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Sensory and optogenetically driven single-vessel fMRI

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Abstract

Magnetic resonance imaging (MRI) sensitivity approaches vessel specificity. We developed a single-vessel functional MRI (fMRI) method to image the contribution of vascular components to blood-oxygenation-level-dependent (BOLD) and cerebral-blood-volume (CBV) fMRI signal. We mapped individual vessels penetrating the rat somatosensory cortex with 100 ms temporal resolution by MRI with peripheral sensory or fiber-optic mediated optogenetic stimulation. BOLD signal originated primarily from venules and CBV signal from arterioles. The optogenetic fMRI signal propagates through the local vascular network with similar kinetics to that of sensory stimulation in deep cortical layers. The single-vessel fMRI method and its combination with optogenetics provide a platform to map the hemodynamic signal through the neurovascular network with specificity to individual arterioles and venules.

Editorial Summary

High-field fMRI with single vessel resolution allows deciphering the contribution of different vessel types to hemodynamic activity evoked by sensory or optogenetic stimulation in the rat brain. Similar vascular kinetics underlie the sensory and light-driven fMRI signal.

Functional MRI (fMRI) maps brain activity based on the tight coupling of hemodynamic responses in the local vasculature to neural activity^{1–4}. But this neurovascular coupling also limits fMRI specificity due to the complexity of the hemodynamic response^{3, 5–7}. Given the large voxels (on the order of millimeter) and slow acquisition time (on the order of seconds) for conventional fMRI, detailed high-resolution hemodynamic response is usually not sampled^{2–4}. The typical voxel size of fMRI corresponds to a large number of neurons and vascular elements. The spatial discrepancy between the vascular origin of the fMRI signal

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Contributions

X.Y. and A.P.K. initiated the work, developed the method, and wrote the paper. A.C.S. and S.D. helped setup the *k*-space reconstruction. H.M. and S. D. designed the RF coil. X.Y. performed experiments on 11.7T and 14T. Y.H., and M.W. helped for data acquisition and analysis under 14T.

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and the neural source that produces the vascular response remains a concern for fMRI brain mapping.

Recently, increased magnetic field strength has made it possible to acquire high-spatiotemporal resolution fMRI images with increased signal-to-noise ratio for both human ($>7\text{T}$) and animal MRI ($>9.4\text{T}$)^{8, 9}. The increased signal-to-noise has been used to increase spatial and/or temporal resolution to localize BOLD signal to venules in the rat somatosensory cortex¹⁰ and to correlate a punctate pattern of CBV signal to arterioles in the cat visual cortex¹¹. Furthermore, very high temporal resolution fMRI has detected fMRI onset at specific cortical layers coinciding with neural projection inputs⁵. In human brains, the layer-dependent fMRI signal was mapped by gradient-echo BOLD and vascular-space-occupancy (VASO)-based CBV methodologies at submillimeter resolution¹². However, no studies map the fMRI signal from individual vessels (distinct arterioles and venules) in deep cortical layers with sampling rates sufficient to define the response. Here, we developed a strategy to enable direct imaging of both arteriole and venule responses to neural activity. The BOLD and CBV-based fMRI signal acquired from individual vessels makes it possible to detect hemodynamic signal propagation through distinct cerebrovascular components at 100ms resolution.

Previously, a conventional fMRI-EPI method¹³ was used to characterize BOLD signal from distinct vascular components in mid-cortical layers of the rat somatosensory cortex¹⁰. Different vascular responses can be mapped by CBV-fMRI with iron oxide particle injection using the EPI method (Supplementary Figure 1, Supplementary Note). Recently, we adapted a line-scanning scheme to the Fast-low-angle-shot (FLASH) fMRI method with very high temporal resolution (Supplementary Figure 2)^{5, 9}. We now used the FLASH-fMRI method to map BOLD and CBV fMRI signal from 2D slices perpendicular to the vessels penetrating the mid-cortical layers of somatosensory cortex of anesthetized rats (Supplementary Figure 3–5, Supplementary Video 1, Supplementary Note). In contrast to the positive BOLD signal mainly due to increased oxy/deoxy ratio in venules, the negative CBV signal was caused by activity-evoked vasodilation leading to increased blood volume, more iron oxide particles, a shorter T_2^* relaxation time, and therefore, less signal. Vasodilation has been attributed to arterioles and there are reports of veins and venule dilation as well¹⁴. In addition, capillary diameter changes could also serve as a major contributor or initiator for changes in cerebral blood volume¹⁵. To detect the single-vessel responses driven by sensory (electrical stimulation of the forepaw or whisker pad) or optogenetic stimulation through fiber optic directly targeting the cortex, we used a multi-gradient-echo (MGE) sequence to anatomically map individual arterioles and venules penetrating the mid-cortical layers of somatosensory cortex. The blood flow delineates vessels because un-saturated MRI signal from blood flowing into the slice can be detected as brighter signal in vessels¹⁶. The specific in-flow effect resulted in brighter signal in both arterioles and venules than in surrounding voxels (Fig 1A). The deoxygenated blood in venules has shorter T_2^* than surrounding tissues or arterioles, which leads to less signal only in venules at the longer TEs (time to echo). Arterioles and surrounding tissues had a similar but longer T_2^* and thus have brighter signal than venules (Fig 1A, Supplementary Figure 6). A similar observation has been reported previously using an in-flow based time-of-flight microangiography to map vessels¹⁷. MGE images at different TEs enabled anatomical mapping of penetrating arterioles and venules

(A-V map) (Fig 1B). We could detect both BOLD and CBV fMRI signal with the FLASH-fMRI method from the same 2D slice for the A-V map. Peak BOLD signal primarily overlapped with penetrating venule ROIs and peak CBV signal primarily overlapped with penetrating arteriole ROIs (Figure 1C, Supplementary Video 2--9). 3D and 2D plots of the vessel-specific spatial distribution of the BOLD and CBV fMRI signal as a function of signal intensity of individual voxels from the A-V map show peak CBV signals on arteriole voxels, but peak BOLD signals on venule voxels (Fig 1D, E, Supplementary Video 10;). The spatial correlation coefficient of BOLD functional maps with venule ROI maps was significantly higher than that with arteriole ROI maps, and vice versa for CBV functional maps (Figure 1F). Spatial correlation was negative for BOLD with arterioles and CBV with venules. Negative spatial correlation coefficients were also detected between BOLD vs. CBV functional maps and between arteriole vs. venule ROI maps (Fig 1G), indicating that most of the BOLD responses came from venules in agreement with recent work¹⁰ and most of the CBV responses came from arterioles^{11, 12}. Thus, the single-vessel method makes it possible to identify the hemodynamic signal from individual arterioles and venules.

The discovery of Channelrhodopsin-2 (ChR2), as a light sensitive cation membrane channel, has enabled control of neural activity optogenetically by targeting specific cell types in neural circuits^{18, 19}. Optogenetics has been implemented to initiate fMRI signals²⁰. The temporal and spatial features of the hemodynamic signal evoked by optogenetic stimulation were reported by optical measurement of the oxy/deoxy hemoglobin in the somatosensory cortex²¹, as well as with BOLD-fMRI^{22,23}. However, the detailed optogenetic hemodynamic signal propagation through the cerebrovascular network at the deep cortical layers has not been studied at the single-vessel level. Combining single-vessel fMRI with optogenetics makes it possible to determine if the hemodynamic features of such fMRI signals are similar to those evoked with more physiological stimulation. We optogenetically evoked neural activity with a fiber optic inserted into the rat whisker barrel cortex expressing ChR2 (Fig 2A). Local field potential (LFP) recordings indicated robust LFP responses upon optical stimulation (Supplementary Figure 7). We mapped the BOLD and CBV signal with EPI-fMRI close to the fiber-optic tip (Fig 2B and Supplementary Figure 8) and aligned the anatomical A-V map to specify individual arterioles and venules near the fiber tip (Fig 2C). The light-driven BOLD signal was primarily located at venule voxels and CBV signal was primarily located at arteriole voxels (Fig 2D, Supplementary Video 11, 12). The averaged time courses of BOLD and CBV signal from venule and arteriole ROIs show similar temporal patterns with both sensory and optogenetic stimuli (Fig 2E, F).

To quantitatively compare the optogenetically activated and sensory-evoked hemodynamic signal, BOLD and CBV signals from individual arterioles and venules were fit to estimate the onset time (t_0), the time-to-peak (ttp), and the full-width-at-half-maximum (FWHM) (Supplementary Figure 9–11). The CBV signal of individual arterioles shows earlier onset (t_0) and time-to-peak (ttp) than BOLD signal of individual venule for both sensory stimulation and optogenetics (Fig 3A, Supplementary Figure 9). However, there are a few very early BOLD responding venules and a few arterioles with late onset of CBV signal (Supplementary Figure 12, Supplementary Note). We analyzed the BOLD signal from individual arterioles (Supplementary Figure 10) and CBV signal from individual venules (supplementary Figure 11) to characterize specific vascular contribution to the BOLD and

CBV fMRI signal, respectively (discussion in Supplementary Note). The t_0 , t_{tp} and FWHM plots of the single-vessel BOLD and CBV signals readily separated into distinct clusters of arterioles and venules (Fig 3B, C). The temporal parameters of the optogenetically activated fMRI signal showed little difference in comparison to the sensory stimulation-evoked fMRI signal acquired from either 11.7T or 14T MRI (Supplementary Figure 13, Supplementary Table 1, Student's t -test). Thus, the optogenetically driven neural activity displayed similar temporal features of hemodynamic signal propagating through the cerebrovascular network as sensory stimulation did.

Two-photon microscopy is increasingly used to image vessels in the cortex^{24–26}. The single-vessel fMRI method has three potential benefits as compared to optical imaging. First, we could detect fMRI signal from individual penetrating vessels in mid-cortical layers. Using this strategy, it should be possible to map single vessels located at subcortical brain regions such as the hippocampus. Second, it is possible to use the full range of MRI techniques to enable anatomy, connectivity and fMRI to be readily co-registered for complementary information. It may even be possible to extend the single vessel mappings to the human brain as sensitivity increases with the emerging high field MRI. Third, fMRI can be performed without any perturbation to animals as opposed to optical imaging that requires window or thin skull preparations and possibly the insertion of optical equipment into the brain. Moreover, the fMRI signal could be acquired at similar temporal resolution as optical microscopy. In this work, we acquired data at a 100ms temporal interval, which represents a similar sampling rate to the optical brain imaging methods whose temporal resolution ranged from 9–18Hz for field-of-view on the sub-millimeter scale.

A question during this work was how well individual penetrating arterioles or venules were characterized in the cortex. The average distance between venule voxels measured in the present work was $372 \pm 33\mu\text{m}$ and that between arterioles was $286 \pm 15\mu\text{m}$. This agrees with known spacing of these vessels indicating a majority of these penetrating vessels were detected²⁶. The established size of penetrating vessels separated by a few hundred microns ranges from 30 to 70 microns^{26, 27}. We acquired the A-V map with in-plane resolution of $75 \times 75\mu\text{m}$ or $50 \times 50\mu\text{m}$, which is close to the mean size of the main penetrating vessels (Fig 2, Supplementary Figure 8). The brighter voxels of A-V maps were usually detected within a 2×2 voxel matrix implying that the in-flow effect was sufficient to highlight individual arterioles smaller than the voxel sizes. The darker voxels representing penetrating venules were larger than the actual size of venules due to the extravascular dephasing effect of the deoxygenated hemoglobin from venule blood²⁷. Different vessel sizes with potentially different orientation angles likely led to varied signal intensity of vessel voxels. The identified arterioles and venules were based on a nearest neighborhood variation analysis; however it is likely that some smaller penetrating vessels were not identified. Future work can compare MRI maps to optical imaging or vessel casting with high resolution CT to quantitatively assess this issue. Thus, it is important to note that the term “single vessel” used in this study designates the arteriole or venule voxels in the A-V map that can be detected under the imaging condition used.

This work shows that peak BOLD signal aligned with venule voxels and the peak CBV signal aligned with the arteriole voxels. The t_0 and t_{tp} of the CBV signal from arterioles was

comparable to the temporal onset estimates of arteriole dilation detected at a 0.5mm cortical depth with two-photon microscopy²⁵. This observation was also consistent with the BOLD and CBV studies performed in cats¹¹ and human subjects¹² to show the early onset of CBV signals. The mean t_0 of BOLD in venules (0.96 ± 0.04 s) was slightly faster than what was previously reported from macro-venules detected by EPI-fMRI methods¹⁰. In a previous study, the transit time (calculated by the time to half-maximal, $t_{1/2}$) from surface arterioles to venules were measured to be 0.8–1.2s by tracing a vascular fluorescent dye with two-photon microscopy during control and mild hypercapnia²⁴. The average transit time estimated in the present study was ~0.8s based on the mean time-to-peak difference from arteriole CBV to venule BOLD signal in the mid-cortical layers (Supplementary Note). The MRI-defined transit time was on the shorter end of the transit time measured by optical microscopy²⁴. A few reasons may explain the shorter transit time: 1) neural activity may drive hemodynamic signal propagation faster than hypercapnia-induced arteriole dilation; 2) there may be layer-specific differences in the transmit time delays from mid-cortical layers which have a denser capillary network than the upper layers imaged optically; 3) different criteria were used to define the transit time in the optical imaging as compared to the present study. The estimate of the transit time by comparing arteriole and venule fMRI signals illustrates that vessel-specific hemodynamic signal propagation can be measured with the single-vessel fMRI method.

In conclusion, distinct arteriole CBV and venule BOLD signals could be characterized with 100ms temporal resolution. The similar vascular kinetics between light-driven and sensory-evoked fMRI signal in the deep cortical layers was consistent with previous optogenetic fMRI studies^{22, 23}, which we extended to single-vessel level in the present study. The single-vessel fMRI method and its combination with optogenetics should be able to decipher the individual vascular coupling events within the neuron-glia-vessel network in both normal and diseased brain states.

Online Methods

MRI image acquisition

All images were acquired with an 11.7 T/31cm and 14.1 T/26cm horizontal bore magnet (Magnex), interfaced to an AVANCE III console (Bruker) and equipped with a 12 cm gradient set, capable of providing 100 G/cm with a rise time of 150 μ s (Resonance Research). For the 11.7 T scanner, a custom-built 9cm diameter quadrature transmitter coil was placed in the gradient. Surface receive-only coils were used during image acquisition. For the 14T scanner, a transceiver surface coil with 6mm diameter was used to acquire fMRI images.

Line-scanning fMRI: A 2D FLASH sequence was applied to map the fMRI signal with the following parameters: TE 4ms (CBV)/16ms (BOLD), TR 100ms; matrix 80×32, in plane resolution 150×150 μ m for 11.7T; matrix 96×64, in plane resolution, 100×100 μ m for 14T; slice thickness 500 μ m, flip angle 20°. As previously described^{5, 9}, the single k space line was acquired for each image of the block-design stimulation pattern. The on/off stimulation trials were repeated for the number of phase-encoding steps (Supplementary Figure 2). The

field of view (FOV) along the phase-encoding direction was aligned to cover the deep layers of the cortical regions of interest (Supplementary Figure 2B) as previously described¹⁰. To reduce the potential aliasing effect along the phase-encoding direction, two saturation slices were applied to null the signal out of the FOV as previously established for line-scanning fMRI⁵. The 2D FLASH slice image was reconstructed from the reshuffled k space data with 100ms sampling rate⁹. As described previously¹⁰, the in-flow effect has little contribution to the BOLD signal detected from the individual venules. For the CBV-fMRI signal, the in-flow effect from blood with high concentration of iron oxides would likely be negligible.

Single vessel multiple gradient echo (MGE) imaging: To detect individual arterioles and venules, a 2D-MGE sequence was used with the following parameters: TR, 30ms; TE, 1.8, 4.3, 6.8, 9.3ms; flip angle, 50°, matrix: 160×128, in-plane resolution: 75×75µm; slice thickness, 500µm for 11.7T. TR: 50ms, TE, 2.5, 5, 7.5, 10, 12.5, 15ms; flip angle 40°, matrix: 192×128, in-plane resolution: 50µm×50µm, 500 µm thickness for 14T. The single vessel map is acquired by averaging the MGE images acquired from the second echo to the forth echo, where the venule voxels showed as dark dots due to fast T2* decay, but arteriole voxels remain bright dots due to the in-flow effect.

Echo-planar Imaging (EPI) fMRI: For the EPI sequence, fastmap shimming, adjustments to echo spacing and symmetry, and B₀ compensation was first setup. Using the single surface coil, a single shot sequence with a 64 × 64 matrix was run with the following parameters: effective TE 18/9.6ms, TR 0.8s, bandwidth 138/300kHz, flip angle 45°, in plane resolution: 150×150. The slice thickness is 500µm. A 3D gradient-echo, EPI sequence with a 64 × 64 × 32 matrix was run with the following parameters: effective echo time (TE) 16ms, repetition time (TR) 1.5s, bandwidth 170kHz, flip angle 12°, FOV 1.92 × 1.92 × 0.96 cm.

For fMRI studies, electrodes were placed on the forepaw or whisker pads to deliver a 2.0mA pulse sequence (300µs duration repeated at 3Hz) by an isolated stimulator (A360LA, WPI)¹⁰. For optical stimulation, the 473nm laser (CNI, China) with build-in FC/PC coupler was used to deliver the light pulse. The light pulse was triggered through an analog module to deliver optical stimulation at different duration ranging from 0.3ms to 20ms. The multimode optical fiber is 200µm (FT200EMT, Thorlabs, NJ). The light power from the fiber tips was calibrated by optical power meters (PM20A, Thorlabs, NJ) and was controlled from 0.3 to 10mw. The power levels used for light-driven fMRI studies (2/5s 10Hz, 20ms light pulse, 3.2mw) did not induce pseudo-BOLD signal due to heating effects, which were tested in the cortical regions without Chr2 expression, as well as in the Chr2-expressing cortical area after the rats died.

The 2D slice covered the forepaw and barrel S1 areas based on the Paxinos atlas. The horizontal slice angle was set at 15 and 40° and the center of the slice was positioned 1mm from the cortical surface to cover layers 4 and 5. For FLASH-fMRI, the forepaw and whisker pad stimulation experiment consisted of 60 dummy scans to reach steady state; followed by 10 pre-stimulation scans, 20 scans during electrical stimulation, and 100 scans post-stimulation (total 13 second for each on/off epoch for three times). The time for each trial was total 42 min. Each trial was repeated 3–4 times for both BOLD and CBV fMRI mapping. After the second trial for CBV-fMRI study, a small dosage of iron oxide particles

(3–4 mg/Kg) would be injected to compensate the potential washout issue of the iron particles from the blood. The single vessel map was acquired at the same slice orientation for further imaging registration. For the EPI-fMRI, the forepaw and whisker stimulation experiment consisted of 10 dummy scans to reach steady state; followed by 10 pre-stimulation scans, 3 scans during stimulation, and 12 scans inter-stimulation for 8 epochs or 20 pre-stimulation scans, 5 scans during stimulation, and 20 scans inter-stimulation for 5 epochs. The pulse sequence-based trigger and stimulation control was established by BioPac system (Goleta, USA) and AD instruments (Oxford, UK).

Animal Surgeries

All animal work was performed according to the guidelines of the Animal Care and Use Committee and the Animal Health and Care Section of the National Institute of Neurological Disorders and Stroke, National Institutes of Health (Bethesda, MD, USA) and the protocol approved by the animal protection committee of Tuebingen (Regierungspräsidium Tuebingen). Total 24 male *Sprague-Dawley* rats were imaged at 2–3 months of age. 8 rats were imaged under 11.7T (both BOLD and CBV FLASH-fMRI data with A-V maps were acquired from 5 of 8 rats) at NIH, 7 rats were imaged under 14T at MPI (both BOLD and CBV FLASH-fMRI data with A-V maps were acquired from 4 of 7 rats), and 9 rats were imaged for optogenetic studies (both BOLD and CBV FLASH-fMRI data with A-V maps were acquired from 4 of 9 rats). The number of animals was calculated by a power analysis with parameters acquired from our previous studies^{5, 10}. During the fMRI experiments, if the rats died during the experiments, data acquired were not included for statistical analysis.

Animal preparation for functional MRI:

A detailed procedure was described in the previous study²⁸. Briefly, rats were initially anesthetized with isoflurane. Each rat was orally intubated with a mechanical ventilator throughout the whole surgical and imaging procedures. Plastic catheters were inserted into the right femoral artery and vein to allow monitoring of arterial blood gases and administration of drugs (anesthetics and Iron oxide particles). Following catheterization, all rats were given i.v. bolus of α -chloralose (80mg/kg). Isoflurane was discontinued in 3–5 minutes. Constant infusion of α -chloralose was set with rate 26.5mg/kg/hr. The rats' rectal temperature was maintained at $\sim 37^{\circ}\text{C}$ while in the magnet. Rats were secured in a head holder with a bite bar to prevent head motion. All relevant physiological parameters, such as, end-tidal CO_2 , rectal temperature, heart rate, and arterial blood pressure, were continuously monitored during imaging. Arterial blood gas contents were checked regularly and adjustment were made by tuning respiratory volume or administering sodium bicarbonate to maintain normal pH levels when required. An i.v. injection of pancuronium bromide (4 mg/kg) was administered to reduce the motion artifacts upon request. BOLD and CBV-fMRI was performed in α -chloralose anesthetized rats. CBV-fMRI was performed directly following BOLD fMRI. CBV-weighted signals were obtained after intravenous administration of 15 mg Fe/kg dextran coated iron oxide (Biopal, MA).

Viral vector injection and fiber optic implantation:

The viral vectors (AAV5.CaMKIIa.hChR2 (H134R)-eYFP.WPRE.hGH) were obtained from UPenn vector core. Three-four week old rats were injected with the 200nl of original viral vector solution into the barrel cortex with stereotactic coordinates: Bregma -2.35mm, lateral -4.8mm, and ventral 1.5 and 0.7mm. For the stereotactic injection procedure, rats were initially anesthetized by isoflurane. A small bur hole was drilled after exposing the skull. A nanoliter injector (WPI, FL) was used to place the 35 gauge needle at the proper coordinates in the stereotactic frame. Injections were performed slowly over 5–6min and the needle was slowly removed after being kept in the injection site for 10min after ending the injection. In 5–6 weeks after the viral injection, 200um fiber optic was inserted into the rat barrel cortex at the stereotactic coordinates: Bregma -2.7mm, lateral -5.1mm, and ventral 1.3mm, tilt 4°. The fiber optic was glued to the skull and skin was sutured after the glue was solidified in 20–30 min.

In vivo electrophysiological recordings:

Rats were placed in a stereotaxic frame for the *in vivo* recordings under similar anesthesia and surgical procedures to the fMRI experiments. The 200µm fiber optic was first inserted to target the barrel cortex previously injected with AVV viral vectors. The electrodes (Plastics One Inc, Roanoke, VA) was positioned to the barrel cortex (Bregma -2.7, lateral -5.1, and ventral 0.8 mm, tilt 5–6°). The intact whisker pad was electrically stimulated at 2.0mA (3Hz, 300µs) by an isolated stimulator (A360LA, WPI). Or, the barrel cortex was directly activated by the light pulse exposure (0.3–20ms, 1, 3Hz, 10Hz, and 0.3 to 20mw). Stimulation trigger were delivered through the M150 Biopac system using the STM100C stimulator module with 10K sampling rate. The evoked potential was acquired through the EEG module of the Biopac system (gain factor: 5000, the band-pass filter 0.1–100 Hz). Mean profiles of evoked potential responses were subsequently obtained by averaging over the entire series with a time-window step of 300ms (synchronized to the start of a stimulation pulse). AcqKnowledge software package (Biopac Systems) was used to calculate the averaged profiles of evoked potential responses.

Animal perfusion and brain slice microscopic imaging: Immediately after the fMRI imaging, rats were deeply anesthetized with sodium pentobarbital (60mg/kg, per rat, SQ). Then, animals were secured dorsally, and an incision was made along the chest. The heart was exposed, by incising through the rib cage and diaphragm. The left ventricle was punctured with a sterile catheter, the right atrium was cut to allow fluid to drain, and heparinized saline was fed into the heart with a perfusion pump. Following the saline, 4% paraformaldehyde, 10% buffered formalin, was fed into the heart. Following perfusion, the brain was carefully extracted from the skull. 30µm brain slice was cut from the frozen rat brain using the cryostat (Leica-CM1860, Wetzlar, Germany). The floating slice was mounted on the glass slide with cover slip. The fluorescent imaging was acquired using the Zeiss Axio Imager 2 (Zeiss GmbH, Göttingen, Germany).

Imaging Processing and Statistical Analysis

Both FLASH- and EPI-fMRI data analysis was performed using Analysis of Functional NeuroImages (AFNI) software (NIH)²⁹. The relevant source codes can be downloaded through <https://www.afni.nimh.nih.gov/afni/>. Detailed description of the processing was provided in a previous study¹⁰.

The FLASH-fMRI data stored in the k -space format (Supplementary Figure 2) were first reshuffled with a matlab script for reconstruction using the built-in function of Bruker Paravision software. For the AFNI analysis, a 2D registration function was first applied to register the reconstructed FLASH images to a template for multiple datasets acquired in the same orientation setup. To register the FLASH fMRI images with the single vessel maps, we applied the tag-based registration method. Ten to twelve tags were chosen from venule voxels distributed around the 2D slices of FLASH and single vessel images. All time-series FLASH-fMRI images was normalized by scaling the baseline images to 100. Multiple trials of block-design time-courses were averaged for each animal. No smoothing procedure was included in the image-processing steps so that the single vessel fMRI signal can be specified from the high-resolution FLASH-fMRI images. The hemodynamic response function (HRF) was derived from linear regression using a tent function.

$$f(x) = \begin{cases} 1 - |x| & \text{for } -1 < x < 1 \\ 0 & \text{for } |x| > 1 \end{cases}$$

The tent function is also called “piecewise linear spline”, which is used for deconvolution of the HRF response with magnitude estimated as beta coefficient.

$$H(t) = \sum_{k=0}^n \left(\beta_k * \left(\frac{t - k * L}{L} \right) \right)$$

$$\text{Or, } H(t) = \beta_0 * T(t/L) + \beta_1 * T((t-L)/L) + \beta_2 * T((t-2*L)/L) + \dots + \beta_k * T((t-k*L)/L)$$

Here, $H(t)$ is the HRF response, k is the total number of tent parametric fitting. β_k is response (tent height) at time $t = k \cdot L$ after stimulation; L : tent radius (L can be equal to TR). The beta value (β) was calculated to estimate the amplitude of fMRI response at each TR ($L=TR$). The voxel-wise beta map is presented to illustrate the spatial pattern of the fMRI response at different time points after the stimulus onset.

The individual arterioles and venules were characterized based on the signal intensity of the voxels detected in the single vessel map. The single vessel map was acquired by averaging the MGE images from the second echo to the forth echo. The individual vessel voxels were determined based on their signal intensity either higher (arterioles) than the mean signal intensity plus 2 times standard deviation or lower (venules) than the mean signal intensity minus 2 times standard deviation of the local area in a 5×5 kernel³⁰ (Supplementary Figure 6A). A `3dLocalstat` function from AFNI library was used to normalize the signal intensity of single vessel maps. This allows to plot the BOLD and CBV beta values of all voxels to their

normalized signal intensity of A-V maps (Figure 1). In addition, a 2D spatial correlation was performed between the single-vessel maps and the corresponding BOLD and CBV fMRI functional maps using the matlab script (corr2). The fMRI onset profile was determined based on the full hemodynamic response function. As previously reported, a two-gamma-variate fitting step was applied to estimate onset time from the averaged and normalized fMRI signal^{5, 29, 31}.

$$f(x) = a * \left(\frac{x}{p * q}\right)^p * e^{\left(p - \frac{x}{q}\right)} - b * \left(\frac{x}{r * s}\right)^r * e^{\left(r - \frac{x}{s}\right)}$$

(x is the variable; a, p, q, b, r, s are the coefficients for two-gamma-variate-function)

Two-gamma-variate function with optimized coefficients was fit to the hemodynamic responses of individual voxels. The fitting curve was plotted with the raw BOLD and CBV hemodynamic signal from individual vessels (Figure 3, Supplementary Figure 9–12). The mean r^2 value for each individual fitting of venule BOLD and arteriole CBV signal was shown in a scatter plot graph for individual rats (Supplementary Figure 13). The time-to-peak and full-width-of-half-maximum was estimated from the fitting function. The onset time was derived from an t_0 coefficient from the modified two-gamma-variate function: $f(t) = \dots \left(\frac{x - t_0}{p * q}\right)^p \dots \left(\frac{x - t_1}{r * s}\right)^r \dots$, which showed a reliable estimate of the hemodynamic signal onset in comparison to the noise threshold-based onset estimates⁵.

Finally, Student's t-test was performed for group analysis and the error bar is the standard error in each graph. No blinding design was needed in this work.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig 1. Single-vessel fMRI overlaps with the arteriole-venule (A-V) map.

A. A 2D MGE slice from the deep layer cortex of forepaw S1 cortex (1.0–1.5mm, left) at different TEs (representative from 5 rats). **B.** The A-V map derived from A. Arterioles and venules appear as bright and dark voxels, respectively. **C.** A zoomed area (box in B) with venule (blue) and arteriole (red) ROIs highlighted (left panel), BOLD (up) and CBV (bottom) fMRI maps at the same 2D slice (middle panel) and the overlays (active voxels in purple). **D.** BOLD and CBV signal distribution as the function of voxel signal intensity in A-V maps ($n=5$, with different colors). **E.** A 2D plot of the BOLD (left, blue) and CBV (right, red) fMRI signal from individual voxels as the function of voxel signal intensity in A-V maps (representative of one dataset from D). **F.** The spatial correlation coefficients of BOLD (upper panel) and CBV (lower panel) functional maps with arteriole (red) and venule (blue) ROIs (black dots: the coefficients of individual rats, $n=5$; mean $\pm$ SEM, BOLD, * means $p=0.00002$; CBV, * means $p=0.0003$, Student's t -test). **G.** The spatial correlation coefficients of the functional maps (BOLD vs. CBV) and the single vessel ROI maps (arteriole vs. venule) (mean $\pm$ SEM).

Fig 2. Optogenetically induced fMRI signal from single vessels penetrating the barrel cortex.

A. Overview of optogenetically driven single-vessel fMRI and a histological section (from the boxed area in B), showing ChR2 expression. Blue arrow, fiber optic entry point. Black arrow, penetrating vessel. Yellowbox, area imaged by single-vessel fMRI (representative from 9 rats). **B.** T2*-weighted image with the fiber optic (red arrow) inserted into the barrel cortex (left). Overlay of the optogenetically induced fMRI signal with the T2*-W image (right). Averaging time course from the indicated cortical area shows corresponding fMRI signal changes with the block-design paradigm (bottom; Illumination: 5s on/30s off, 5 epoch, 20ms light pulse, 10Hz, 3.2mw). **C.** A-V map with venule (bright) and arteriole (dark) voxels. The dark area in the center indicates the position of the fiber optic. Green box area shown in D. **D.** Venule (top) and arteriole (bottom) ROIs on the A-V maps (left), the BOLD (top) and CBV (bottom) fMRI maps (center) and the respective overlays (BOLD, top; CBV, bottom). **E.** The averaged BOLD and CBV fMRI time course from individual arterioles (red) and venules (blue) by sensory stimulation of the forepaw (BOLD, $n=7$, CBV, $n=5$ rats, all ROIs were averaged for each rat. Mean value is from the average of all rats in each group, error bar is $\pm$ SEM). **F.** The averaged BOLD and CBV fMRI time course from individual arterioles (red) and venules (blue) driven by optogenetic stimulation of the barrel cortex ($n=4$ rats, error bar is $\pm$ SEM; illumination: 20ms, 10Hz, 3.2mw light pulse for 2s on).

Fig 3. Temporal features of sensory and optogenetically driven BOLD and CBV fMRI signal from individual arterioles and venules.

A. A-V maps of individual arteriole and venule voxels, onset time (t0) maps and time-to-peak (ttp) maps of a representative rat each with either sensory stimulation (representative from 5 rats) or fiber optic mediated optical stimulation (representative from 4 rats) (left panel). **B.** 3D plots of t0, ttp, and full-width-of-half-maximum (FWHM) of sensory-evoked fMRI signal from individual arteriole (red diamonds, $n=61$, $r^2>0.4$) and venule (blue circles, 69 , $r^2>0.5$) voxels ($n=5$, rats), and of t0, ttp, and FWHM of light-driven fMRI signal from individual arteriole (red diamonds, $n=33$, $r^2>0.3$) and venule (blue circles, 37 , $r^2>0.35$) voxels ($n=4$, rats). **C.** Distribution of the number of venule (blue) and arteriole (red) voxels with different t0, ttp and FWHM in rats with sensory stimulation ($n=5$) or with fiber-optic mediated optical stimulation by optogenetics ($n=4$, rats).