Supplementary file 2 (Additional methodological details)

Microvessel isolation and fractionation. Rat cortical microvessels were isolated as previously described (Tome et al. 2015. J. Neurochem. 134:200). Briefly, animals were euthanized, the cortex was excised and the meninges and choroid plexus removed from the cortex. The cortex was minced and homogenized in ice cold buffer and the microvessels separated from the lipids using a centrifugation step (20 min at 5800 x g) in 30 % Ficoll. The pellet containing the microvessels was resuspended and filtered through a series of nylon mesh filters so that vessel segments between the sizes of 40 µm and 300 µm were retained. Each sample contained vessels from a pool of 3 rats. Multiple 3 rat pool samples were combined (as indicated in specific figure legends) when additional material was needed for a particular measurement. This procedure results in a 9-fold enrichment of endothelial cell GLUT1 over that found in brain homogenates (McCaffrey et al2007. J. Neurochem. 103:2540). The samples also contain pericytes, astrocyte end feet and red blood cells, but little other identifiable tissue.

Aliquots of microvessel homogenate were diluted in buffer and subsequently mixed with 60% OptiPrep to make a final concentration of 35% OptiPrep. The resulting OptiPrep/microvessel homogenate was overlaid with a discontinuous stepwise gradient consisting of 30/25/20/15/10% OptiPrep in buffer. Gradients were centrifuged and fractionated from the top into 24 aliquots as previously described (Tome et al. 2015. J. Neurochem. 134:200). Each gradient contained 300 µg microvessel homogenate protein.

Microscopy sample preparation. Isolated microvessels from a pool of 3 rats were fixed in 10% neutral buffered formalin and then embedded in paraffin. The paraffin block was cut into 3 µm sections and the sections mounted on glass slides by the Tissue Acquisition Cellular/Molecular Analysis Shared Resource (TACMASR) at the University of Arizona on a fee for service basis.

Integrity of the tissue was confirmed on a section that was stained with hematoxylin and eosin (H & E) and inspected via brightfield microscopy.

Cholesterol assay. An equal volume aliquot of each fraction was removed. Each aliquot was incubated with cholesterol esterase and cholesterol oxidase to release H_2O_2. The H_2O_2 was detected via fluorescence (EX = 550 nm/EM = 590 nm; Tecan GENios, Mannedorf (SWI) following coupling to the Amplex® Red reagent.

Immunoprecipitation. Immunoprecipitation experiments were designed as shown below (Immunoprecipitation experimental design).

Immunoblots. Gradient fractions or the eluates from the co-immunoprecipitations were separated on SDS-PAGE gels and transferred to membranes as previously described (Tome et al. 2015. J. Neurochem. 134:200). Blots were incubated with primary and secondary antibodies specific for the protein target. The blots were then incubated with chemiluminescence reagents and the bands visualized by exposing to film or detecting the signal with a ChemiDoc Touch Imaging system (Bio-Rad) according to the manufacturer's instructions.

Immunoblot and microscopy image processing and analysis. Films or ChemiDoc printouts were scanned or the ChemiDoc images were exported as TIF files. Bands were quantitated using the algorithm in FIJI (Schindelin et al. 2012. Nat. Methods 9:676). The images were cropped and auto contrast was applied to the entire cropped portion prior to compiling the figures. All images for immunofluorescence or PLA were acquired using the same settings as the no primary antibody controls using the Leica DMI6000B microscope. Images were exported as TIF files containing a micron scale. Images were cropped in Photoshop (Adobe Systems, San Jose, CA). The immunofluorescence images were split into single color images using FIJI (Schindelin

et al. 2012. Nat. Methods 9:676). The figures were assembled from the single color images. In some cases, the brightness and contrast were increased in the merged image, prior to channel splitting, to enhance printing. Images from samples with primary antibodies received the same adjustments of brightness and contrast as the corresponding no primary antibody controls. Colocalization of signal in the images was measured using the Coloc2 plug in in FIJI on the z-slice that represented the best possible focal plane in each of the three channels (RGB) as described in detail previously (Tome et al. 2016. J. Cereb. Blood Flow Metab. 36:1913). Representative deconvolution images for the figures were prepared by creating a MAX intensity projection of 5 slices centered around the z-slice with the best focal plane. Brightness and contrast were enhanced for illustration of a particular feature.