SUPPLEMENTARY METHODS

Free hemoglobin analysis

The free hemoglobin content was estimated by preparing blood or clots in an identical manner as for animal injection, but preparations were then expelled into a 1.5-ml Eppendorf tube and centrifuged at 1,000*g* for 10 min within 2 h. The serum was collected and frozen at -80°C in light-protected tubes. After thawing to 25°C, samples were exposed to Drabkin's reagent (Sigma Aldrich), which converts hemoglobin moieties to cyanmethemoglobin, according to manufacturer's instructions. Absorbance at 540 nm was then measured by spectrophotometry. Each sample was measured in triplicate in 2 plates and absorbance measures were averaged. Total hemoglobin concentration was determined from a standard calibration curve.

Immunoblotting

Brain slices were thawed and 50-100 mg of cortical gray matter was homogenized in 200 µl of RIPA buffer (Thermo Fisher Scientific) in an Eppendorf tube fit with a small homogenizer, followed by pipetting 10 times with a syringe and 27-g needle. 100 µl of lysate was saved for RNA isolation, and the remaining 100 µl was diluted with 400 µl RIPA buffer and continuously lysed at 4°C for 20 min on a rotator, then centrifuged at 11,000*g* at 4°C for 10 min. The supernatant was collected into a new 1.5-mL tube and the pellet discarded. Protein concentration was measured by BCA assay kit (Thermo Fisher). 15 µg of protein underwent electrophoresis in a 4-15% SDS gradient polyacrylamide gel and was then transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Membranes were blocked in 5% non-fat dry milk in 15 mM Tris-HCl, pH 7.4, 150 mM NaCl and 0.1% Tween 20 (TBST) for 4 hr at room temperature (RT) followed by overnight incubation with the primary antibody (COX2, Abcam, 1:1000; GAPDH, Calbiochem, 1:10,000) in the blocking solution at 4°C. After washing three times with TBST, the membranes were incubated in horseradish peroxidase-conjugated secondary antibody (1:3000) for 2 hr at RT. Immunoreactivity was visualized using enhanced chemiluminescence (Thermo Fisher). Protein bands were scanned and quantified with ImageJ software.

RNA Isolation and Real-Time PCR

The 100-µl tissue lysate was mixed well with 1 ml of TRIzol reagent (Life Technologies) and incubated for 5 min at RT. After adding 200 µl of chloroform (Fisher Scientific), the mixture

was shaken vigorously by hand for 15 s followed by 2-min incubation at RT. The homogenate was then centrifuged at 12,000*g* for 15 min at 4°C. The upper phase was carefully transferred to a new Eppendorf tube and mixed with an equal volume of 70% ethanol, then loaded in a spin cartridge from PureLink RNA mini kit (Ambion). RNA was purified and DNA-free following the kit protocol. The RNA concentration was measured by Nanodrop and 1 µg RNA was used to make 20 µl of cDNA by using Superscript III RT-PCR kit (Invitrogen) according to manufacturer's guidelines. Then, 1.5 µl of cDNA was used to set up each 20-µl RT-PCR reaction mixture following SYBR Green Master Mix manual (Thermo Fisher). Primers for RT-PCR were as follows: GAPDH forward 5'-GCTTCTACTGGTGCTGCCAAGG-3' and reverse 5'-TCAGGTCCACAACCGACACG-3'; COX-2 forward 5'-CCCGATTCAAAGGAAGTTGTGG-3' and reverse 5'- CTGATGGGTGAAGTGCTGGG-3'. Real-time PCR was performed using the ABI StepOne Plus system.

Immunofluorescence staining

Brain tissue was fixed in 4% paraformaldehyde overnight and then processed serially in 10%, 20%, and 30% sucrose followed by 1:1 ratio of 40% sucrose and OCT freezing compound. Frozen tissue was then sectioned at 12 µm by cryostat. Sections were warmed to 25°C and permeabilized with 0.25% triton in PBS for 10 min. They were then blocked by 10% goat serum for 1 hr at 25°C and incubated overnight at 4°C in primary antibody (rabbit anti-cyclooxygenase-2, 1:500 in 10% goat serum, Abcam). Sections were washed with 0.1% triton in PBS and incubated with fluorescence secondary antibody (goat anti-rabbit Alexa Fluor 488, Invitrogen) at 25°C for 2 hr in the dark, followed by washing and staining with DAPI (10 µg/mL in PBS) for 10 min in the dark. Sections were mounted using DPX mounting media (Electron Microscopy Sciences) and images were obtained with an EVOS FL auto fluorescence microscope (Thermo Fisher).