SUPPLEMENTAL MATERIAL

Microglial Calcium Waves During the Hyperacute Phase of Ischemic Stroke

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Expanded Materials & Methods

Mice

The Cre-dependent GCaMP5- and tdTomato-expressing (PC::G5-tdT) mouse line has been maintained as a homozygous colony; the homozygous PC::G5-tdT females were mated with the homozygous Aif1(Iba1)-IRES-Cre (Iba1-Cre) males to generate doubly heterozygous experimental animals. The Cx3cr1-CreER animals were purchased from the Jackson laboratories and maintained as a compound double homozygous Cx3cr1-CreER; PC::G5-tdT colony. These males were crossed with homozygous PC::G5-tdT females to generate experimental animals that were heterozygous for the Cx3cr1-CreER allele, and homozygous for the PC::G5-tdT reporter. Genotyping was performed using Transnetyx services. All experiments were reviewed and approved by the University of Utah and University of Virginia IACUC Committees.

Middle cerebral artery occlusion (MCAo)

Permanent middle cerebral artery occlusion was performed by the Koizumi method.[45] Under isoflurane anesthesia, the right side arteries involved in the blood supply of the brain were exposed, including the common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA). The ECA was tied with a 6-0 silk suture, then proximal trunk of the CCA was tied, and a microclip (15911, WPI) was placed on the ICA. Next, a small incision was made in the CCA using microscissors and a 6.0 monofilament (6022910PK10, Doccol Corp) was inserted into the ICA. The filament was advanced until detecting resistance, indicating occlusion of the middle cerebral artery (MCA). The occluding filament in the CCA was tied to prevent repositioning. The skin was closed with 4-0 suture, the animal received Buprenorphine SR (0.5-1.0 mg/kg s.c.) and was allowed to recover on a thermal blanket. Next, the stroked and cranial window-implanted mouse was sedated with isoflurane, placed in the headpost holder and transferred on the platform with the headpost holder to the microscope stage and maintained under light anesthesia with 1-1.5% isoflurane.[15]

Histology

The 2,3,5-triphenyltetrazolium chloride (TTC) staining procedure was performed as previously described.[15] Twenty four hours after the MCAo occlusion, at the end of the imaging session, the mice were euthanized with an intraperitoneal injection of Euthasol. The brains were harvested and sliced at 1 mm-thick coronal slices using the brain matrix (ASI Instruments). Next, the slices were placed in the TTC solution (500 mg of TTC mixed with 25 ml of PBS) for 25 minutes. Finally, the slices were moved to a 4% paraformaldehyde. The stroke region was defined as a white portion of the brain's slice. The stained slices were photographed with an Olympus stereoscope equipped with a CCD camera.

Drug administration

Lipopolysaccharides (LPS, Sigma L4391) were dissolved at 0.5 mg/mL in sterile saline and frozen at -20°C. Twelve hours before the two-photon imaging of microglial calcium, the reporter mice were injected LPS i.p. at 1mg/kg, while the controls received saline.

The CM-EX-137-SDD CRAC channel inhibitor, provided by CalciMedica, was prepared and administered as follows: First, a solution of 0.5% methyl cellulose (Sigma M0262) + 1% Tween 80 (Sigma P8074) was made by heating the appropriate amount of water to 80°C. Methylcellulose (MC) was gradually added under constant stirring, then Tween 80 and sufficient amounts of water were added to achieve the desired final concentration of 0.5% MC and 1% Tween 80 (w/w). The final clear solution was kept at 4°C until use. A suitable amount of spray-dried dispersion (SDD) of CM-EX-137 substance (typically 5-10 mg) was weighed and suspended in 0.5% MC, 1% Tween 80 (at 3 mg drug per 200 µL vehicle). A homogeneous suspension was achieved by repeated sonication (5-6 bursts of 1-2 second duration) using Qsonica Q55 Sonicator with the CL-188 microtip probe. Control vehicle was prepared with HPMCAS (Hypromellose Acetate Succinate, also known as Shin-Etsu AQOAT). (HPMCAS is the inert material that CM-EX-137 is spray dried onto, it provides a backbone for compound adherence that dissolves in the small intestine). The control vehicle was prepared at the same concentration as drug (3 mg HPMCAS in 200 µL 0.5% MC, 1% Tween 80) and sonicated to emulsify the suspension. The drug or vehicle were administered by oral gavage using disposable 20ga polypropylene feeding tubes with soft elastomer tips (Instech Laboratories FTP-20-38) 4-5 hours before the calcium imaging experiments. The bioavailability of the SDD is much greater than other forms. CalciMedica previously showed in the rat that after oral administration of CM-EX-137-SDD the plasma level of CM-EX-137 first plateaued at 4 hours and the concentration of the compound was significantly higher in the brain than in plasma.[22] All animals (4 males and 2 females) used in these experiment were of Cx3cr1_CreER/+; PC::G5-tdT/PC::G5-tdT genotype. To induce GCaMP5 and tdTomato expression, they received two consecutive tamoxifen injections, within 48 h, at 6 weeks of age (100 mg/kg in corn oil, i.p.) and they were 15 weeks old when used in the experiment. The body weight of the animals ranged between 25-32 g. The CM-EX-137 drug concentration was adjusted to 0.75 mg per 30 g bodyweight (i.e. 25 mg/kg CM-EX-137). Because the active compound only represents 25% of the total weight of the SDD formulation (the other 75% is inert HPMCAS material) we dosed the mice at 3.0 mg of CM-EX-137-SDD per 30 g body weight in 200 µL of 0.5% MC, 1% Tween 80 vehicle (corresponding to 100 mg/kg CM-EX-137-SDD). The drug was very well tolerated at this dosage, the animals recovered from imaging experiments showing normal ambulatory activity and feeding behavior.

Two-photon imaging and image processing

The initial high-resolution, low-speed (1000 x 1000 pixel, 0.2 Hz) two-photon recordings were acquired with the Prairie Ultima IV system in the Cell Imaging core at the University of Utah. The Coherent Chameleon infrared laser was tuned to 920 nm and the signal with acquired with multi-alkali detectors in the green and red channels. The 16x 0.8 NA Nikon objective was used with 1x or 3x digital zoom.

The high-speed imaging experiments were performed with the Olympus FVMPE-RS multiphoton system in the Brain Immunology and Glia (BiG) Center at the University of Virginia.

The system was equipped with sensitive GaAsP detectors and a resonant scanner; the Mai Tai DeepSee laser was tuned to 920 nm. The cranial window-implanted mice were anaesthetized with 1% isoflurane and mounted on the microscope stage. The body temperature was maintained at 35-37 °C with a heating pad throughout the experiment. The images were acquired with an Olympus 25X NA 1.05 objective lens. The line averaging on the resonant scanner was set to 6 to obtain images with 512 x 512 pixel resolution at 2.5 Hz acquisition speed. A target field of view was selected at a depth between 100 nm and 200 nm. For stroke imaging, multiple 20-min long recording sessions were acquired with each animal. For imaging of KCl-induced CSD, continuous 5-minute long T-series were recorded. To chemically induce spreading depolarization, micropipettes with 20-μM diameter tips were pulled from borosilicate glass capillaries (Sutter instruments) and filled with 1 M KCl. The micropipettes were then inserted in the Nanoliter injector (WPI), mounted on a precise manual manipulator situated on the breadboard platform next to the headpost holder.[1] The micropipette was then lowered into the burr hole. First, baseline was recorded for 1 minute. At 60 sec, 100 nL of 1 M KCl was injected within 1 second though the burr hole to induce spreading depolarizations. Fifteen minutes later, after full recovery from depolarization, another CSD recording in a different field of view was performed, and the procedure was repeated 2-3 times.

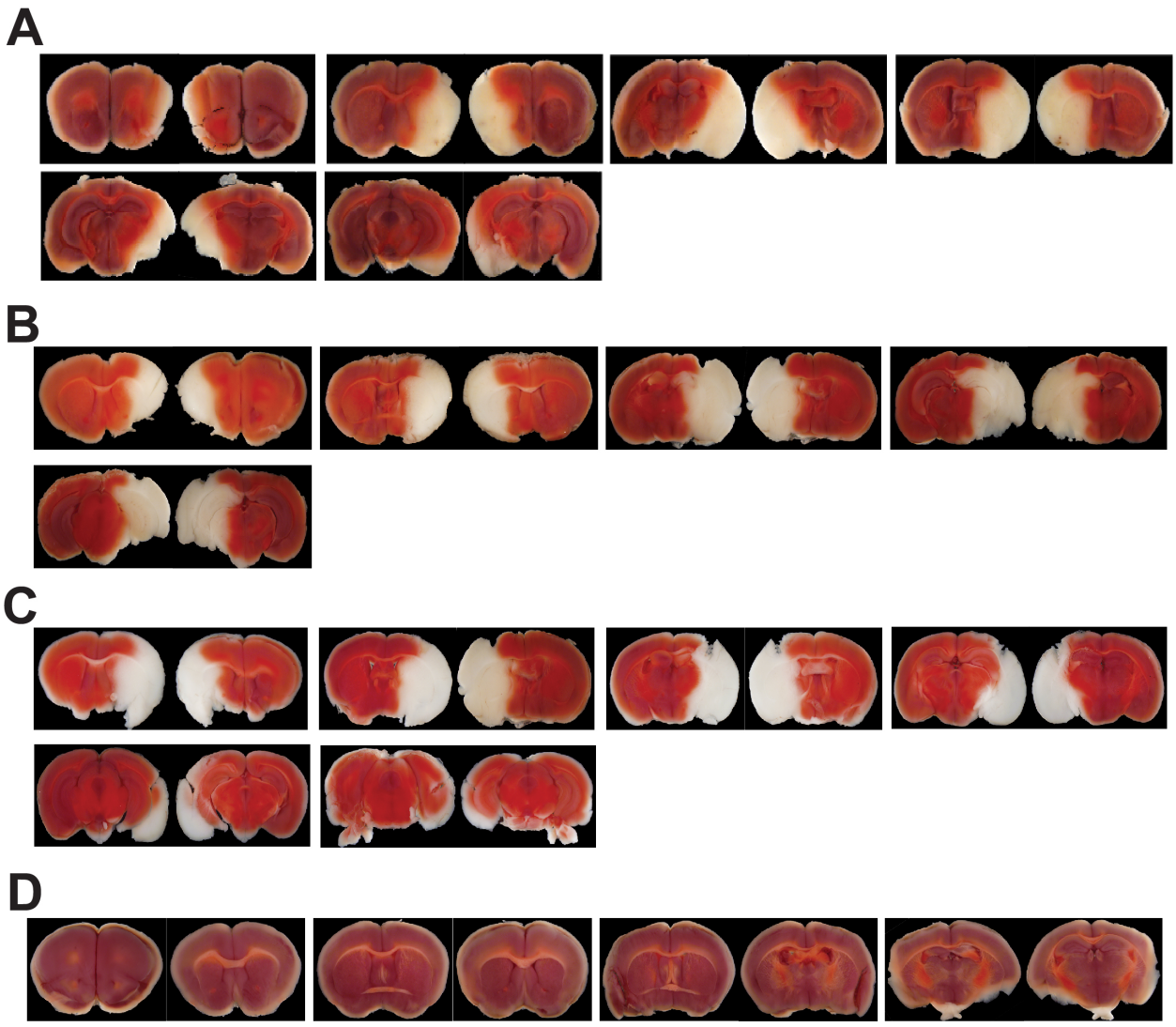
The two-photon T-series were processed with Imaris 9.5 (Bitplane). All recordings were first carefully examined; for each recording, 3-5 microglial cells that responded to CSD with the strongest Ca^{2+} transients were selected. A dynamic region of interest (ROI) was selected in the red (tdTomato) channel to track the somata of selected microglial cell over time. Imaris tracked the ROI using the signal of red (tdTomato) fluorescence, which represents cell morphology. These ROIs perfectly matched the changing shapes of the selected microglial cell bodies. The ROIs were then used to calculate the mean green (GCaMP5) fluorescence intensities, indicating intracellular calcium ($[Ca^{2+}]i$) changes over time. Finally, the fluorescence intensities along with other parameters for all time points were exported in excel spreadsheets.

GCaMP fluorescence signal analysis

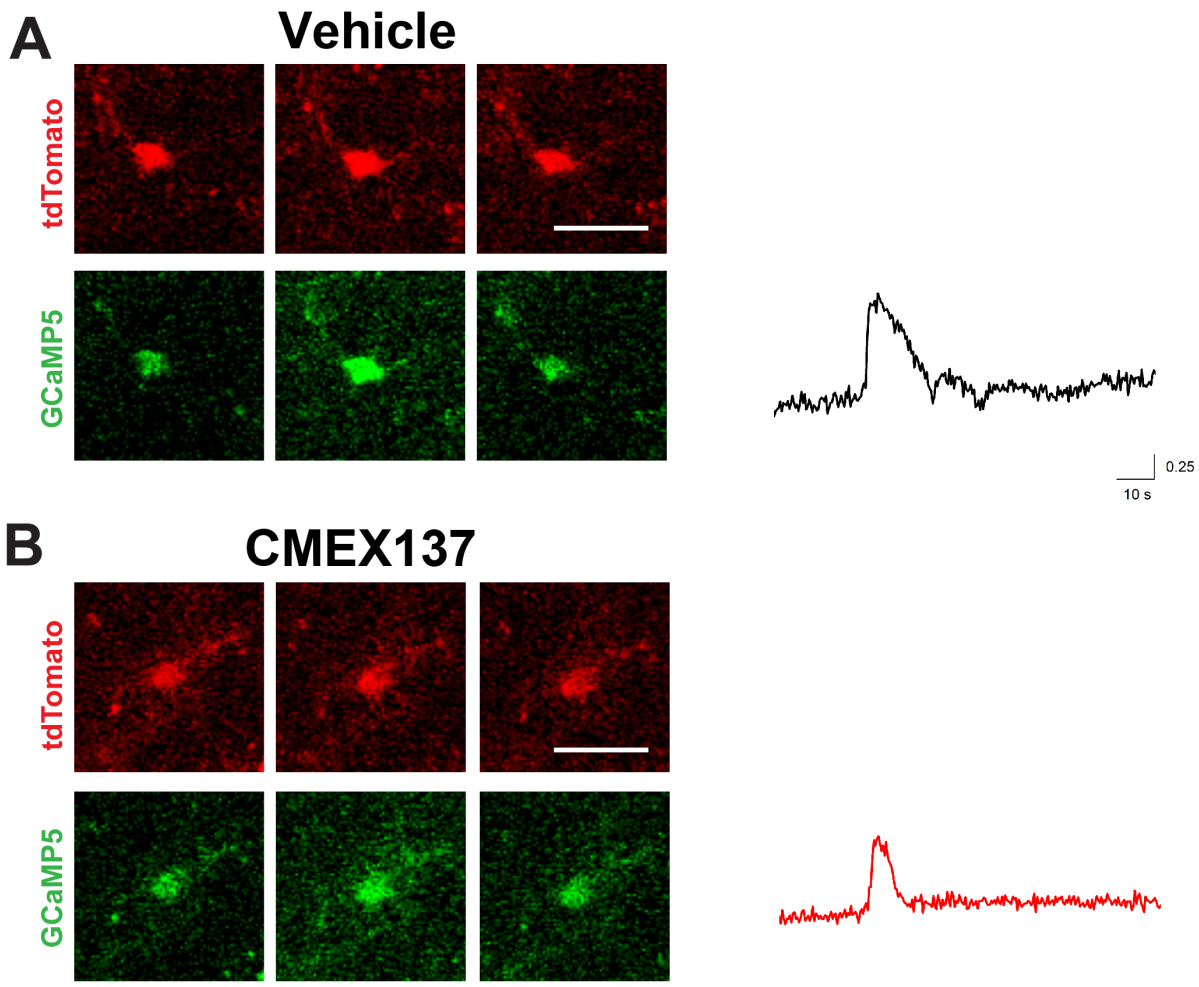
The GCaMP fluorescence signals displayed a high signal to noise ratio (SNR), thus the combined use of a mean filter and wiener filter were used as an initial pre-processing step to reduce noise. Both filters had a kernel size of 5 timepoints (~3.9s), and for the Wiener filter noise was modeled as the standard deviation in the soma's mean fluorescence for each timepoint. Typically, baseline fluorescence is calculated through a simple average of the signal prior to stimulus, but the signals recorded frequently contained fluctuations in background fluorescence before and after the passage of the CSD. Therefore, baseline fluorescence was treated as a function over time, $F_0(t)$, with the final signal trace being $F_{trace}(t) = \frac{F(t)-F_0(t)}{F_0(t)}$, where $F(t)$ is the filtered GCaMP signal. To model background fluorescence, first a median filter that had a kernel size of 101 timepoints (~78s) was used on the signal, which removed the CSD response from the signal. Then a wiener filter with a kernel size of 71 timepoints (~55.38s) was used to remove the remaining high-frequency noise. This resulted in a smooth signal which estimated the background fluorescence independent of stimulation from the CSD. The measurements of microglial responses to CSD stimulation were calculated over a window from 60s to 200s after the imaging began. The peak trace intensity was measured as the maximum value during this window. The duration of the microglial response was measured as the time between the last timepoint where the trace was bellow 0 prior to the peak

value and the first timepoint was below 0 afterwards. The area under curve (AUC) was measured over this same period. We conducted the signal processing and analysis using custom scripts in Python and Jupyter Notebook. All signal processing was done with filters and implementations found within the Python package *scipy*. The algorithm was written by M. Filip Sluzewski and the Jupyter Notebook is available upon request.

Online Figures I – II



Online Figure I. Histological staining of the brains subjected to *in vivo* imaging in Figure 1. **A-C**, TTC staining of the MCAo1, MCAo2 and MCAo3 brains shows infarcted areas (white) in the cortex, striatum, hippocampus and thalamus at the end of the imaging sessions outlined in Figure 1D. Both the front and back side of the stained 1-mm sections are shown in mirror orientations. **D**, Representative sham control brain stained analogously to the MCAo1-3 brains. No infarcted tissue was seen in the brains after sham operation and complete imaging procedures.



Online Figure II. Two photon images of microglia in Cx3cr1-CreER; PC::G5-TdT mice undergoing CSD with pharmacological blockade. A, Representative microglial cell from an animal receiving vehicle p.o. Both the red channel (tdTomato) and green channel (GCaMP5G) signals are shown. The middle panels were captured at peak GCaMP5G fluorescence during CSD, the panels to the left and to the right were acquired 10 s before, and 10 s after the peak, respectively. **B**, Representative images from an animal treated with CM-EX-137 four hours before CSD induction. GCaMP5G signal traces of both cells are shown on the right; control vehicle in black and CM-EX-137 inhibitor in red. Scale bars, 20 µm.

Online Video I - VII

Online Video I. Stroke-induced microglial calcium wave 2 h after MCA occlusion. This video presents a microglial calcium wave from the early phase of ischemic stroke, with limited calcium loading in microglial cell bodies. The video covers 1 minute of the real time recording at 0.2 Hz, played at 5x accelerated speed. It shows an overlay of the red (structural reporter tdTomato) and the green (Ca^{2+}-indicating GCaMP5G) signals. The imaging was obtained with a 16x NA 0.8 lens and 3x digital zoom.

Online Video II. Stroke-induced microglial calcium wave 20 h after MCA occlusion. In the late hyperacute phase of ischemic stroke, substantial calcium transients were observed in the activated microglia. This video presents 2 minutes of the real time recording at 0.2 Hz, accelerated 5 times. It shows an overlay of the red (structural reporter tdTomato) and the green (Ca^{2+}-indicating GCaMP5G) signals. (Captured with 16x NA 0.8 lens, 1x digital zoom).

Online Video III. Stroke-induced microglial calcium wave 12 h after MCA occlusion. The high-speed (2.5 Hz) two-photon recording shown in this video presents a late calcium wave, captured 12 hours after MCAo surgery, with robust calcium transients in microglia. The lag of microglial calcium responses relative to the passing wave front is also clearly visible. This video represents a 1-minute real time recording, played at 2x speed. It shows an overlay of the red (structural reporter tdTomato) and the green (Ca^{2+}-indicating GCaMP5G) signals. Objective: 25x NA 1.05 lens, 1x digital zoom.

Online Video IV. KCl-induced microglial calcium wave (saline control). High-speed imaging of a cortical spreading depolarization (CSD), triggered with distal KCl microinjections, in the naïve, uninflamed cortex. Moderate calcium loading in microglial somas and processes occurred regularly in this setting. The video represents a 1-minute real time recording at 2.5 Hz, played at 2x speed. It shows an overlay of the red (structural reporter tdTomato) and the green (Ca^{2+}-indicating GCaMP5G) signals. Objective: 25x NA 1.05 lens, 2x digital zoom.

Online Video V. KCl- induced microglial calcium wave following LPS administration. Twelve hours after LPS administration, microglial calcium transients become strongly enhanced during the evoked CSD. The video represents a 1-minute real time recording at 2.5 Hz, played at 2x speed. It shows an overlay of the red (structural reporter tdTomato) and the green (Ca^{2+}-indicating GCaMP5G) signals. Objective: 25x NA 1.05 lens, 2x digital zoom.

Online Video VI. KCl-induced microglial calcium wave (vehicle control). Microglial calcium transients during an evoked CSD in the control animal receiving vehicle p.o. 4 hours before the imaging. Moderate calcium activity in the cell bodies as well as in the processes is clearly evident. The genotype of this animal was Cx3cr1-CreER/+; PC::G5-tdT/PC::G5-tdT; it harbored two copies of the tdTomato/CGaMP5G reporter, enabling a higher sensitivity. The video covers a 1-minute real time recording at 2.5 Hz, played at 2x speed. It shows an overlay of the red (structural reporter tdTomato) and the green (Ca^{2+}-indicating GCaMP5G) signals. Objective: 25x NA 1.05 lens, 2x digital zoom.

Online Video VII. KCl-induced microglial calcium wave following the CM-EX-137 inhibition. This animal received CM-EX-137 (25 mg/kg p.o.) 4 hours before the imaging. KCl-evoked CSD stimulus elicited much smaller calcium transients in the microglial cells. Similar to the vehicle control, this mouse was of Cx3cr1-CreER/+; PC::G5-tdT/PC::G5-tdT genotype; it harbored two copies of the tdTomato/CGaMP5G reporter, enabling a higher sensitivity. The video spans a 1-minute real time recording at 2.5 Hz, played at 2x speed. It shows an overlay of the red (structural reporter tdTomato) and the green (Ca^{2+}-indicating GCaMP5G) signals. Objective: 25x NA 1.05 lens, 2x digital zoom.

*** Preclinical Checklist**

Preclinical Checklist: Prevention of bias is important for experimental cardiovascular research. ***This short checklist must be completed, and the answers should be clearly presented in the manuscript.*** *The checklist will be used by reviewers and editors and it will be published. See "Reporting Standard for Preclinical Studies of Stroke Therapy" and "Good Laboratory Practice: Preventing Introduction of Bias at the Bench" for more information.*

This study invovles animal models:
Yes

Experimental groups and study timeline

The experimental group(s) have been clearly defined in the article, including number of animals in each experimental arm of the study:	Yes
An account of the control group is provided, and number of animals in the control group has been reported. If no controls were used, the rationale has been stated:	Yes
An overall study timeline is provided:	Yes

Inclusion and exclusion criteria

A priori inclusion and exclusion criteria for tested animals were defined and have been reported in the article:	Yes

Randomization

Animals were randomly assigned to the experimental groups. If the work being submitted does not contain multiple experimental groups, or if random assignment was not used, adequate explanations have been provided:	N/A
Type and methods of randomization have been described:	N/A
Methods used for allocation concealment have been reported:	N/A

Blinding

Blinding procedures have been described with regard to masking of group/treatment assignment from the experimenter. The rationale for nonblinding of the experimenter has been provided, if such was not feasible:	N/A
Blinding procedures have been described with regard to masking of group assignment during outcome assessment:	N/A

Sample size and power calculations

Formal sample size and power calculations were conducted based on a priori determined outcome(s) and treatment effect, and the data have been reported. A formal size assessment was not conducted and a rationale has been provided:	Yes

Data reporting and statistical methods

Number of animals in each group: randomized, tested, lost to follow-up, or died have been reported. If the experimentation involves repeated measurements, the number of animals assessed at each time point is provided, for all experimental groups:	Yes
Baseline data on assessed outcome(s) for all experimental groups have been reported:	Yes
Details on important adverse events and death of animals during the course of experimentation have been provided, for all experimental arms:	Yes
Statistical methods used have been reported:	Yes
Numeric data on outcomes have been provided in text, or in a tabular format with the main article or as supplementary tables, in addition to the figures:	Yes

Experimental details, ethics, and funding statements

Details on experimentation including stroke model, formulation and dosage of therapeutic agent, site and route of administration, use of anesthesia and analgesia, temperature control during experimentation, and postprocedural monitoring have been described:	Yes
Different sex animals have been used. If not, the reason/justification is provided:	Yes

Statements on approval by ethics boards and ethical conduct of studies have been provided:	Yes
Statements on funding and conflicts of interests have been provided:	Yes

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