In Vivo Imaging of the Mouse Neurovascular Unit Under Chronic Cerebral Hypoperfusion

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Background and Purpose—Proper brain function is maintained by an integrated system called the neurovascular unit (NVU) comprised cellular and acellular elements. Although the individual features of specific neurovascular components are understood, it is unknown how they respond to ischemic stress as a functional unit. Therefore, we established an in vivo imaging method and clarified the NVU response to chronic cerebral hypoperfusion.

Methods—Green mice (b-act-EGFP) with SR101 plasma labeling were used in this experiment. A closed cranial window was made over the left somatosensory cortex. To mimic chronic cerebral hypoperfusion, mice were subjected to bilateral common carotid artery stenosis operations using microcoils. In vivo real-time imaging was performed using 2-photon laser-scanning microscopy during the preoperative period, and after 1 day and 1 and 2 weeks of bilateral common carotid artery stenosis or sham operations.

Results—Our method allowed 3-dimensional observation of most of the components of the NVU, as well as dynamic capillary microcirculation. Under chronic cerebral hypoperfusion, we did not detect any structural changes of each cellular component in the NVU; however, impairment of microcirculation was detected over a prolonged period. In the pial small arteries and veins, rolling and adhesion of leukocyte were detected, more prominently in the latter. In the deep cortical capillaries, flow stagnation because of leukocyte plugging was frequently observed.

Conclusions—We established an in vivo imaging method for real-time visualization of the NVU. It seems that under chronic cerebral hypoperfusion, leukocyte activation has a critical role in microcirculation disturbance. (Stroke. 2014;45:3698-3703.)

Key Words: astrocytes ■ leukocytes ■ microcirculation ■ microscopy, fluorescence, multiphoton ■ pericytes

The neurovascular unit (NVU) is a conceptual framework that integrates responses in all cell types, including neuronal, glial, inflammatory, and vascular elements.1-5 This cell–cell integrated response is an indispensable factor used to maintain brain function and homeostasis. In fact, dysfunction of the NVU is the basis for many diseases.2–4 The concept of NVU emphasizes that maintenance of integrated cellular function is more important than just salvaging an individual cell alone. Although the intricate molecular pathway of neuronal death has been dissected in detail, the mechanisms of how the entire NVU responds to cerebral ischemia are not completely understood. Understanding this concept may provide a comprehensive framework for investigating mechanisms and therapies of ischemic brain damage.5

The NVU is a dynamic structure assembled by endothelial cells, basement membranes, perivascular astrocytes, pericytes, and neurons. Therefore, it is difficult to understand the whole structure (including the anatomic relationship between cells) and the dynamic changes that occur within a single specimen. To understand the NVU more easily, an imaging method that can detect the whole NVU component at one time in vivo should be introduced. Thus, the aim of the present experiments was (1) to establish an in vivo imaging method for the NVU, which has extraordinarily spatial and time-dependent features. A spatial feature would require in-detail, 3-dimensional (3D) observation of the intricate NVU structure, whereas a time-dependent feature would require repeated longitudinal observation to capture real-time events, such as dynamic microcirculation in capillaries and (2) to clarify the stress response of the NVU in the bilateral common carotid artery stenosis (BCAS) model of chronic cerebral hypoperfusion.

Materials and Methods

Animal Model

All procedures were performed in accordance with the guidelines for animal experimentation from the ethical committee of Mie University. Male green fluorescent protein transgenic mice (C57BL/6

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TgN [b-act-EGFP] Osb were used in this experiment (aged 9–14 weeks; Japan SLC, Inc, Shizuoka, Japan).6

Two-Photon Laser-Scanning Imaging Experiment
Mice were initially anesthetized with isoflurane (1%–2%), and a custom-made attachment device for holding the head in place was fixed to the skull. A closed cranial window was made by removing part of the skull (4 mm in diameter) over the left somatosensory cortex while leaving the dura intact, and the exposed cortex was sealed with a cover glass (Figure 1A).7 These animals were then allowed to recover from anesthesia and were housed in a cage with free access to food and water. Mice were also kept in the cage in-between scheduled experiments. For plasma labeling, sulforhodamine 101 (SR101) dissolved in saline (0.01 mol/L) was injected intraperitoneally (8 mL/kg body weight) just before beginning the imaging experiment. Each animal was placed on a custom-made apparatus under 1.2% isoflurane, and imaging was conducted with a 2-photon laser-scanning microscope (FV1000-2P; Olympus, Japan; For more detailed methodology see in the online-only Data Supplement).

Surgical Procedure of Chronic Cerebral Hypoperfusion
To replicate chronic cerebral hypoperfusion, mice were subjected to BCAS using microcoils.9 In brief, under anesthesia with 1.3% isoflurane, the common carotid arteries (CCA) were exposed through a midline cervical incision, and a microcoil (inner diameter: Rt CCA, 0.18 mm; Lt CCA, 0.16 mm) was applied to the bilateral CCA. We applied a 0.18 mm×0.16 mm combination coil (purchase from SAMINI Co, Ltd., Japan) to elicit neurovascular response as much as possible. This combination has been selected because that of 0.16 mm×0.16 mm results in many mice dying over the observation period. Sham-operated animals underwent bilateral exposure of the CCA without applying the microcoil.

Measurement of Cerebral Blood Flow
Cerebral blood flow (CBF) was determined by a laser speckle flowmetry (Omegazone, laser speckle blood flow imager, Omegawave), which obtains high-resolution 2D images in a matter of seconds, as previously described.9 Briefly, we measured CBF through the cranial window in the same physical condition in terms of anesthesia and body temperature as during 2-photon laser-scanning. A 780-nm laser semiconductor illuminated the area of interest, and light intensity was accumulated in a charge-coupled camera device and transferred to a computer for analysis. Image pixels were then analyzed to produce average perfusion values.

Experimental Design
To exclude the effect of damage from the procedure involved in creating the cranial window, mice were allowed to rest for 1 week after the surgery. Laser speckle flowmetry and 2-photon laser-scanning were then performed as part of the preoperation period (Pre). Mice were randomly assigned to a sham (n=7) or BCAS (n=7) group, and 3 to 5 days after the preoperation period, sham or BCAS operations were performed (3 mice from the Sham group and 2 mice form the BCAS group were excluded because the cranial window became dim during the observation period.). Laser speckle flowmetry and 2-photon laser-scanning were then performed 1 day after BCAS or sham operations, and repeated again after 1 and 2 weeks. Anatomic morphology of the NVU (vessel structures, astrocytes, and pericytes) and microcirculation profiles (kinetics of erythrocytes and leukocytes) were evaluated at each time point for both sham and BCAS groups (for more detailed Methods see in the online-only Data Supplement).

Statistical Analysis
Results are expressed as the mean± SD. The Mann–Whitney U test was used to evaluate differences between groups, and an ANOVA followed by a post hoc Tukey–Kramer test was used to evaluate differences over time. Two-sided P<0.05 was considered statistically significant.

Results
NVU in the Normal Brain
In the operated cranial window, we were able to observe and clearly identify small pial vessels (see Figure 1A for a representative image). We identified volumes of interest and were then produced a maximum intensity projection from the surface of the brain to an imaging depth of 550 μm (Figure 1B). We 3D reconstructed these volumes of interests and then visualized whole vascular compartments (from pial small arteries to pial small veins) as is demonstrated in Figure 1B. In addition, zoom scanning of each vessels allowed us to identify the shapes of entire vessels (Figure 1C), and the NVU compartment from arterioles to capillaries and from capillaries to postcapillary venules (Figure 1D). In the transgenic mouse, only 4 cell types including astrocytes, pericytes, leukocytes, and platelets could be detected as cells positive for green fluorescent protein fluorescence under 2-photon laser-scanning microscope; therefore, each cell type could be identified based on their morphology (Methods and Figure I–III in the online-only Data Supplement).6,11 Thus, astrocytes,
which extend their foot processes around the arterioles and capillaries, could be clearly identified (Figure 1D, arrows), and pericytes, which wrap around the capillaries, could also be clearly observed (Figure 1D, arrowheads). Plasma labeling with SR101 allowed us to visualize erythrocytes, which were detected as nonfluorescent particles within the plasma stream. The kinetics of erythrocytes in microcirculation could be detected dynamically by repeated volume scanning (Movie I in the online-only Data Supplement).

**Regional CBF Under Chronic Cerebral Hypoperfusion**

Figure 2 shows the mean CBF values, as measured by laser speckle flowmetry, in the both sham and BCAS groups (Figure 2A). In the sham group, CBF was not changed significantly throughout the entire observation period. In contrast, CBF values after the BCAS operation were significantly decreased when compared with those of preoperative baselines and sham controls (Figure 2B).

Figure 3 shows the inflow (pial artery) and outflow (pial vein) velocity changes in the 2 groups. Specifically, we measured the slope of line scan images of pial arteries (Figure 3A) and veins (Figure 3B) to indicate inflow and outflow velocity, respectively, at each time point. In the sham group, inflow velocity was not changed significantly throughout the entire observation period. In contrast, the inflow velocity after BCAS operation was significantly decreased when compared with those of preoperative baselines and sham controls (Figure 3C). Similarly, outflow velocity was not changed in the sham group, and significantly decreased after BCAS operation when compared with those of preoperative baselines and sham controls (Figure 3D).

**Anatomic Morphology Under Chronic Cerebral Hypoperfusion**

Under chronic cerebral hypoperfusion, the small pial vessels and the deep cortical capillaries did not show obvious morphological changes such as vascular loss or angiogenesis throughout the entire observation period (Figure 4A). Vascular shape such as tortuosity and size for arterioles (Figure 4B) and venules was not changed throughout the observation period, and obvious changes of wall thickness for arterioles (Figure 4B, Lt lower inset) and venules were also not detected. Although the arteriolar lumen (Figure 4B, Rt lower inset) slightly increased in post-BCAS operation, there was no significant difference in luminal area over time (1 day, 119.9±49.5%; 1 week, 102.2±14.1%; 2 weeks, 104.9±23.3% compared with the preoperative baseline). The venular lumen in the BCAS group also remained unchanged throughout the entire observation period (1 day, 95.2±12.5%; 1 week, 95±12.4%; 2 weeks, 106±15.1% compared with the preoperative baseline). In the sham group, wall thickness and luminal area did not show obvious changes throughout the observation period. Figure 4C show that astrocytes extended their foot process around the vessels but showed no obvious morphological differences between pre- and post-BCAS operative periods. In addition, pericytes were found to wrap around capillaries at the both periods with no morphological changes. Taken together, astrocytes and pericytes remained unchanged morphologically between BCAS and sham groups during the entire observation period.

**Microcirculation in Deep Cortical Capillaries Under Chronic Cerebral Hypoperfusion**

Figure 5A and Movies II to V in the online-only Data Supplement show representative microcirculation in the deep cortical capillaries under chronic cerebral hypoperfusion. Under low magnification, stagnation of capillary flow, which was detected as high fluorescent plasma segments without nonfluorescent erythrocytes (Figure 5A, white arrows; Movie III, in the online-only Data Supplement), was observed under chronic cerebral hypoperfusion, whereas it was rarely found under

![Figure 2](http://stroke.ahajournals.org/)

**Figure 2. Temporal profile of cerebral blood flow (CBF) in the sham-operated and bilateral common carotid artery stenosis (BCAS) mice.** Mean CBF was analyzed by laser speckle flowmetry through the same cranial window as was used for 2-photon laser-scanning. The circle indicates the cranial window. A, CBF changes in the sham and BCAS group. The line graph (B) indicates CBF changes in the sham (◼) and BCAS (▲) groups. Data are presented as means±SD. *P<0.05 vs sham value. #P<0.05 between different time points. 1D indicates 1 day; 1W, 1 week; and 2W, 2 weeks.

![Figure 3](http://stroke.ahajournals.org/)

**Figure 3.** Inflow velocity of the pial arteries and outflow velocity of the pial veins analyzed by repetitive line-scans. A, Inflow velocity of the pial artery. The white line in a reference image indicates the central longitudinal axis of a selected small pial artery. Slopes of the lines in line-scan images indicate the inflow velocity. Line graphs (C) indicate arterial inflow changes in the sham (◼) and bilateral common carotid artery stenosis (BCAS; ▲) groups. B, Outflow velocity of the pial vein. The line in a reference image indicates the central longitudinal axis of a selected small pial vein. Slopes of the lines in line-scan images indicate the outflow velocity. Line graphs (D) indicate venous outflow changes in the sham (◼) and BCAS (▲) groups. Data are presented as means±SD. *P<0.05 vs sham value. #P<0.05 between different time points. 1D indicates 1 day; 1W, 1 week; and 2W, 2 weeks.

![Figure 5](http://stroke.ahajournals.org/)

**Figure 5** shows that astrocytes extended their foot process around the vessels but showed no obvious morphological differences between pre- and post-BCAS operative periods. In addition, pericytes were found to wrap around capillaries at the both periods with no morphological changes. Taken together, astrocytes and pericytes remained unchanged morphologically between BCAS and sham groups during the entire observation period.
normal microcirculation (Movie II in the online-only Data Supplement). We also observed leukocyte plugging (Figure 5A, blue arrow) in the high fluorescent plasma stagnated capillary (Figure 5A, blue arrowheads) under higher magnification (Movies IV and V in the online-only Data Supplement). Plugged leukocytes did not tightly obstruct capillaries, but slowly moved downstream (Figure 5A, purple arrows) and eventually were released (plugging time was usually <120 s), resulting in reflow of plasma streams with erythrocytes (Figure 5A, yellow arrow). Long-term and complete leukocyte plugging, as well as leukocyte infiltration, in the perivascular parenchyma could not be identified during the observation period. On the contrary, platelet activation (Methods and Figure III in the online-only Data Supplement), which is a pivotal mechanism of focal cerebral ischemia, could not be found during the observation period. Frequency of capillary flow stagnation in the BCAS group was markedly higher than that of the sham group throughout the entire observation period (Figure 5B and 5C).

Leukocyte Rolling and Adhesion in the Cortical Vessels Under Chronic Cerebral Hypoperfusion

In the sham-operated group, rolling and adhesion were rarely detected throughout the entire observation period (Figure 6A). After BCAS operation, however, rolling and adhesion of leukocytes were typically observed in the pial venules although rarely in the pial arterioles under chronic cerebral hypoperfusion (Figure 6A, arrows). From 1 day to 2 weeks after the BCAS operation, the frequency of rolling and adherent leukocytes decreased in the both small pial veins and pial arteries (Figure 6B and 6C). However, 2 weeks after BCAS operation, the frequency of rolling and adhesion was still higher significantly in the pial veins.

Discussion

Neuron-targeted therapies against brain ischemia have been uniformly unsuccessful in clinical trials. This is thought to be a consequence of lack of understanding of the integrated response among neuronal, glial, and vascular elements.1-5 Each of these elements responds to an ischemic event by activation...
and individual features of specific cell responses are well studied, but it remains unknown how they respond as a NVU. In this study, we developed an in vivo imaging method for the NVU. This method allows repeated longitudinal observation of the NVU in 3D, which is helpful to understand anatomic morphology of microvessels with varying sizes as a whole. In addition, this method allows identification of cellular components (astrocytes, pericytes, circulating leukocytes, and platelets) and allows investigation of the anatomic relationship between these cells. This imaging approach also provides semiquantitative information on the kinetics of erythrocytes and leukocytes to understand microcirculation alteration.

Chronic cerebral hypoperfusion induces subcortical white matter lesions and vascular cognitive impairment. This condition is often an initial trigger of a cascade of pathophysiological changes in the NVU and can be experimentally replicated in animal models of chronic cerebral hypoperfusion such as BCAS model. In these models, gray matter exhibits more slight pathophysiological changes than white matter under chronic cerebral hypoperfusion. In this study, we could not detect any morphological change in the cortical gray matter. The morphological changes as stress response of NVU in the cerebral cortex may be limited and under the detection level of 2-photon laser microscopy method. However, additional studies will be required to address this point finally.

As for the dynamic microcirculation, there was stagnation of capillary flow and plugging by leukocytes under chronic cerebral hypoperfusion. The phenomenon of capillary plugging has been postulated to explain no-reflow phenomenon during early reperfusion after ischemia. After several hours of ischemia, an incomplete restoration of blood flow, no-reflow phenomenon, has been reported also in other organs. Many pathophysiological mechanisms have been implicated in this process, however, the exact mechanisms responsible for this phenomenon have been unclear. Leukocytes are large stiff cells, which physiologically adhere to vascular endothelium, and are known to express adhesion molecules under a variety of conditions. The present study has provided the first evidence that leukocyte transiently plugs capillaries under chronic cerebral hypoperfusion, and this plugging has induced stagnation of capillary flow. In this stagnated plasma, no oxygen-transporting erythrocytes were found, implying a viscous cycle for further exacerbation of parenchymal hypoxia. On the contrary, platelet activation was not observed throughout the observation period. Antiplatelet treatment has been the target of therapeutic strategies for chronic cerebral hypoperfusion, but this strategy should be substituted for the agents that have the potential to suppress plugging of leukocytes and restore insufficient CBF. Indeed, beneficial effect of induced immune tolerance against adhesion molecules has been revealed in chronic cerebral hypoperfusion previously.

Leukocyte rolling and adhesion in the pial vessels have been reported in various organs under a variety of conditions, appearing more prominently in the venules than in the arterioles. In accordance with these observations, leukocyte rolling and adhesion were observed typically in the pial veins during chronic cerebral hypoperfusion. Our results may further indicate that leukocyte activation may be the first step eventually leading to stress response of the whole NVU to chronic cerebral hypoperfusion.

Conclusions

In this study, we established an in vivo imaging method for the NVU and provided evidence that leukocytes activation may play a critical role in microcirculation disturbance under chronic cerebral hypoperfusion.

Acknowledgments

The study was conceived by Dr Tomimoto, and the protocol was drafted by Drs Suzuki, Tanaka, Mizoguchi, and Tomimoto. Drs Yata, Nishimura, Unekawa, and Tomita performed the experiments. Dr Yata was responsible for data analysis, data interpretation, and preparation of the report. All authors contributed to data interpretation and approved the final version.

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Disclosures

None.

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Supplemental Methods

Identification cell type for the GFP-positive cells in green mouse brain
To identify all cells existing in the VOI (same VOI as in the in vivo imaging) of mouse brain, we preformed the nuclear staining in our green mouse brain. To clearly show all lesions of the VOI and to protect the intrinsic GFP fluorescence, we performed the SCALEVIEW-A2 method according to instructions set forth by the distributor (SCALEVIEW-A2: Olympus, Japan).\(^1\)

Briefly, the anesthetized mouse was transcardially perfused with 4% paraformaldehyde /phosphate-buffered saline (PFA/PBS). The whole brain was removed and was subjected to post-fixation in 4% PFA/PBS at 4 °C for 10 hr. A 1-mm coronal slice (parietal level which included the same VOI as in the in vivo imaging approach) was cut, embedded in OTC compound, and frozen in liquid nitrogen. Next, the slice was incubated in SCALEVIEW-A2 until it became transparent. For nuclear staining, TO-PRO-3 (Invitrogen, USA 20 diluted 1:200 in SCALEVIEW-A2) was used. We performed VOI imaging by using two-photon microscopy (Supplemental Fig I).

Of the cells that exhibited nuclear staining, we detected only 4 types of cells that were GFP-fluorescent. The first cell types were star-shaped (astrocyte-like) cells that exist in the parenchyma. The second types were fusiform cells that wrapped around the capillary (pericyte-like). In these fusiform cells, highly fluorescent GFP proteins were co-localized within the nucleus. The other two cell types were fusiform cells present inside the penetrating arteriole wall and those present inside the venule wall, respectively.

To validate the cell type for the first two cells, double immunofluorescence staining was performed. After transcardial perfusion with 4% PFA/PBS, the whole brain was post-fixed in 4% PFA/PBS for 24 hr and embedded in paraffin. Coronal brain sections (5-μm thick) were prepared at the parietal level. Immunofluorescence staining followed a standard protocol as described previously\(^2\), and included rabbit polyclonal anti-glial fibrillary acidic protein (GFAP, 1:1000; Abcam), rabbit monoclonal
anti-aminopeptidase-N (APN, 1:250; Abcam), mouse monoclonal anti-green fluorescent protein (GFP, 1:500; Sigma), and appropriate secondary antibodies: Alexa Fluor 488 goat-anti mouse IgG (Invitrogen, Carlsbad, CA, 1:250), and Alexa Fluor 594 goat-anti rabbit IgG (Invitrogen, 1:250). All of the first type of cells were co-localized with GFAP, while the second type of cells were colocalized with APN (Supplemental Fig I).

Platelet activation
To verify the kinetics for activated platelets in this transgenic mouse brain, we performed laser-induced vascular injury. The wall of the pial small vessel was irradiated over a period of < 10 min at the two-photon wavelength of 910 nm. Just after irradiation, platelets adhered and aggregated (Supplemental Fig II).

Two-photon imaging
A 910-nm excitation wavelength was used. An emission signal was separated by a beam splitter (570 nm) and simultaneously detected through a band-pass filter for GFP (495–540 nm) and SR101 (575–630 nm). The objective used in this study was an Olympus XLUMPL 20× water-immersion plan fluorite lens, with numeric aperture = 1.0. A single-plane image consisted of 540 × 540 pixels and the in-plane pixel-size was 0.41–1.23 μm depending on the instrumental zoom factor. Because of the time limitation with plasma labeling, scanning time was limited to within 1.5 h. First, we verified the major MCA branch, and we selected a volume of interest (VOI) in the watershed area of the somatosensory cortex (avoid the cortical large vessels). This included the pial small artery branching from the MCA and the pial small veins (rectangle (630 μm × 630 μm) in Fig. 1A). Volume images in VOI were acquired up to a maximum depth of 0.5–0.6 mm from the cortical surface with a Z-step size of 2 μm (Fig. 1B). Next, we selected an appropriate small pial artery (diameter, 20–30 μm; arrow in Fig. 1B) and a small pial vein (diameter, 20–30 μm; arrowhead in Fig. 1B) in the VOI. We tracked the artery from the surface of the cortex to a depth of 0.5–0.6 mm via 3× zoom scanning (Fig. 1C). In the small pial artery, we evaluated the frequency of leukocyte rolling and adhesion, and measured inflow velocity as determined by line scanning. Repetitive line scans (0.244 ms/line) were performed along the central longitudinal axis of the selected small pial artery (Fig. 3A). Linear shadows produced by non-fluorescent erythrocytes within the plasma stream permitted computation of vessel flow velocity, which was proportional to the slope Δx/Δt. To evaluate microcirculation from the arterioles to the capillaries, we selected a VOI on a portion of the arteriole branch (VOI of the artery: depth of about 400–450 μm; cuboid in Fig. 1C).
Capillary microcirculation was difficult to evaluate using single-plane images because of complicated direction that the capillaries run in; therefore, we repeated volume scanning for the VOI of the artery (5-μm interval depth; 50-μm thickness and 11 slices; time for one set of volume scans, 12.1 s). After 30 volume scans, each volume image was reconstructed as a maximal intensity projection (MIP) (Fig. 1D), and each MIP was combined to form a time-lapse video (12.1-sec interval; Supplementary Video I). We also tracked veins from the surface of the cortex to a depth of 0.5–0.6 mm via 3x zoom scanning (Fig. 1C). In the small pial veins, we evaluated frequency of leukocyte rolling and adhesion, and measured outflow velocity by line scanning. To evaluate the microcirculation from the capillaries to the venules, we selected the VOI on a portion of the branched venule (VOI of the vein: depth of about 250–300 μm; cuboid in Fig. 1 C), and repeated the volume scanning in the same sequence as was used for the VOI of the artery scanning (Fig. 1D). Follow-up imaging at different time-points was performed at the same location and on the same vessels. All volume images were reconstructed and analyzed offline using the software FluoRender (Scientific Computing and Imaging Institute, University of Utah, USA), FluoView (Olympus, Japan), or Image J (National Institutes of Health, USA).

**Analysis of anatomical morphology under chronic hypoperfusion**

To evaluate the shape of each vessel (elongation, meandering, tortuousness (coiled form)), 3D reconstruction was performed for each artery and vein at each time-point. Luminal narrowing (or autoregulatory vasodilatation) and vessel wall thickening were evaluated using single-plane images (horizontal axis of the vessels) of the VOI of arteries and veins at each time-point. Lumen area of horizontal axis of the vessels was measured manually. To evaluate capillary density (capillary loss and angiogenesis), maximal intensity projections were performed at 50-μm depth from the surface of the cortex to 500-μm depth for the VOI. The morphology of astrocytes and pericytes was evaluated in each VOI for arteries and veins.

**Analysis of microcirculatory parameters**

To evaluate microcirculation in the capillaries, the kinetics of erythrocytes and leukocytes within the SR101-labelled plasma stream were evaluated. Stagnation of capillary flow was defined as high-fluorescent plasma segments without non-fluorescent erythrocytes, and capillary plugging of leukocytes was defined as slowly moving leukocytes in the high-fluorescent plasma segment. The frequency of capillary flow stagnation for each VOI of arteries and veins was measured (frequency/30 frames).
Leukocyte rolling was defined as rolling on the endothelium slower than freely moving erythrocytes, while adhesion was defined as leukocytes attaching to the vessel walls. Frequency of rolling and adhesion was measured in each pial vessel (number/100 \( \mu \text{m/min} \)).
Supplemental Fig I

The first column shows MIP (700-μm thick) for intrinsic GFP and nuclear staining (TO-PRO 3) for the parietal cortex (the same VOI as in the in vivo imaging approach). The arrow indicates the penetrating artery. Arrowhead indicates the penetrating venule. Scale bar = 100 μm
The second column shows a zoomed-in image of the rectangle in the first column. Arrows indicate the first type of cell’s nuclei expressing GFP fluorescence. The arrowhead indicates the second type of cell nucleus expressing GFP fluorescence as fusiform in the capillary wall. Scale bar = 20 μm

Third column shows a zoomed-in image of the penetrating arteriole (arrow in the first column). Arrows indicate the cell’s nuclei which express GFP fluorescence in the vessel wall. Scale bar = 30 μm

Fourth column shows a zoomed-in image of the penetrating venule (arrowhead in first column). Arrows indicate the cell’s nuclei which express GFP fluorescence in the vessel wall. Scale bar = 30 μm
Supplemental Fig II

Upper column shows double immunofluorescence histochemistry for GFP, GFAP, and their merge in the transgenic mouse. The arrow indicates GFP-positive star-shaped cells, all of which co-localized with GFAP. The lower column shows the double immunofluorescence histochemistry for GFP, APN, and their merge in the transgenic mouse. The arrow indicates GFP-positive fusiform cells in the capillary wall. APN was stained around the highly fluorescent fusiform cells. Scale bar = 20μm
Supplemental Fig III

Laser-induced vascular injury. The rectangle in the Lt image indicates the irradiated area. The arrow indicates activated platelets. Just after irradiation, platelets adhered to the vessel wall and aggregated.

Scale bar = 20 μm
Supplemental Reference


Legends for the Video files

Supplementary Video I
Time-lapse video showing GFP (green) and SR101 (red) fluorescence of the deep cortical capillaries (in the VOI of an artery) in a sham mouse. Erythrocytes can be observed as non-fluorescent particles within the plasma stream.

Supplementary Video II
Time-lapse video showing SR101 fluorescence of the deep cortical capillaries (in the VOI of a vein) during the preoperative period in a BCAS mouse. Stagnation of capillary flow, which was detected as a high-fluorescent segment without non-fluorescent erythrocytes, was rarely observed under normal conditions.

Supplementary Video III
Time-lapse video showing SR101 fluorescence 2 weeks after chronic cerebral hypoperfusion in the deep cortical capillaries (in the VOI of a vein). Stagnation of capillary flow is obvious under chronic cerebral hypoperfusion.

Supplementary Video IV
Time-lapse video showing SR101 fluorescence of capillary plugging by leukocytes. Capillary plugging can be observed in stagnant capillaries as non-fluorescent particles in the plasma stream.

Supplementary Video V
Time-lapse video showing GFP and SR101 fluorescence of capillary plugging by leukocytes. Plugging by leukocytes was detected as GFP-positive fluorescence. The leukocyte is not tightly plugged in the capillary; rather it slowly moves downstream and is finally released.