

Supplementary Methods for: “Neutrophil adhesion in brain capillaries reduces cortical blood flow and impairs memory function in Alzheimer’s disease mouse models,” by J.C. Hernandez, et al.

Variations in surgical preparation and *in vivo* two-photon microscopy for TgCRND8 mice

Cranial window implantations were also performed in TgCRND8 mice (41-51 weeks of age, all female)¹⁷. These animals were housed at The Rockefeller University’s Comparative Biosciences Center and treated in accordance with IACUC-approved protocols. The window implantation followed the same protocol as used for other animals, except that mice were anaesthetized using avertin (50 mg/100 g, intraperitoneal) and were given atropine (0.004 mg/100 g). For TgCRND8 mice, imaging was performed using a Fluoview 1000MPE two-photon laser scanning microscope (Olympus) equipped with a SpectraPhysics MaiTai DeepSee laser and a 25x/1.05 NA objective at The Rockefeller University Bio-Imaging Resource Center.

Awake imaging

A subset of mice was imaged with 2PEF without anesthesia. During the craniotomy surgery, a 3D-printed skull-attached mounting frame was secured on top of the cranial window to allow for head fixation during anesthesia-free imaging. The 3D-printed frame was flanked by 4 screws (TX000-1-1/2 self tapping screws, Small Parts Inc., Miami Lakes, FL) inserted into the skull. The screws and appropriate parts of the frame were glued to the skull using Loctite and dental cement to firmly attach the mounting frame.

We adapted and modified the awake imaging system from Dombeck et al.⁵², in which a large (8 inch diameter) Styrofoam ball (Floracraft) was levitated using a thin cushion of air between the ball and a custom made (3D printed) casting containing eight 0.25 inch diameter air jets, arranged symmetrically. The air pressure was adjusted to just float the ball when the mouse was on top of it.

We trained mice to remain in a calm state during awake, head-fixed imaging. During the first training session, mice were handled, with the room lights on, by a trainer wearing gloves for ~10 min or until the mice routinely ran from hand to hand. The mice were then transferred to the ball and allowed to move freely for ~10 min with the room lights on while the handler rotated the ball to keep the mice centered near the top. The second training session consisted of again allowing the mice to move freely on the ball for ~10–15 min, again with the room lights on. The third training session began by head restraining the mice on the ball in complete darkness for ~15–20 min. Typically it would take 5–10 min for the mouse to learn to balance and then begin to walk or run. Mice were then head-fixed and placed on the ball during imaging under the microscope. Awake imaging lasted less than 30 min. Following awake imaging, mice were anesthetized as described above and imaging was repeated over the same cortical area to compare capillary physiology between the awake and anesthetized states.

Variations in quantification of capillary network topology and capillary segment stalling for 5xFAD mice

For the data on the fraction of capillaries stalled in the 5xFAD mice, individual capillary segments for categorization as flowing or stalled were identified using a more automated approach. Briefly, the image stack was preprocessed to remove motion artifacts and noise. Then the vasculature network in the image stack was segmented using deep convolutional neural network followed by a post-processing procedure to extract the vectorized vasculature network³². Finally, each identified capillary segment was manually classified as either flowing or stalled.

Measurement of volumetric blood flow in penetrating arterioles

To quantify blood flow in cortical penetrating arterioles, we measured the vessel diameter from image stacks and the centerline RBC flow speed from line-scan measurements, as described in Santisakultarm *T.P.* et al.⁵³. The volumetric blood flow, F , was calculated as:

$$F = \frac{\pi v r^2}{2} \quad \text{Eq. 1,}$$

where v is the time-averaged centerline RBC speed and r is the vessel radius. To correlate the impact of the number of capillaries stalled on penetrating arteriole blood flow, we imaged the same capillaries and measured blood flow in the same six to eight penetrating arterioles in young APP/PS1 and wt mice (age 3-4 months), older APP/PS1 mice (age 11-14 months), and in 5xFAD and wt mice (age 5-7 months) treated with antibodies against Ly6G or with isotype control antibodies. Images to determine capillary stalling and line scans to determine penetrating arteriole blood flow speed were taken at baseline and at 60-90 min after treatment. All analysis was conducted blinded to the animal genotype, age, treatment, and imaging time point.

Amyloid plaque segmentation and density analysis

2PEF images of methoxy-X04 labeled amyloid plaques were filtered and binarized. Briefly, we first linearly interpolated the image stacks to have an isotropic 1 μm voxel size and then reduced the background signal in a line-by-line fashion by subtracting the median of each line. Salt and pepper image noise was reduced using the adaptive Wiener method with a 3 x 3 pixel kernel⁵⁴. The image was then binarized using a manually-determined threshold (99% of the intensity distribution) and smoothed with a 3 x 3 pixel median filter. Objects smaller than 25 voxels were then removed, with object connectivity here defined as voxels sharing a face. The volume fraction of amyloid either globally or in a tube that follows the centerline of each capillary segment was then calculated from this binarized image. The tube volume was generated by swaying a sphere with a specified radius along the centerline of the capillary segment from one end to the other.

Kinetics of capillary stalling

To determine the short-term fate of capillaries that stalled, we repeatedly imaged the same capillary bed at baseline and at 5, 10, and 15 min later in APP/PS1 mice (n= 6 animals), and tracked the fate of all the capillaries that were stalled at baseline. If a vessel was observed as

stalled at all subsequent imaging time points, it was scored as remaining stalled, and if flow had resumed the stall was scored to have resolved. If the originally stalled capillary resumed flow, then re-stalled at a later time point that was scored as re-stalled. In some animals, we further determined the cause of capillary stalls at each of these time points.

To evaluate the longer-term fate of capillaries that were stalled, we imaged APP/PS1 mice (n= 5 animals) at baseline and then 1, 3, 7, and 14 days later and determined what fraction of the capillaries stalled at baseline were stalled at any subsequent imaging session.

We estimated how frequently we would observe capillaries stalled at baseline to be stalled at any subsequent imaging session assuming that no stalls lasted long enough to stay stalled between imaging sessions and that each capillary segment was equally likely to stall. With this model, the probability, P_c , of the capillaries stalled at baseline to be stalled at any subsequent imaging session is:

$$P_c = 1 - (1 - r)^N \quad \text{Eq. 2,}$$

where r is the fraction of capillaries with stalled blood flow and N is the number of observations after the baseline imaging.

Flow cytometry of circulating neutrophil counts

To determine the impact of α -Ly6G on neutrophil number, we used flow cytometry to determine neutrophil counts 3, 6 and 24 hr after a single treatment (4 mg/kg) and after one month of treatment every three days (2 mg/kg).

Blood from APP/PS1 and wt mice was collected from the submandibular vein and mixed with 1x RBC lysis buffer (00-4300-54, ThermoFisher Scientific). After incubation at room temperature for 10 min, the sample was centrifuged at 500 g for 5 min and the supernatant was removed. The cell pellet was re-suspended in 500 μ L of Hank's balanced salt solution (HBSS) supplemented with 1% bovine serum albumin (BSA) and centrifuged again; this washing procedure was repeated 3 times. Following isolation, neutrophils were re-suspended at a density of 10^7 cells per ml in HBSS supplemented with 1% BSA. The cell samples were labeled at room temperature for 45 min with the following anti-mouse

antibodies: anti-CD45 (560695, BD Bioscience), anti-CD11b (557686, BD Bioscience) and anti-Ly6G (551460, BD Bioscience). After washing the samples with HBSS samples have been re-suspended in FACS buffer (1% BSA and 2mM EDTA in PBS), the remaining leukocytes were analyzed by flow cytometry using a Guava easyCyte Flow Cytometer (EMD Millipore Corporation). Data were analyzed using FlowJo software (FlowJo LLC). The neutrophil population was identified based on the side and forward scatter and later gated for CD45^{high}, CD11b^{high}, and Ly6G^{high} using FlowJo.

Measurement of global blood flow using ASL-MRI

Imaging was performed on a 7.0 Tesla small animal MRI system with 450 mT/m gradient amplitude and a 4500 T/m/s slew rate (Biospec 70/30, Bruker). The animals were anesthetized with isoflurane in oxygen and immobilized in the MRI using a nose cone and bite ring. A volume coil was used for transmission and a surface coil for reception. We imaged APP/PS1 and wt mice (age 7-9 months) at baseline. About 48 hrs later, animals were given an intraperitoneal injection of α -Ly6G or isotype control antibodies (4 mg/kg) and a second set of images were acquired between 2-6 hr after injection. Mice were randomly assigned to receive treatment or isotype control antibodies and the experimenter was blinded to both mouse genotype and whether the antibody was the treatment or control during the experiment.

Anatomical images were acquired to find a coronal slice at a location approximately 1 mm caudal to Bregma⁵⁵. This position was used for subsequent ASL imaging, which was based on a FAIR-RARE pulse sequence that labeled the inflowing blood by global inversion of the equilibrium magnetization⁵⁶. In this method, inversion recovery data from the imaging slice are acquired after selective inversion of the slice and after inversion of both the slice and the surrounding tissue. The difference of the apparent R1 relaxation rate images then yields a measure of the CBF⁵⁷. Three averages of one axial slice were acquired with a field of view of 15 × 15 mm, spatial resolution of 0.23 × 0.23 × 2 mm³, echo time TE of 5.36 ms, effective TE of 26.84 ms, repeat time TR of 10 s, and a RARE factor of 36. This resulted in a total scan time for the CBF images of about 25 min. Turbo-RARE anatomical images were acquired with the following parameters: 10 averages of 14 slices with the same field-of-view and orientation

as the ASL images, resolution = $0.078 \times 0.078 \times 1 \text{ mm}^3$, TE = 48 ms, TR = 2000 ms, and a RARE factor of 10. The total scan time was about 6 min.

For computation of CBF, the Bruker ASL perfusion processing macro was used. It uses the model and includes steps to mask out the background and ventricles described in Kober, et al.⁵⁸. The masked CBF images were exported to Analyze format on the MRI console. We then used the anatomical image to create a mask that outlined the entire cortical region, excluding the sinus, and averaged the CBF measurement across this region for each animal at each imaging time point. Analysis of ASL-MRI data was conducted by a researcher blinded to animal genotype, imaging time point, and treatment.

Multi-Exposure Laser Speckle Imaging

Mice were anesthetized for imaging using 3% isoflurane for induction and were given atropine and glucose each hour as described above. Depth of anesthesia was monitored using a force-sensitive square resistor placed below the mouse to detect respiration. For imaging, anesthesia was reduced to 1.5% in medical air and then adjusted to maintain a respiration rate between 65-75 breaths per minute. The mouse temperature was maintained at 37°C with a feedback-controlled heating pad.

Multi-exposure laser speckle contrast imaging quantifies the degree of blurring of a laser speckle pattern due to moving scatterers for different image exposure times⁵⁹. In the cortex, moving red blood cells are the primary moving scatterers, so this technique yields a measure of cerebral blood flow. Images were taken using a near-IR camera (Basler) through a 4x objective (NA=0.28, Olympus) with a 786-nm stabilized laser diode (LD785-SEV300, ThorLabs), intensity modulated using an acousto-optic modulator (AOMO 3100-125 and AODR 1110AF-AEFO-1.5, Gooch & Housego). Laser speckle images were taken at 15 exposure times ranging across three decades from 50 μs to 80 ms, as described by Kaszmi et al.⁶⁰, with a calibrated laser intensity for each exposure time. Images were taken at ~ 10 fps with 30 images for each exposure time at each time point.

Mice were imaged for 20 minutes prior to antibody injection in order to ensure stabilization under anesthesia. Animals received either α -Ly6G or isotype control antibodies (4mg/kg,

intraperitoneal). The mice were imaged for 3 hours post-injection.

Speckle contrast values, K , were fitted to the equation:

$$K^2(T, \tau_c) = \beta \rho^2 \frac{e^{-2x} - 1 + 2x}{2x^2} + 4\beta \rho(1 - \rho) \frac{e^{-x} - 1 + x}{x^2} + v_e \quad \text{Eq. 3}$$

$$\text{with } x = \frac{T}{\tau_c}$$

where τ_c is the correlation time, considered to be inversely proportional to average speed of moving scatterers in the sample, T is the exposure time, ρ is the fraction of dynamically scattered light, v_e accounts for any noise in the system, and β is a normalization constant indicating the mismatch between speckle and pixel sizes. The β parameter is estimated using the simpler form of the equation where ρ is considered to be 1:

$$K^2(T, \tau_c) = \beta \frac{e^{-2\frac{T}{\tau_c}} - 1 + 2\frac{T}{\tau_c}}{2(\frac{T}{\tau_c})^2} + v_e \quad \text{Eq. 4}$$

Fractional blood flow changes are proportional to the inverse of the correlation time image divided by a baseline image taken immediately before antibody injection.

Additional behavior experiments

Forced swim test: The forced swim test measured depression-like behavior. Mice were individually placed in a 4-L glass beaker filled with 2.5 L of 25°C water. Mice were allowed to adjust for 1 min and then were evaluated for 6 min. An experimenter blind to the genotype and treatment analyzed the videotaped behavior and scored the immobility time, defined by the absence of active, escape-oriented behaviors such as swimming, jumping, rearing, sniffing, or diving.

Balance beam walk: The balance beam walk measured motor coordination and balance by scoring the ability of the mice to traverse a graded series of narrow beams to reach an enclosed safety platform. The beams consisted of long strips of wood (80 cm) with a round cross section of 12- or 6-mm diameter. The beams were placed horizontally, 40 cm above the floor, with one end mounted on a narrow support and the other end attached to an enclosed platform. Bright light illuminated the end of the beam where the mice started. Mice received

three consecutive trials on each of the round beams, in each case progressing from the widest to the narrowest beam (15 min between each trial). Mice were allowed up to 60 s to traverse each beam. The time to traverse each beam and the number of times either hind paw slipped off each beam were recorded for each trial. Analysis of each measure was based on the mean score across all trials for that mouse at that time point. Experimenters were blinded to the genotype and the treatment of the mice.

Extraction of network topology and vessel diameters from mouse anatomical dataset

One large postmortem dataset from the vibrissa primary sensory (vS1) cortex in mouse previously obtained by Tsai et al²¹ and Blinder et al.⁴⁴, was used for this study ($\sim 1 \text{ mm}^3$ and $\sim 15,000$ vessel segments). In brief, this dataset was obtained by filling the vessels with a fluorescent indicator, extracting the brain and imaging with 2PEF from the pial surface to near the bottom of cortex. In this dataset, penetrating arterioles and ascending venules that reached the pial surface were identified by following their connections to a large cerebral arteriole or venule. We further labeled subsurface vessels in three classes: arterioles, capillaries, and venules. Starting with the surface and penetrating arterioles (venules) vessels were classified by iteratively seeking all vessels with diameter above $6 \mu\text{m}$ connected to any previously labeled arteriole (venule). All remaining vessels were labeled as capillaries. The diameter threshold was manually chosen as the smallest integer diameter value which resulted in arteriolar and venular trees that exhibited no loops, in contrast to the very looped capillary network.

Due to post-mortem shrinkage, vessel diameters in this mouse dataset were smaller than those measured *in-vivo*, so required rescaling^{21,44,53}. As blood flow is highly dependent on vessel diameters, two successive corrections were applied. First, a monotonically increasing function, which tends to one at large diameters, was applied to all vessels diameters:

$$d = d_0 + A \cos\left(\tan^{-1}\left(\frac{d_0}{B}\right)\right), \quad A > 0, \quad B > \frac{2}{3}A \sin\left(\tan^{-1}\left(\frac{1}{\sqrt{2}}\right)\right) \quad \text{Eq. 5}$$

where d is the corrected diameter and d_0 is the diameter extracted from the image stack. A and B are constrained parameters calculated so that the corrected vessel diameter

distribution matched *in vivo* measurements from two photon microscopy²¹, as shown in Supplementary Fig. 15b. This function ensures that the hierarchy of diameters in the network is preserved and the larger vessels are not rescaled. For the network represented in Fig. 6a, $A=1.4$ and $B=10.3$, so that the diameter threshold for capillary vessels becomes $7.2 \mu\text{m}$. A second depth-dependent correction was then applied to the diameter of arterioles and venules:

$$d = d_0(az + b) \quad \text{Eq. 6}$$

where z is the depth below the cortical surface and a and b are parameters determined so that the diameters of the trunks of the penetrating arterioles and ascending venules matched *in-vivo* measurements⁶¹. For the network represented in Fig. 6a, these parameters were $a=-0.0014 \mu\text{m}^{-1}$ ($-9.36\text{e-}4 \mu\text{m}^{-1}$) and $b=2.54$ (2.02) for arterioles (venules).

Extraction of network topology and vessel diameters from human anatomical dataset

The dataset used was previously obtained by Cassot et al.⁶² and Lauwers et al.²² from thick sections ($300 \mu\text{m}$) of a human brain injected with India ink from the Duvernoy collection⁶³. The brain came from a 60-year old female who died from an abdominal lymphoma with no known vascular or cerebral disease. It corresponds to a large volume (6.4 mm^3 of cerebral cortex) extending across 20.8 mm^2 along the lateral part of the collateral sulcus (fusiform gyrus) extracted from Section S2 in Lauwers et al.²², and includes a total of 27,340 vessel segments. The mean radius and length of each segment were rescaled by a factor of 1.1 to account for the shrinkage of the anatomical preparation. The main vascular trunks were identified manually and divided into arterioles and venules according to their morphological features, following Duvernoy's classification^{63,64}. Following Lauwers et al.²² and Lorthois et al.²³ as in the mouse data sets, arterioles (venules) were defined by iteratively seeking all vessels with diameter above $9.9 \mu\text{m}$ connected to any previously identified arteriole (venule), so that no loops were present. All remaining vessels were classified as capillaries.

Synthetic network generation

The synthetic periodic network of order three (i.e. three edges per node) was generated to match the mouse network parameters. A 1-mm³ vascular network was constructed by replication of a simple periodic network (Supplementary Fig. 15c). Capillary diameters and lengths were uniform and were set to the averages for the mouse network. A single penetrating arteriole and ascending venule (with diameters set to the averages from the mouse network) served as inlet/outlet. The distance between the inlet and outlet corresponded to the average distance between penetrating arterioles and ascending venules from the mouse dataset.

Blood flow simulations

The methodology for simulating blood flow in these intra-cortical vascular networks has been presented in detail in Lorthois et al.²³. Briefly, the network was represented by a graph in which edges represent vessel segments between branches that are characterized by an average diameter and length. We used a one-dimensional (analogous to electric circuit models) nonlinear network model that was slightly modified from Pries et al.⁶⁵ to handle large networks for the flow simulations. Using an iterative procedure, the model takes into account the complex rheological properties of blood flow in the microcirculation (Fåhræus, Fåhræus-Lindqvist, and phase separation effects). These effects are modeled using empirical descriptions^{66,67} deduced from experiments in rats. The model was used to calculate the flow and hematocrit in each vessel and the pressure at each intersection of vessels. For the human dataset, the parameters for the empirical descriptions of the Fåhræus, Fåhræus-Lindqvist and phase separation effects were re-scaled in order to account for the difference in characteristic size between human and rat RBCs, as proposed by Lorthois et al.²³ and Roman et al.⁶⁸. This simulation approach has no free parameters.

Boundary conditions: Physiologically realistic pressure drops of 60 mmHg, as measured in rats⁶⁹ and estimated in humans²³, were imposed between all arteriolar and venular trunks feeding and draining the computational volume, while a no-flow condition was imposed on deeper arteriolar or venular vessels that intersected the lateral boundaries of the simulated

volume. A constant discharge hematocrit of 0.45, corresponding to a typical value of the systemic hematocrit, was also imposed in arteriolar trunks. Moreover, a pseudo-periodic boundary condition was applied to all capillaries at the lateral boundaries, as illustrated in Supplementary Fig. 15c. Fictitious vessels were created that link capillaries intersecting opposing faces in a semi random fashion. A grid was created on the two faces and refined until, for a given cell, each capillary on one face was matched with at most 2 capillaries on the opposing face, allowing the creation of fictitious bifurcations. Once the optimal grid was found, the closest neighboring vessels from the opposing faces were connected together. The length of the resulting fictitious vessels was set to 50 μm and their diameters to the average diameters of the connected capillaries. This pseudo-periodic boundary condition is similar in spirit but simpler and more computationally effective than the one recently introduced by Schmid et al.⁷⁰. Finally, a no-flow boundary condition was applied to all vessels intersecting the bottom face of the domain. We also compared the results with no-flow boundary conditions for all capillaries at the lateral boundaries.

Simulating stalls: In order to study the influence of capillary stalling on cerebral blood flow, a given proportion of capillaries in each network was randomly occluded. To simulate occlusion, the radius of the selected vessels was divided by 100. This resulted in a large increase of the hemodynamic resistance, of order 10^8 , and a similar decrease of the computed flow through these vessels. At least five repetitions were performed for each proportion of stalled capillaries and each set of conditions considered. On the mouse data, about 1000 simulations in total were run on a 32-core Intel(R) Xeon E5-2680 v2 @ 3.3 GHz for a total computational time of ~ 170 hours. For human data set, about 100 simulations were run on the same machine for a total computational time of ~ 50 hours.

Histopathology

Immunohistochemistry was performed on the brains of mice chronically treated every third day for 4 weeks with either α -Ly6G antibody or isotype control (Iso-Ctr n=5, α -Ly6G n=4). A single paraformaldehyde-fixed hemisphere of each brain was cut into 40 μm thick sagittal sections.

Every sixth section from each mouse was stained with 1% Thioflavin-S (T1892, Sigma) for 10 min at room temperature and washed twice with 80% ethanol for 2 min. The sections were mounted using Fluoroshield with DAPI (F6057, Sigma). Images were taken using confocal microscopy (Zeiss Examiner.D1 AXIO). For each image, the background was subtracted using the ImageJ background subtraction plugin (Rolling ball with 7 μ m radius). Images were then manually thresholded, using the same threshold for all sections from a given mouse. Appropriate thresholds varied mouse to mouse and were set to ensure that the smallest Thioflavin-S labeled objects that morphologically appeared to be an amyloid plaque remained above threshold. Cortical and hippocampal regions of interest were defined in each section anatomically, and the fraction of pixels above threshold was determined across all sections for these regions of interest. All image processing was done blinded to treatment group. As a second measure of amyloid deposition, we manually counting the number of Thioflavin-S positive amyloid plaques in the cortex and hippocampus, again across all sections and while blinded to the treatment group. All sections were stained and imaged in parallel. Artifacts such as bubbles were eliminated from analysis by manually excluding these regions.

Sample size determinations

In most cases, sample sizes for comparisons across groups were determined from power analysis calculations. The data on capillary stall incidence in Fig. 1c and d in APP/PS1 vs. wt mice is significantly overpowered, as this data includes experiments that yielded the initial discovery of elevated capillary stalling in APP/PS1 mice as well as all subsequent baseline data. For measurements of the cellular cause of capillary stalls (Fig. 2a and b), we anticipated finding ~20 stalls per mouse (~2% of 1,000 characterized capillaries). Assuming each possible stall cause was equally likely and wanting to resolve these percentages to $\pm 10\%$ at a 95% confidence interval, then binomial statistics implied the need for ~100 capillary stalls, or about five mice. For 2PEF measurements of penetrating arteriole blood flow (Fig. 3b and c), ASL-MRI measurements of cortical perfusion (Fig. 3d and e), and behavioral experiments (Fig. 4) comparing APP/PS1 and wt mice before and after antibody treatment, we used a matched-pairs study design, estimates of measurement variability from published and

preliminary data, and estimates of minimal biologically-relevant effect size to determine minimum group sizes using G*Power. This yielded minimum group sizes of 5 mice for 2PEF measurements of penetrating arteriole blood flow and 10 mice for ASL-MRI and behavioral experiments. In subsequent experiments with other mouse models and other treatments we continued using these group sizes.

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