patterns of stimulus-evoked arterial dilation that form the basis of our findings (Figure 2e) could not be the result of systemic effects.

Discussion

Our results provide strong evidence that the vascular endothelium is a critical pathway in functional neurovascular coupling. We showed that selective interruption of endothelial signaling blocked retrograde dilation of pial arteries in response to stimulus in the somatosensory cortex. Wide-field disruption of the vascular endothelium at the pial surface led to a significant attenuation of the hemodynamic response, particularly its initial peak.

These findings have important implications for earlier models of functional neurovascular coupling, as well as our understanding of the role that endothelial signaling might play in the brain.^{7,8} Figure 5 illustrates known mechanisms for endothelial control of vasodilation alongside some of the brain-specific neurovascular coupling mechanisms proposed to date. The addition of the vascular endothelium to this picture appears to explain many of the anomalous findings in neurovascular coupling and fMRI research to date and raises a number of important new questions, as detailed below.

Which Types of Endothelial Mechanisms Might be Involved?

As illustrated in Figure 5, it is well established that in peripheral vasculature, stimulation of the vascular endo-

thelium can initiate 2 types of propagated vasodilation: (1) endothelial hyperpolarization, which exhibits long-range and almost unattenuated propagation over distances exceeding a millimeter, and is insensitive to cyclooxygenase (COX) pathway and nitric oxide (NO) inhibitors,^{20,22} and (2) a slower-moving ($\approx 116 \mu\text{m/s}$) calcium wave-associated propagated vasodilation that mediates vasodilation through endothelium-derived NO and prostanoids, such as prostacyclin (PGI₂), which decays over a distance of approximately 500 microns.⁴⁶ This “slow” mechanism has been shown to generate secondary vasodilation upon reaching arteriole segments already predilated by “fast” endothelial hyperpolarization, maintaining local dilation even after more-distant segments recover.⁴⁶ One study has suggested that higher levels of intracellular calcium release within the endothelium are required to generate endothelial hyperpolarization, compared to NO and prostanoid-dependent relaxation (340 vs. 220 nmol/L^{47,48}).

The properties of these 2 “slow” and “fast” endothelial mechanisms fit well with the central/distant patterns of functional hyperemia shown in Figure 1, as well as the presence of residual parenchymal hyperemia after light-dye treatment (Figure 3e). We hypothesize that the initial, rapid, long-range dilation of pial arteries is consistent with long-range propagation of vasodilation by endothelial hyperpolarization. Consistent with this model, the more localized and sustained parenchymal hyperemia seen during prolonged stimulation could be dominated by slower and more spatially restricted NO and prostanoid-dependent mechanisms. This slower mechanism might more strongly influence diving arterioles and perhaps capillaries beyond the penetration depth of light-dye treatment (see Data S1).⁴⁶ Moreover, we note that endothelial hyperpolarization appears to be prominent at the onset of the response, short-lived, and largely independent of stimulus duration, whereas parenchymal hyperemia is sustained and more dependent on stimulus duration. We posit that this pattern may be representative of a thresholding effect, whereby endothelial hyperpolarization occurs only at the onset of stimulation, whereas slower NO and prostanoid-dependent dilation is repeatedly triggered by continuing stimulation.⁴⁷ This model appears to fit well with the nonlinearities routinely observed in fMRI blood-oxygen-level dependent (BOLD) data, as discussed further below.⁴⁹

Can Endothelial Involvement Explain Previous Pharmacological Results?

To date, almost all attempts to pharmacologically impair functional hyperemia have observed some residual hyperemic response. In particular, studies seeking to block COX-1, COX-2, or NO pathways in order to isolate astrocyte and neuronal contributions, respectively, have found that neither

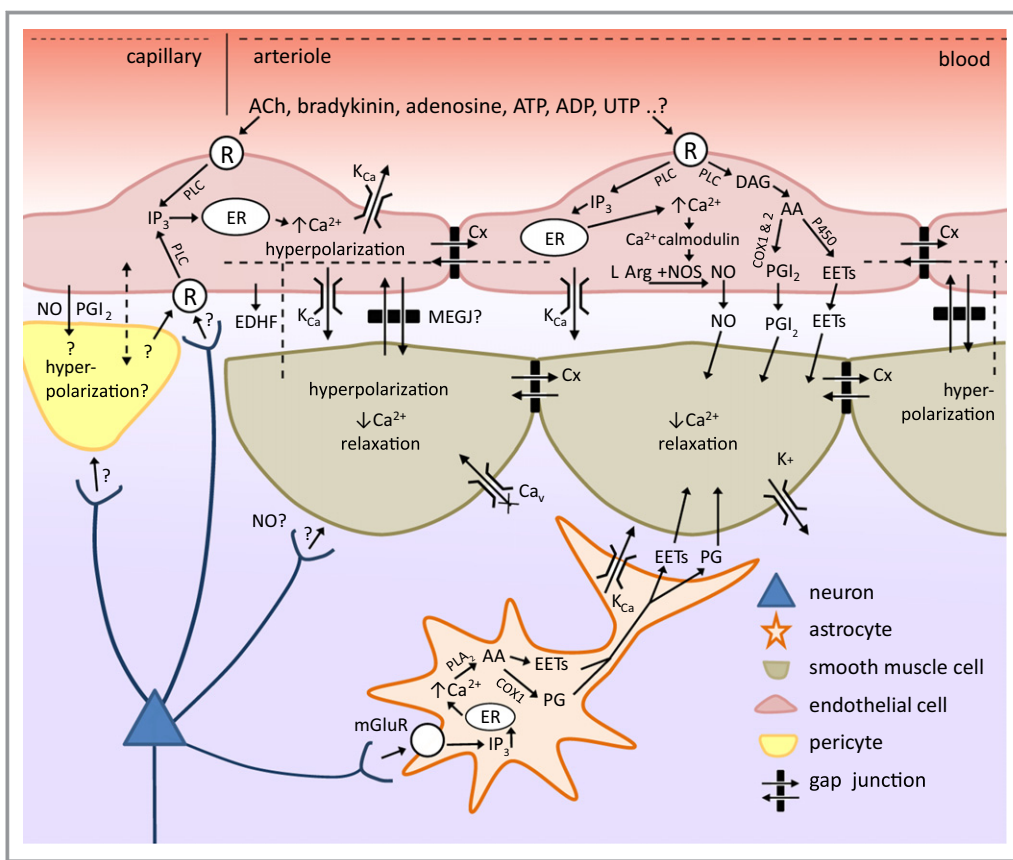


Figure 5. Incorporating the vascular endothelium into neurovascular coupling (adapted from refs. 44 and 7). Astrocyte: hypothesized to sense glutamate through metabotropic glutamate receptors (mGluR), increasing intracellular calcium $[Ca^{2+}]_{ia}$ and generating arachidonic acid (AA) from phospholipase A₂ (PLA₂) which is converted by COX1 (or 3) to prostaglandins (PG) and by P450 epoxygenase to epoxy-eicosatrienoic acid (EETs).^{7,9} Both PGs and EETs can relax smooth muscle cells (SMCs). Endothelial cells can increase their intracellular calcium $[Ca^{2+}]_{ie}$ in response to receptor (R) binding (targets include acetylcholine (ACh), bradykinin (BK), adenosine triphosphate (ATP), adenosine diphosphate (ADP), uridine triphosphate (UTP), and adenosine^{21,24–26}) causing inositol triphosphate (IP₃)-mediated release of calcium from the endothelial endoplasmic reticulum (ER).¹⁹ Phospholipase C (or PLA₂), through diacyl-glycerol (DAG), can also produce EETs and AA derivatives, including prostacyclin (PGI₂) within endothelial cells, both of which can drive SMC relaxation whereas increased $[Ca^{2+}]_{ie}$ can also drive production of endothelial nitric oxide (NO), which can also relax SMCs. $[Ca^{2+}]_{ie}$ increases can also lead to endothelial hyperpolarization through opening of calcium-dependent potassium channels (K_{Ca}). Endothelial hyperpolarization can spread rapidly to adjacent endothelial cells through gap junctions and is coupled to encircling SMCs either through myoendothelial gap junctions (MEGJs) or some other “endothelium-derived hyperpolarizing factor” (EDHF).¹⁹ SMC hyperpolarization causes relaxation through closure of voltage-dependent calcium channels (Ca_v). Signaling from excitatory and inhibitory cortical neurons^{10,11} as well as afferents from regions such as the thalamus, basal forebrain, and locus coeruleus^{4,45} to astrocytes, pericytes, SMCs have also been proposed.^{2,7} Potential signaling pathways, yet identified, are indicated by question (?) marks, and include the possibility of signalling between astrocytes, pericytes and endothelial cells, or even direct neuronal signalling to the vascular endothelium at the capillary level.

(nor both) completely block functional hyperemia.^{9,11,50} If both slow and fast endothelial pathways (as described above) are involved in functional hyperemia, then, as shown in Figure 5, components of stimulus-evoked vasodilation would be dependent on both endothelial-derived NO and prostanoids generated by endothelial COX pathways. Equally, endothelial hyperpolarization-mediated vasodilation, which is unaffected

by COX and NO inhibition, would remain.²² No previous neurovascular coupling studies have attributed a loss of attenuation of vasodilation under COX or NO inhibition to action on the vascular endothelium.⁷ We propose that endothelial involvement in neurovascular coupling can explain many of the seemingly complex and paradoxical effects of these agents on functional neurovascular coupling, including

the presence of residual responses.^{9,11} Studies relying on such pharmacological manipulations as evidence for the role of a particular cellular component in neurovascular coupling may have overlooked the possible action of these agents on the vascular endothelium.

Implications for Earlier Models of Neurovascular Coupling

Propagation of vasodilation within the vascular endothelium provides a mechanism to explain the retrograde selection and dilation of specific diving arterioles and pial artery branches. Studies proposing a role for astrocytes in functional neurovascular coupling have focused on interactions between astrocyte end-feet and the outer surface of perivascular smooth muscle cells on diving arterioles. However, recent studies challenging the role of astrocytes in neurovascular coupling have focused on discrepancies in the timing of measurable increases in intracellular calcium in astrocytes, which appear later than the onset of dilation in adjacent to diving arterioles.¹⁷ Others have noted the lack of connectivity between astrocytes and reactive pial arteries above the glia limitans.^{17,27,51} We propose that propagated vasodilation might explain these discrepancies by removing the requirement for astrocyte control of blood flow at the level of diving arterioles.^{15,17} We note that the generally slow intracellular calcium responses observed in astrocytes may implicate astrocyte involvement in the later phases of functional hyperemia.^{15,40,52} However, we also note that a recent study suggesting that more-rapid astrocyte responses may be present around capillaries¹² might imply a direct relationship with the vascular endothelium at the capillary level.

Additional recent studies that have begun to explore the role of pericytes in the active modulation of capillary tone.^{6,13,53} Pericytes have been shown *in vitro* to relax *in response* to PGI₂, NO, and bradykinin,⁶ making them an intriguing partner for ECs, either to assist in initiation of propagated vasodilation or to receive signals *from* ECs in a similar way to arterial smooth muscle cells (Figure 5).

How Might Endothelial Signaling be Initiated in the Brain?

The involvement of endothelial signaling in functional neurovascular coupling implies that this signaling must, at some point, be initiated. In the peripheral vasculature, endothelial hyperpolarization can be initiated through activation of endothelial G-protein-coupled receptors sensitive to signaling molecules and neurotransmitters, including ACh, adenosine, adenosine triphosphate (ATP), adenosine diphosphate (ADP), uridine triphosphate, K⁺ ions, and bradyki-

nin.^{8,21,24–26} Although innervation of the microvasculature by ACh-expressing afferents has been explored,^{54,55} many other pathways might be viable candidates for initiation of endothelially driven vasodilation and could feasibly involve direct neuronal signaling to the endothelium or signaling through intermediaries, such as astrocytes, pericytes, or smooth muscle cells.^{7,9,11–13,40} These potentially neurogenic mechanisms for the initiation of functional hyperemia have not been explored to date.

Endothelial propagation also obviates the need for some extravascular network to dictate *which* penetrating arterioles should be chosen to dilate in order to increase capillary flow at a given site of initiation.^{10,17,18} The endothelium provides an integrative conduit for signaling, which would be expected to find the shortest route to larger arteries, selecting the optimal arteriolar branches to generate a localized increase in blood flow.⁵⁶ ATP-evoked endothelial propagation of vasodilation has been shown to be able to fully traverse the capillary bed from its venous to arterial side in a hamster cheek pouch model.⁵⁷ Our results therefore suggest that initiators and mediators of neurovascular coupling could be located at the capillary bed and do not necessarily need to contact the abluminal surface of penetrating arteriole smooth muscle cells.⁵⁷ Initiation at the capillary bed would also be consistent with signaling originating at the location of highest metabolic demand.

Implications for Interpretation of fMRI Studies

A primary concern in the analysis and interpretation of fMRI data is whether observed responses can be inferred to directly represent neuronal activity.² Numerous studies have noted nonlinearities in the spatiotemporal properties of the fMRI response showing, for example, invariance in response amplitude and temporal shape for stimuli ranging between 5 ms and 2 seconds.^{39,49} Several previous studies of the nonlinear properties of the fMRI response have led some to explore and model the possibility of a 2-component (or more) mechanism for neurovascular coupling, although without an explicit mechanistic basis.^{38–40,49,58}

Although more work is needed to carefully characterize the properties and dynamics of endothelium-driven vasodilation in the brain, we propose that many aspects of the BOLD response might be explained through consideration of the spatiotemporal and, possibly, threshold-dependent properties of endothelial signaling, both fast and slow.^{20,22,46} Uncovering the mechanisms initiating endothelial hyperpolarization during functional hyperemia would provide critical insight into whether responses to short (or long) duration stimuli, or to spontaneous activity in resting state fMRI, can indeed be interpreted as directly neurogenic.² The specific properties of

endothelium-dependent mechanisms could also provide a basis for the finest achievable “functional” spatial resolution of fMRI.^{59,60}

Synergy Between Vascular Control in the Brain and Body

Our findings suggest a closer relationship than previously considered between functional hemodynamic regulation in the brain and the rest of the body. Consistent with our findings, we note that spatiotemporal properties of endothelial hyperpolarization in peripheral vasculature closely match the behavior of pial arteries during functional hyperemia, including the speed and range of initial retrograde vasodilation, and the timing of the peak and return to baseline after short stimuli.^{8,18–20,22,23,25} Studies of blood flow control in skeletal muscle have already introduced the idea of an integrative vascular signaling pathway that fine-tunes local perfusion through retrograde vasodilation from capillaries (in areas of demand) to larger arterioles.^{18,60–62}

The primary studies providing evidence *against* endothelial propagation of vasodilation in neurovascular coupling have proposed that signaling from the glia limitans might mediate stimulus-evoked dilation of pial arteries.^{27,63} In Xu et al. 24-hour exposure to the gliotoxin, L- α -amino adipic acid, was found to eradicate in vivo pial arterial dilations in vivo in response to bicuculine suffusion and sciatic nerve stimulation, whereas light-dye treatment failed to significantly attenuate the same dilations. The major difference between this and our study is the intensity and duration of the stimulation used. Bicuculine suffusion induced seizure-level neuronal firing and arterial dilations of over 30% of baseline diameters over the course of up to 20 minutes (compared to approximately 6% dilations for <16 seconds in our study). The strong, direct sciatic nerve stimulation used by Xu et al. lasted for 20 seconds, but induced massive arterial dilations exceeding 35% of baseline diameters, peaking around 40 seconds after stimulus onset. Similarly, Kanu et al., who studied the influence of L- α -amino adipic acid on pial artery dilation in newborn piglets, used direct cortical application of ADP in order to induce \approx 30% increases in pial artery diameter over the course of 3 minutes.⁶³ We conclude that pial arterial dilations induced by stronger, more prolonged stimuli may be governed by a different mechanism than responses to the more physiological levels of native stimulus presented in our experiments. Other studies have explored the presence of specific connexins on cerebral vessels as evidence for endothelial involvement. Kao et al.²⁸ examined vessels in the brainstem and found little evidence of connexin 40 and 37 in the microvasculature, but noted strong expression in brainstem pial arteries and larger penetrating arterioles. However, cortical vessels were not

studied, and it should be noted that questions remain regarding the specific gap junction components involved in endothelial propagation of vasodilation.^{20,32,48} Moreover, numerous studies have independently verified the ability of cerebral vessels to conduct vasodilation within their endothelium.^{8,23–26}

Implications for Brain Health

Stimulus-evoked cortical hyperemia serves to tune local blood flow levels to the demands of active neurons. This dynamic modulation of blood flow may be as critical to maintaining normal brain function as baseline flow levels.

Impairment of neurovascular coupling has been implicated in Alzheimer’s disease and neurodegeneration.^{4–6} However, a wider range of systemic pathologies are known to involve the dysfunction of the vascular endothelium, including cardiovascular disease and diabetes, many of which have associated neural deficits that go beyond simple factors, such as cardiac output.⁶⁴ A number of studies have noted alterations in fMRI BOLD responses, and therefore possible neurovascular dysfunction, in aging,³ diabetes,⁶⁵ hypertension,⁶⁶ and microangiopathy.⁶⁷ Exploration of a link between neurovascular dysfunction and cognitive decline could provide new avenues for the development of diagnostic tools and therapeutic interventions.

We also note that the vascular endothelium is directly exposed to the bloodstream, suggesting that even drugs that do not cross the blood–brain barrier, but act on the endothelium, could influence neurovascular coupling.^{3,9} One study demonstrated that intravenous indomethacin and rofecoxib (a nonsteroidal anti-inflammatory) both cause attenuation of the cortical hemodynamic response to somatosensory stimulation.⁶⁸ The data shown are consistent with a reduction in the later slow phase of functional hyperemia, suggesting impairment of prostanoid/NO signaling, consistent with our 2-phase model. The possible involvement of multiple endothelial pathways, dependent on COX-1, COX-2, NO, and, perhaps, ACh or bradykinin, significantly increases the number of anti-inflammatory, analgesic, antihypertensive, and other drugs that could potentially influence functional neurovascular coupling. Involvement of the endothelium in neurovascular coupling provides a mechanistic basis for drug-induced alterations to the fMRI response, but also highlights the potential for systemic drugs to have either positive or negative influences on brain health through alteration or enhancement of neurovascular coupling.

In summary, our results demonstrate that an intact vascular endothelium is critical for the generation of a full hemodynamic response to stimulus in the brain. This result provides a new roadmap for understanding neurovascular coupling as well as for interpreting functional imaging signals. Our findings also highlight the arterial endothelium as a possible component to be explored in relation to brain

pathologies, such as neurodegeneration, as well as a potentially accessible therapeutic target.

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Disclosures

None.

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