Occlusion of cortical ascending venules causes blood flow decreases, reversals in flow direction, and vessel dilation in upstream capillaries

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The accumulation of small strokes has been linked to cognitive dysfunction. Although most animal models have focused on the impact of arteriole occlusions, clinical evidence indicates that venule occlusions may also be important. We used two-photon excited fluorescence microscopy to quantify changes in blood flow and vessel diameter in capillaries after occlusion of single ascending or surface cortical venules as a function of the connectivity between the measured capillary and the occluded venule. Clotting was induced by injuring the target vessel wall with femtosecond laser pulses. After an ascending venule (AV) occlusion, upstream capillaries showed decreases in blood flow speed, high rates of reversal in flow direction, and increases in vessel diameter. Surface venule occlusions produced similar effects, unless a collateral venule provided a new drain. Finally, we showed that AVs and penetrating arterioles have different nearest-neighbor spacing but capillaries branching from them have similar topology, which together predicted the severity and spatial extent of blood flow reduction after occlusion of either one. These results provide detailed insights into the widespread hemodynamic changes produced by cortical venule occlusions and may help elucidate the role of venule occlusions in the development of cognitive disorders and other brain diseases.

Keywords: collateral flow; hemodynamics; nonlinear microscopy; stroke; vasoregulation

Introduction

There is increasing evidence that suggests cerebral venous insufficiency may have a role in many brain diseases. Small ischemic lesions in the cortical gray matter and in the periventricular and subcortical white matter have been linked to the development of cognitive dysfunction (Kovari et al, 2004; Mok et al, 2004; Vermeer et al, 2003). These lesions, which have previously been attributed to arteriolar clots or hemorrhages (Fisher, 1965), have recently also been found to correlate with venule pathology and occlusion (Black et al, 2009; Moody et al, 1995). Furthermore, venular occlusions have long been believed to have either a primary or secondary role in multiple sclerosis (Putnam, 1937; Singh and Zamboni, 2009). However, due in part to limitations of existing animal models of microvascular occlusion, very little is known about the impact of venule occlusions on cerebral hemodynamics.

Previous studies have focused on blood flow deficits resulting from thrombosis in large cerebral veins. Occlusion of the superior sagittal sinus (Ungersbock et al, 1993) and its bridging veins (Nakase et al, 1997a,b) in animal models caused severe blood flow reductions in underlying capillaries and resulted in ischemic infarction. Additional studies of occlusion of the superior sagittal sinus have shown that blood flow reductions were not as severe when more bridging veins were present, suggesting that vascular topology may be critical for blood flow redistribution (Ueda et al, 2000). Although these studies provide insights into the effects of thrombosis of larger-surface veins, the techniques used for blocking these large vessels do not translate to the production of clots in smaller (<50 μm diameter) surface venules (SVs) or
in ascending venules (AVs). The recent finding that femtosecond laser ablation (Vogel et al, 2005) can be used to initiate clotting in individual vessels has permitted targeted occlusion of single cortical penetrating arterioles (PAs) (Nishimura et al, 2007, 2010) and capillaries (Nishimura et al, 2006). This nonlinear optical technique is an ideal tool for producing occlusions in targeted cortical venules to investigate whether small venules in the cerebral vasculature lead to physiologically important blood flow deficits.

In this study, we quantified the effects of single venules occlusions on blood flow in the rat neocortex. We used femtosecond laser ablation to damage the endothelium and induce clotting in targeted venules and used two-photon excited fluorescence (2PEF) microscopy to investigate the resulting blood flow and vessel diameter changes as a function of both topological separation and spatial distance from the occluded vessel. We found that occlusion of a single AV caused dramatic decreases in blood flow and increases in vessel diameter in capillaries that were upstream from the targeted venule. Similar results were observed after the occlusion of a SV, but flow decreases were almost eliminated when a surface collateral vessel that provided a new drain was present. We mapped the cortical microvascular topology in 600–500 μm-sized volume and used these data to predict the spatial extent and severity of blood flow reduction after an AV occlusion and found these predictions to agree well with in vivo measurements. These results highlight the dominant influence of vascular topology in determining blood flow rearrangements that result from a vascular occlusion and provide the first comprehensive, quantitative picture of blood flow rearrangements that result from venular occlusions in the neocortex.

**Materials and methods**

**Animals and Surgical Procedures**

Experiments were performed on 27 male Sprague Dawley rats (Harlan Inc., South Easton, MA, USA) ranging from 265 to 375 g in mass. Ascending venule occlusions were induced in 16 animals, SV occlusions were induced in 8 animals (with collateral vessel: 3, no collateral: 5), and the rest were used in sham experiments. Only one venule was occluded in each animal. All rats were anesthetized with urethane (1.5 g/kg rat), and glycopyrrolate (0.5 mg/kg rat) was administered intramuscularly to help prevent secre-

body temperature was kept at 37°C using a heating blanket controlled by a rectal thermometer (50–7053; Harvard Apparatus, Holliston, MA, USA). Heart rate and arterial oxygen saturation were monitored using a pulse oximeter (MouseOx; Starr Life Sciences Corp., Oakmont, PA, USA) clipped to the rat’s hind paw. Hydration was maintained with hourly subcutaneous injections of 5% glucose (wt/vol) in saline (1 mL/kg rat). Physiologic parameters were manually recorded every 20 minutes to ensure that the physiologic state of the rat was stable.

Craniotomies were performed to gain optical access to the brain. A subcutaneous injection 0.1 mL bupivicaine (0.125% wt/vol in deionized water) was administered to reduce pain at the incision site. A ~3 × 6 mm2 craniotomy was made over the parietal cortex and the dura was removed. The brain was then covered in 1.5% agarose (A9793; Sigma, St Louis, MO, USA) in artificial cerebral spinal fluid (Kleinfeld and Delaney, 1996), and an 8-mm diameter no. 1.5 glass coverslip (50201; World Precision Instruments, Sarasota, FL, USA) was placed over the craniotomy and sealed using dental cement (Co-Oral-It Dental Mfg Co.). To visualize the vasculature using 2PEF microscopy, we intravenously injected ~0.3 mL of 5% (wt/vol) 2-MDa fluorescein-conjugated dextran (FD2000S; Sigma) in saline. All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee of Cornell University.

**Two-Photon Imaging of the Cortical Vasculature**

*In vivo* images were obtained using a custom-designed 2PEF microscope that used a low-energy, 100-fs, 800-nm, 76-MHz repetition rate pulse train generated by a Ti:sapphire oscillator (Mira-HP; Coherent Inc., Santa Clara, CA, USA) that was pumped by a continuous wave diode-pumped solid state laser (Verdi-V18; Coherent Inc.). Data acquisition and laser scanning were controlled using MPSCOPE software (Nguyen et al, 2006). Fluorescence was detected through an interference filter centered at 517 nm with a 65-nm bandpass. A 0.28 numerical aperture, ×4 magnification, air objective (Olympus, Center Valley, PA, USA) was used to obtain images of the entire cranial window. In these large-area 2PEF images, surface vessels were identified and large venules were followed upstream to identify candidate AVs and SVs for occlusion (Figure 1A). A 0.95 numerical aperture, ×20 magnification, water-immersion objective (Olympus) was used for high-resolution imaging (Figure 1B), measurements of vessel diameter, and blood flow speed (Figure 1C), and vessel occlusion (Figure 2).

The vasculature surrounding the target venule was mapped with 1-μm-spaced image stacks. These high-resolution image stacks were used to map the topological relationship and spatial distance between measured vessels and the targeted venule (Figure 1B). Diameters were measured by averaging at least 10 movie frames and manually selecting a segment of the vessel. The selected region was then thresholded (20% of maximum intensity), and the average width across the length of the selected segment was calculated as the vessel diameter (Figure 1C1). Centerline red blood cell (RBC) velocities were obtained by tracking the motion of RBCs, which do not take up the intravenously injected dye and show up as dark patches inside the vessel (Figure 1C2). Repetitive linescans along the axis of the vessel, at a rate of 1.7 kHz for ~1 minute, were used to form a space-time image in which moving RBCs produce streaks with a slope that is equal to the inverse of the speed (Figure 1C3; characteristic diameter...
and RBC flow speed of all measured vessels shown in Supplementary Figure 1). The slope was calculated using an automated image-processing algorithm (Schaffer et al., 2006). Measured parameters were then mapped to vascular networks that included the targeted venule (Figure 1D).

Vessel Identification

Ascending venules were defined as vertically oriented, subsurface vessels that transported blood from the capillary bed to the brain surface, as confirmed through measurement of blood flow direction. Vessels directly downstream from AVs and on the brain surface were defined as SVs. Surface arterioles, which form a net-like network on the brain surface, as well as PAs, which transport blood vertically into the brain, were also identified (Figure 1A). The PAs fed an extensive network of capillaries that eventually connected to and drained into AVs. We defined all subsurface vessels between a PA and an AV as capillaries. We determined the dependence of blood flow and vessel diameter changes after venule occlusion on topological separation by counting the number of vessel branches between a measured vessel and the occluded venule. Measured capillaries that could not be traced back to the targeted venule were grouped as ‘not connected’.

For this study, we occluded individual AVs and SVs. For SV experiments, we found two topologically distinct vascular patterns for SVs immediately upstream from AVs. Both patterns were composed of two AVs that merged into one SV, forming a ‘Y’-shaped architecture (outlined in blue; Figures 5A and 5B). The two patterns were distinguished by the presence or absence of a ‘collateral’
SV that connected to another SV (purple line and dashed blue lines, respectively; Figure 5A). In both topologies, occlusions were induced in the merged venule (red circles; Figures 5A and 5B) and vessel diameter and blood flow measurements were made in upstream SVs and capillaries.

**Targeted Clotting of Venules by Femtosecond Laser Ablation**

Femtosecond laser pulses were tightly focused on the wall of the targeted vessel (characteristic diameter and RBC flow speed of target venules shown in Supplementary Figure 2), causing nonlinear absorption of laser energy that drives photodisruptive damage (Nishimura et al., 2006). This injury initiated the natural clotting cascade, forming a clot that stopped blood flow in the targeted venule (Figure 2A).

As nonlinear absorption occurred only at the focus at which laser intensity was the highest, photodisruption was confined to the focal volume, leaving the surrounding vessels and tissues intact. Venules were irradiated with a 50-fs, 800-nm, 1-kHz pulse train produced by a Ti:sapphire regenerative amplifier (Legend 1k USP; Coherent Inc.) pumped by a Q-switched laser (Evolution 15; Coherent Inc.) and seeded by a Ti:sapphire oscillator (Chinhook Ti:sapphire laser; Kapteyn-Murnane Laboratories Inc., Boulder, CO, USA, pumped by Verdi-V6; Coherent Inc.). Both 2PEF imaging and ablation laser beams were combined along the same beam path using a polarizer beam splitter within the custom 2PEF microscope and focused to the same plane, allowing for simultaneous imaging and ablation (Nishimura et al., 2006). Pulse energy for ablation was varied with neutral density filters, and the number of pulses focused in the vessel lumen was varied.

**Figure 2** Targeted occlusion of cortical ascending venules through clotting initiated by femtosecond laser photodisruption. (A) Schematic of venule clotting process. (B) Example of an AV occlusion. Gray planes in schematics on the left indicate the imaging plane, with the red ‘X’ indicating the location of the targeted ablation in the AV. Images on the right show a time-lapse series of the surface segment (top) and the 20-μm deep ascending segment (bottom) of the target vessel during clotting. This example shows clotting of an AV by ablation of the ascending segment of the vessel, approximately halfway between the first branching capillary and the brain surface. At baseline, flow of red blood cells was evident in the ascending and surface segments of the targeted venule. The vessel wall of the ascending segment was irradiated with 100-nJ pulses, until we observed extravasation of fluorescently labeled blood plasma, indicating that the vessel wall was injured (15 minutes). Further damage was induced along the vessel wall until a clot formed and complete cessation of red blood cell motion in the ascending and surface segments was observed (45 minutes). Nearby surface vessels were not clotted and remained flowing. AV, ascending venule.
controlled using a mechanical shutter with a 2-millisecond minimum opening time (VMM-D4: Uniblitz, Rochester, NY, USA). For AV occlusions, the targeted segment was always located above the first branching upstream capillary and below the brain surface, at a depth of several tens of micrometers. Initially, venules were irradiated with a single pulse with energy below the expected damage threshold (~100 nJ at a depth of ~50 μm). The number of pulses was then increased from a single pulse to 1,000 pulses, by increments of 100, until damage was observed, which was indicated by extravasation of fluorescently labeled plasma outside the vessel. If no injury was observed, the energy was increased by ~25% and the process was repeated. Once extravasation occurred, the vessel was irradiated at multiple locations along the lumen until the motion of RBCs ceased and an accumulation of RBCs in the targeted vessel segment was observed (Figure 2). The procedure for occluding SVs was similar, except that the initial laser energies used were lower because the target vessel was located on the brain surface.

Characterization of Capillary Topology and Prediction of Spatial Dependence of Blood Flow Change After Penetrating Arteriole or Ascending Venule Occlusion

Two-photon excited fluorescence image stacks from six additional rats were used to quantify the locations of AVs and PAs over ~4 mm² of brain surface (Figure 6A) and determine the nearest-neighbor separations (Supplementary Figure 7). In three animals, we traced the connectivity of all vessels within a ~0.1-mm³ volume of brain tissue using custom software (Figure 6B). From these data, we quantified the distance between capillary segments and the nearest AV (2,153 capillaries) or PA (2,203 capillaries) as a function of the number of branches upstream or downstream from the capillary was from the AV or PA (Figure 6C). We then determined the distribution of capillary branch numbers as a function of distance from PAs and AVs in 50-μm radial bins. We used in vivo flow change data from this study for AV occlusions and from previous work for PA occlusions (Nishimura et al. 2010) to determine the average blood flow decrease in different capillary branches stoming from an occluded AV or PA. Combining both the capillary branch number distribution (Figure 6C) and this flow change data, we predicted the postclot blood flow speed change as a function of distance from occluded AVs and PAs (Figure 6D). A step-by-step overview of the procedure for generating data in Figure 6D is given in the Supplementary Text.

Statistical Analysis

Boxplots were generated with Matlab (The Mathworks, Natick, MA, USA) using the boxplot function (Figures 4A, 4E, 5C, and 5E). The dependence of median RBC flow speed change (Figure 4B), reversal rate (Figure 4D), and mean vessel dilation (Figure 4F) in capillaries as a function of distance from occluded AVs were determined using a variable-width spatial window that was constrained to contain 20 data points. Trend lines were further smoothed with a 20-μm moving average and a running 95% confidence interval about the trend line was calculated. Differences were considered significant for P-values < 0.05. For nonparametric data, rank-based analysis was conducted using Dwass–Steel–Critchlow–Fligner (StatsDirect, Altrincham, Cheshire, UK) multiple comparisons test (Figures 4A and 5C), whereas normally distributed data were analyzed using the Tukey–Kramer (StatsDirect) multiple comparisons test (Figures 4E, 5E, and 7B). For binomial data, statistical differences were calculated using a binomial proportions test with a Bonferroni adjustment (Figures 4C and 5D). Medians are reported for nonparametric data, whereas means are reported for normal data.

Results

We used 2PEF imaging of fluorescently labeled blood plasma in anesthetized rats to map vascular topology and quantify blood flow speed and vessel diameter in surface vessel and capillary networks near AVs and SVs that were candidates for occlusion (Figure 1). Irradiation of the targeted venules with high-energy, tightly focused, femtosecond laser pulses caused localized vascular injury, leading to extravasation of fluorescently labeled plasma and RBCs and triggering clotting of the vessel (Figure 2). Nearby surface vessels and capillaries were not clotted and remained flowing. We then measured the changes in diameter and RBC centerline velocity that resulted from venule occlusion in individual vessels throughout the nearby vascular network (Figure 3, Supplementary Figures 3 and 4).

Ascending Venule Occlusions Caused Decreased Blood Flow Speeds in Upstream Capillaries

To understand the impact of AV occlusions on blood flow in nearby and connected blood vessels, we investigated how changes in blood flow speed depend on the topological connection and spatial distance between individual vessels and the occluded venule. The RBC speed in capillaries that were one to four branches upstream from the clotted venule decreased dramatically, with flow speeds of only 6% (16%) of the baseline value for vessels one (two) branch(es) upstream from the occlusion (Figure 4A). The average speed did not change in capillaries that were more than four branches upstream or not connected to the occluded venule, although vessels five to seven branches upstream showed a 9.5 times increased variance in speed after the occlusion when compared with sham experiments (Figure 4A; P < 1.0E-7, F-test). Surface venules downstream from the occluded venule slowed, whereas parallel SVs (i.e., vessels that drained into the same venule as the clotted vessel) did not change speed (Figure 4A). The median RBC speed in measured capillaries located within 100 μm of the occluded venule decreased to ~26% of the baseline value, and returned to baseline with increasing distance from the occluded vessel (Figure 4B).
Blood Flow Directions Reversed in Many Upstream Capillaries After Ascending Venule Occlusions

In addition to changes in blood flow speed, we observed dramatic changes in the routing of blood flow through the capillary bed after AV occlusion. We frequently observed a reversal in blood flow direction in capillaries one to four branches upstream from the occluded venule, with just over half of the first and second upstream branches reversing flow direction (Figure 4C). Neither capillaries not connected to the occluded venule nor downstream or parallel SVs reversed flow direction (Figure 4C). The number of blood flow reversals in capillaries decreased with increasing distance from the occluded venule, with no flow reversals observed >650 μm away from the target vessel (Figure 4D).

The Diameter of Upstream Capillaries Increased After Ascending Venule Occlusions

We further examined the impact of AV occlusions on the diameter of nearby and connected vessels. We found that capillaries up to three branches upstream from the targeted venule dilated after the clot as compared with sham experiments, with an average diameter increase of ~25% (Figure 4E). Capillaries further upstream or not connected to the targeted vessel, as well as downstream and parallel SVs, did not dilate significantly (Figure 4E). The amount of dilation decreased with increasing distance from the clot, with no dilation observed at distances >200 μm (Figure 4F).

Collateral Surface Venules, When Present, Helped Maintain Normal Blood Flow After Surface Venule Occlusions

We also examined the blood flow changes caused by occlusion of SVs in vascular topologies that had (Figure 5A; Supplementary Figure 5) and did not have (Figure 5B; Supplementary Figure 6) a collateral SV. On average, blood flow speeds in capillaries one and two branches upstream from the AVs...
were unaffected by a SV occlusion when a collateral vessel was present, whereas flow was reduced to an average of 12% of the baseline speed when no collateral vessel was present (Figure 5C). Blood flow reversals in the first two upstream capillaries occurred only when no collateral vessel was present (Figure 5D). Diameters increased in the first upstream capillary branch only when no collateral vessel was present, whereas the second upstream capillary branch dilated after a SV occlusion both with and without a collateral (Figure 5E).

**Topological Architecture had a Large Role in Determining Blood Flow Changes**

To gain insights into the role of vascular topology in determining blood flow rearrangements after an occlusion, we used in vivo imaging to map the vascular topology over ~0.1 mm³ volumes in the cortex and combined this with topology-dependent measurements of RBC speed change after vascular occlusion to predict the spatial extent and severity of blood flow decrease. We found that the number of AVs outnumbered PAs by a factor of 1.8 (Figure 6A;
Supplementary Figure 7; 6 animals, 360 PAs to 201 AVs). We used tracings of all vessels (Figure 6B; 4,256 capillaries in 3 animals) to determine the three-dimensional spatial location of each capillary as a function of topological connectivity to the nearest AV or PA. The average distance of capillaries from an AV or PA as a function of the number of branches upstream or downstream, respectively, was comparable (Figure 6C), suggesting a similarity in the capillary branching topology away from AVs and PAs. We then used in vivo measurements of capillary flow speed decreases after AV and PA occlusions as a function of topological separation from Figure 4A and from the study by Nishimura et al (2010), respectively, to calculate the postclot flow speed changes in capillaries as a function of distance from the occluded vessel, weighted according to the topological data from Figure 6C. This calculation predicted both the spatial extent and the severity of the blood flow deficit to be smaller after occlusion of a single AV as compared with a PA (Figure 6D), in agreement with the experimental data from Figure 4B and previous work (Nishimura et al, 2007, 2010).

**Discussion**

We examined vessel-by-vessel hemodynamics after cortical venule occlusions using nonlinear optical techniques. Measurements of centerline RBC speed and direction, vessel diameter, and topological...
connectivity of individual vessels were obtained using 2PEF microscopy and femtosecond laser ablation was used to trigger clotting in targeted AVs and SVs. Vessels were measured before and after the occlusion to determine topologically and spatially dependent changes in RBC speed and direction, as well as in vessel diameter. Capillaries immediately upstream from the target venule experienced a dramatic decrease in RBC speed, reversals in blood flow direction, and an increase in diameter, with each parameter recovering to baseline values in vessels further upstream from the clotted venule. Similar behaviors were observed in upstream capillaries after SV occlusions in topologies that did not contain a collateral vessel, whereas blood flow deficits were alleviated with the presence of a surface collateral venule. Furthermore, we studied the density of AVs and PAs, as well as the topology of the capillaries that branch from them and used these values to predict differences in the spatial extent and severity of the blood flow decreases that result from AV and PA occlusions. These observations revealed the blood flow impact, both as a function of vascular topology and distance through the cortex, associated with cortical venule occlusions.

**Nonlinear Optical Techniques Enable the Creation of Robust Models of Small Cortical Venule Occlusion**

The ability to measure cerebral blood flow during normal and pathologic states is critical for the development of an animal model of venule stroke. Most previous work on cortical venule occlusions has used laser Doppler flowmetry to characterize flow changes that result from occlusion of cortical veins with \( \sim 0.1 \) to 1 mm diameter (Nakase et al, 1997a, b; Ungersbock et al, 1993; Ueda et al, 2000). Although this approach allows for mapping of regional blood flow changes resulting from large vessel occlusions, it would tend to average over the effects associated with occlusions in vessels with \( \sim 10 \) to 50 \( \mu \)m diameter, such as those considered in this study. Quantitative autoradiography has also been used to map tissue perfusion deficits after superior sagittal sinus occlusion (Kurokawa et al, 1990), but this method does not enable the in vivo measurements that could, e.g., connect vascular topology to blood flow change. In this study, we used 2PEF microscopy to characterize, at the vessel-by-vessel level, changes in blood flow and diameter in vessels located deep within the cortical tissue after venule occlusion. Although it is difficult to
characterize a large number of vessels in one experiment with this approach, the limited topological and spatial extent of blood flow rearrangements we observed after AV and SV occlusion suggest that 2PEF imaging is well suited to the study of these microvessel occlusions (Zhang and Murphy, 2007). Other methods that enable simultaneous measurement of blood flow changes across many surface or penetrating/ascending vessels, such as Doppler optical coherence tomography (Srinivasan et al, 2011) or advanced intrinsic optical imaging (Chen et al, 2011), could complement the approach taken here.

Conventional methods for producing vascular occlusions are not well suited to producing occlusions in small venules. Mechanical ligation approaches are limited to large vessels, such as the superior sagittal sinus (Frerichs et al, 1994). Optical methods relying on linear excitation of an intravenously injected photosensitizer (Watson et al, 1985) potentially provide higher precision (Nakase et al, 1997a), but thrombus formation is difficult to localize to a single vessel. Embolus injection models have been used to occlude smaller vessels (Miyake et al, 1993), but this technique is inappropriate for venule occlusions, as the emboli will block arterioles and capillaries before ever reaching the venous system. In this study, we tightly focused high-energy, femtosecond laser pulses onto the vessel lumen to drive nonlinear absorption of the laser energy at the focus, thereby inducing an injury that is confined within the focal volume (Nishimura et al, 2006). This injury triggers clotting in the target vessel, while leaving nearby vessels and the surrounding brain tissue intact (see Supplementary Movie 1). Overall, this approach provides a robust animal model of cortical venule occlusions that allows precise and controlled clotting of single venules at and below the brain surface with minimal collateral damage.

**Topology is a Dominant Factor in the Redistribution of Blood After Vascular Occlusion**

Vascular topology has a crucial role in redistributing blood flow after a cortical vessel clot. Occlusion of communicating surface arterioles does not lead to severe decreases in flow in downstream arterioles and capillaries because the vascular anastomoses within the surface arteriole network provide collateral flow (Blinder et al, 2010; Schaffer et al, 2006). Similarly, after occlusion of a SV with a collateral vessel, we find that blood flow changes in upstream capillaries are minimized, whereas topologies that do not contain a surface collateral venule result in severe blood flow decreases. These studies highlight the important role of redundancy in both the arterial and the venular networks in maintaining flow after surface vessel occlusions. In contrast, occlusions in vessels with topologies that contain no collateral pathways, such as PAs and AVs, lead to flow decreases in the underlying capillaries. Clotting of a PA results in severe decreases in blood flow in downstream capillaries to at least seven branches downstream from the targeted vessel (Nishimura et al, 2010), whereas blood flow is seen to recover after the fourth branch upstream after an AV occlusion. The difference in the topological distance through the capillary network at which blood flow recovers after a PA or AV occlusion can be attributed to the fact that PAs are outnumbered by AVs almost two to one. As the capillary branching pattern is similar for both vessel types, this difference in density, and therefore in distance between two PAs or two AVs (Supplementary Figure 7), suggests that the number of capillary branches between two PAs is greater than the number of branches between two AVs. As a result, blood flow deficits in capillaries downstream from an occluded PA will extend further into the capillary network before another nearby PA is able to effectively provide alternatively sourced blood flow. In contrast, because two AVs are closer, blood in capillaries upstream from an occluded AV requires fewer branches to reach an alternative AV, resulting in less severe blood flow deficits. To further support this idea, we performed a topology-weighted analysis of capillary flow changes as a function of distance from an occluded vessel, which showed that the spatial extent and severity of blood flow decrease was less dramatic after an AV occlusion as compared with a PA occlusion. Taken together, these results not only implicate the role of vascular topology and connectivity in the redistribution of blood flow but also highlight the importance of the occlusion location within the vascular hierarchy.

**Capillary Dilations are a Passive Process After an Ascending Venule Occlusion**

One of the key findings in this study is the large dilation in upstream capillaries after occlusion of an AV. One question is whether the observed dilation is an active or passive process. Previous anatomic studies of the cerebral microvasculature have established the lack of smooth muscle cells in the capillaries upstream from AVs (Rhodin, 1968), suggesting that capillaries do not actively modulate their diameter. This leads to the hypothesis that the capillary dilations we observe are a passive process, induced by an increase in intravascular pressure, caused by the fact that after the venule occlusion, the immediately upstream capillaries are now topologically further away from a low pressure draining venule. To model this scenario, we considered the capillary as a tube embedded in a matrix of brain tissue (Fung et al, 1966), with a Young’s modulus, $E$, of 1.5 kPa and a Poisson’s ratio, $v$, of 0.5 (Gefen et al, 2003). The change in vessel radius, $R$, is then linked to the change in intravascular pressure, $P$, by

$$P_c - P_o = \left(\frac{E}{1 + v}\right) \left(\frac{R_c - R_o}{R_o}\right)$$

where $P_c$ and $R_c$ are postclot and $P_o$ and $R_o$ the baseline pressure and radius, respectively. Using
equation (1), we calculated the pressure increase required to induce a dilation that agrees with our experimental measurements (Figure 7). The 5% to 10% increase in intravascular pressure we calculate compares well with the pressure difference measured between capillary beds and the first draining venules in cat mesentery (Lipowsky, 2005). However, we note that pericytes have been shown to actively modulate the diameter of capillaries (Peppiatt et al., 2006), and their involvement in the diameter changes observed here cannot be ruled out, although the magnitude of the dilation we observed is larger than the typical diameter changes associated with pericyte activation (Fernandez-Klett et al., 2010). Indeed, previous work showed capillaries downstream from an occluded PA dilate (Nishimura et al., 2010), presumably an active process, with diameter changes smaller than those observed here.

Blood Flow Decreases After Small Venule Strokes may be Damaging to Brain Cells

The extensive blood flow and vascular changes we observed after occlusion of single cortical AVs and SVs suggest that these occlusions may lead to dysfunction in brain cells. Previous work has shown that occlusion of large cortical veins leads to regional reductions in blood flow that causes ischemic infarction (Nakase et al., 1997b). We found that blood flow decreased to ~20% of baseline values within 100 μm of occluded AVs, which is at the level at which cellular inhibition of protein synthesis and energy failure begins to occur (Mies et al., 1991). These blood flow reductions are not sufficient to cause acute cell death, but, if protracted in time, may lead to brain dysfunction (Iadecola, 2010). This conclusion is supported by studies in the intestinal mesenteric circulation showing that venular occlusion lead to parenchymal cell death that increased with time (Takase et al., 1999). Furthermore, changes in vascular wall shear stress caused by changes in flow speed and direction could promote inflammation and oxidative stress in endothelial cells (Harrison et al., 2006). Finally, increased intravascular capillary pressures may lead to increased blood–brain barrier permeability (Mayhan et al., 1986), which can cause brain edema and inflammation (Mayhan et al., 1986; Rosenberg, 1999). Venous insufficiency has been associated with an increasing number of brain pathologies, ranging from multiple sclerosis to dementia (Black et al., 2009; Moody et al., 1995; Singh and Zamboni, 2009). Although the role of venous alterations in the pathogenesis of these conditions remains uncertain, these results show that venular occlusions have profound and widespread effects on brain microcirculation, highlighting the pathogenic potential of venous insufficiency.

Disclosure/conflict of interest

The authors declare no conflict of interest.

References

Black S, Gao F, Bilbao J (2009) Understanding white matter disease: imaging-pathological correlations in vascular cognitive impairment. Stroke 40:S48–52
Blinder P, Shih AY, Rafie C, Kleinfeld D (2010) Topological basis for the robust distribution of blood to rodent neocortex. Proc Natl Acad Sci USA 107:12670–5
Chen BR, Bouchard MB, McCaslin AF, Burgess SA, Hillman EM (2011) High-speed vascular dynamics of the hemodynamic response. Neuroimage 54:1021–30
Clendenon JL, Phillips CL, Sandoval RM, Fang SF, Dunn KW (2002) Voxx: a PC-based, near-real-time volume rendering system for biological microscopy. Am J Physiol-Cell Ph 282:C213–8
Fernandez-Klett F, Offenhauser N, Dirmagl U, Priller J, Lindauer U (2010) Pericytes in capillaries are contractile in vivo, but arterioles mediate functional hyperemia in the mouse brain. Proc Natl Acad Sci USA 107:22290–5
Fisher CM (1965) Lacunes: small, deep cerebral infarcts. Neurology 15:774–84
Frerichs KU, Deckert M, Kempski O, Schurer L, Einhaupl K, Baethmann A (1994) Cerebral sinus and venous thrombosis in rats induces long-term deficits in brain-function and morphology—evidence for a cytotoxic genesis. J Cereb Blood Flow Metab 14:289–300

Fung YC, Zweifach BW, Intaglie M (1966) Elastic environment of capillary bed. Circ Res 19:441–61

Gefen A, Gefen N, Zhu QL, Raghupehati R, Margulies SS (2003) Age-dependent changes in material properties of the brain and braincase of the rat. J Neurosci Res 70:365–77

Harrison DG, Widder J, Grumbach I, Chen W, Weber M, Searles C (2006) Endothelial mechanotransduction, nitric oxide and vascular inflammation. J Intern Med 259:351–63

Iadecola C (2010) The overlap between neurodegenerative and vascular factors in the pathogenesis of dementia. Acta Neuropathol 120:287–96

Kleinfeld D, Delaney KR (1996) Distributed representation of vibrissal movement in the upper layers of somatosensory cortex revealed with voltage-sensitive dyes. J Comp Neurol 375:89–108

Kovari E, Gold G, Herrmann FR, Canuto A, Hof PR, Michel JP, Bouras C, Giannakopoulos P, et al. (2004) Cortical microinfarcts and demyelination significantly affect cognition in brain aging. Stroke 35:410–4

Kurokawa Y, Hashi K, Okuyama T, Uede T (1990) Regional energy-state following middle cerebral-artery occlusion assessed by laser Doppler scanning. J Neurosci Meth 30:1914–27

Lipowsky HH (2005) Microvascular rheology and hemodynamics. Microcirculation 12:5–15

Mayhan WG, Faraci FM, Heistad DD (1986) Disruption of the blood-barrier in cerebrum and brain stem during acute hypertension. Am J Physiol 251:H1171–5

Mies G, Ishimaru S, Xie Y, Seo K, Hossmann KA (1991) Sustained decrease in cerebral venous hypertension due to embolic occlusion of the superior sagittal sinus in the rat. Surg Neurol 34:390–5

Moody DM, Brown WR, Challa VR, Anderson RL (1995) Periventricular venous collagenses—association with leuкоaraisis. Radiology 194:469–76

Nakase H, Kempski OS, Heimann A, Takeshima T, Tintera J (1997a) Microcirculation after cerebral venous occlusions as assessed by laser Doppler scanning. J Neurosurg 87:307–14

Nakase H, Nagata K, Ohtsuka H, Sakaki T, Kempski O (1997b) An experimental model of intraoperative venous injury in the rat. Skull Base Surg 7:123–8

Nguyen QT, Tsai PS, Kleinfeld D (2006) MPScope: a versatile software suite for multiphoton microscopy. J Neurosci Meth 156:351–9

Nishimura N, Rosidi NL, Iadecola C, Schaffer CB (2010) Limitations of collateral flow after occlusion of a single cortical penetrating arteriole. J Cereb Blood Flow Metab 30:1914–27

Nishimura N, Schaffer CB, Friedman B, Lyden PD, Kleinfeld D (2007) Penetrating arterioles are a bottleneck in the perfusion of neocortex. Proc Natl Acad Sci USA 104:365–70

Nishimura N, Schaffer CB, Friedman B, Tsai PS, Lyden PD, Kleinfeld D (2006) Targeted insult to superficial cortical blood vessels using ultrashort laser pulses: three models of stroke. Nat Methods 3:99–108

Peppiatt CM, Howarth C, Mobbs P, Attwell D (2006) Bidirectional control of CNS capillary diameter by pericytes. Nature 443:780–4

Patel SS (1937) Lesions of ‘encephalomyelitis’ and multiple sclerosis—venous thrombosis as the primary alteration. J Am Med Assoc 108:1477–80

Rhodin JA (1968) Ultrastructure of mammalian venous capillaries, venules, and small collecting veins. J Ultrastruct Res 25:452–500

Rosenberg GA (1999) Ischemic brain edema. Prog Cardiovasc Dis 42:209–16

Schaffer CB, Friedman B, Nishimura N, Schroeder LF, Tsai PS, Ebner FF, Lyden PD, Kleinfeld D (2006) Two-photon imaging of cortical surface microvessels reveals a robust redistribution in blood flow after vascular occlusion. Plos Biol 4:258–70

Singh AV, Zamboni P (2009) Anomalous venous blood flow and iron deposition in multiple sclerosis. J Cereb Blood Flow Metab 29:1867–78

Srinivasan VJ, Atchoin DN, Radhakrishnan H, Jiang JY, Ruvinsskaya S, Wu W, Barry S, Cable AE, Ayata C, Huang PL, Boas DA (2011) Optical coherence tomography for the quantitative study of cerebrovascular physiology. J Cereb Blood Flow Metab 31:1339–45

Takase S, Delano FA, Lerond L, Bergan J, Schmid-Schonbein GW (1999) Inflammation in chronic venous insufficiency: is the problem insurmountable? J Vasc Res 36:3–10

Ueda K, Nakase H, Miyamoto K, Otsuka H, Sakaki T (2000) Impact of anatomical difference of the cerebral venous system on microcirculation in a gerbil superior sagittal sinus occlusion model. Acta Neuropathol 98:249–55

Ungersbock K, Heimann A, Kempski O (1993) Cerebral blood flow alterations in a rat model of cerebral sinus thrombosis. Stroke 24:563–9; discussion 9–70

Vogel A, Noack J, Huttman G, Palauf G (2005) Mechanisms of femtosecond laser nanosurgery of cells and tissues. Appl Phys B-Lasers O 81:1015–47

Watson BD, Dietrich WD, Busto R, Wachtel MS, Ginsberg MD (1985) Induction of reproducible brain infarction by photochemically initiated thrombosis. Ann Neurol 17:497–504

Zhang SX, Murphy TH (2007) Imaging the impact of cortical microcirculation on synaptic structure and sensory-evoked hemodynamic responses in vivo. Plos Biol 5:1152–67

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