SUPLEMENTAL MATERIAL

Brain cleanup as a target for post-stroke recovery: the role of RXR in phagocytes

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MATERIAL AND METHODS

All animal studies followed the guidelines outlined in *Guide for the Care and Use of Laboratory Animals* from the National Institutes of Health and were approved by the Animal Welfare Committee of the University of Texas Health Science Center at Houston. All studies were performed using a randomization (coin toss) approach and all the analyses were performed by investigators blinded to the treatment assignments (animals were coded for the group allocation). Animals were fed a standard rodent diet, and housed in standard cages on a 12-hour inverted light–dark cycle. Experiments included male and female animals at ages of 4-6 months. Behavioral analyses were conducted from the hours of 10:00 AM to 4:00 PM.

Mac-RXR$\alpha^{-/-}$ mice. To investigate the role of RXRα in ischemic stroke and in MΦ function, we used conditionally-disrupted RXRα mice in myeloid cells. The experimental mice were progeny of M Lyz-Cre^{+}/RXR$\alpha^{-/-}$ mice crossed with RXRα^{LoxP}. The genotypes of mice were age- and gender-matched littermates, either RXRα^{LoxP} wild-type control (M Lyz-Cre^{-}/RXRα^{LoxP}) or Mac-RXR$\alpha^{-/-}$ (M Lyz-Cre^{+}/RXRα^{LoxP}) [1].

Molecular signaling and genotyping. The following primers (5'- AGG TGT AGA GAA GGC ACT TAG C and 5'-CTA ATC GCC ATC TTC CAG CAG G) were used to genotype M Lyz-Cre mice to produce one 510 bp Cre band using the following PCR condition (25 cycles): 94^{o}C for 2 min, 65^{o}C for 50s, and 72^{o}C for 50s. To genotype RXRα^{LoxP} mice, primers (5'- ACC AAG CAC ATC TGT GCT ATC T, 5'- CAA CTG TAT ACC CCA TAG TGT T, and 5'- ATG AAA CTG CAA GTG GCC

TTG A) were used to amplify a wild-type 600 bp-band and an 800-bp floxed-out band using the following PCR conditions (30 cycles): 94^{o}C for 1 min, 55^{o}C for 30 s, 72^{o}C for 1.5 min[1].

Administration of RXR agonist. For *in vitro* experiments, microglial cells were pre-incubated for 24 h with 0.5 μM bexarotene (BEX, Sigma), and then exposed to dead neurons to assess phagocytosis. DMSO (0.1%) was used for vehicle control. For *in vivo* studies, BEX was first dissolved in DMSO at 10 mg/ml and then diluted in saline (final concentration of DMSO < 3%), which was administered intraperitoneally (i.p.) at 5 mg/kg, first at 24 h after surgery and then once a day for a total of 7 days. DMSO (3%) was used for vehicle control.

Ischemia model in mice. Focal ischemia was induced by left middle cerebral artery (MCA) and left common carotid artery (CCA) occlusion as we described previously[2]. Briefly, all animals were anesthetized with 0.35 g/kg i.p. injection of chloral hydrate. The rectal temperature was monitored and maintained at 36.5±0.5^{o}C during ischemia and the first hour of reperfusion using a feed-forward temperature controller (YSI Model 72, Yellow Springs, OH). Under direct visualization with the surgical microscope, a burr hole was drilled at 1.5 mm rostral to the fusion of the zygomatic arch with the squamosal bone to expose the left MCA rostral to the rhinal fissure. A 0.005-inch diameter stainless steel wire (Small Parts Inc, Miami, FL) was placed underneath the left MCA rostral to the rhinal fissure, proximal to the major bifurcation of the MCA, and distal to the lenticulostriate arteries. The artery was then lifted, and the wire rotated clockwise. The left CCA was occluded using atraumatic Heifetz aneurysm clips. Reperfusion was established at 60 min after occlusion by first removing the aneurysm clips from the CCA, and then rotating the wire counterclockwise and removing it from beneath the MCA. Interruption of flow through the MCA was inspected under the microscope. We experienced no procedural or post-stroke mortality when performing experiments in this study.

Brain atrophy or infarction volume measurement. The infarction volume was measured as previously described[2]. Briefly, the mice were deeply anesthetized by intraperitoneal injection of

0.6 g/kg of chloral hydrate, perfused intracardially with 50 ml of ice-cold PBS and then decapitated. The brains were removed and snap frozen by submersion into -80°C 2-methylbutane. Brains were stored in a -80°C freezer before cryosectioning into five 10 μm sections with 1 mm intervals between two sections. The images of each section were captured using a digital camera, and the tissue loss (cavity size) was indirectly determined by subtracting the areas of ipsilateral side of the brain from the areas of contralateral side of the brain, measured by computer-interfaced NIH ImageJ program. The atrophy volume represented the integrated missing area derived from the five sections.

Neurological functional deficits (NDS) measurement. All behavioral tests in mice were conducted in a quiet and low-lit room by an experimenter blinded with respect to the treatment groups. Pre-tests were performed to exclude animals with abnormal behavior. A battery of behavioral tests, including foot fault, postural flexing, and corner turn, were employed to measure the neurological functional deficits and arrive at a combination score, as reported[2]. All animals survived to the terminal endpoint.

RNA isolation and quantitative reverse transcription-polymerase chain reaction (RT-qPCR). The RNA extraction and RT-qPCR were performed as we described previously[3]. Total RNA from harvested cells was isolated using Trizol reagents. Complimentary DNA (cDNA) was synthesized from 2μg of RNA using amfiRivert Platinum One (GenDEPOT) following the manufacturer's protocol. The SYBR Green-based real-time PCR system was used for gene expression profiling in ischemic brain. Each cycle consisted of 5 s of denaturation at 95℃, 15 s of annealing at 60℃, and 10 s of extension at 72℃ (40 cycles). Mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an internal control. The sequences of primers are listed in Table 1. The expression fold-change was calculated using the delta-delta Ct method.

Primary brain glial culture and microglia isolation. The cortices of 1-2 day-old post-natal pups were dissected and dissociated by triturating as we previously described[2]. Briefly, the dissociated

cells were suspended in DMEM with 10% fetal bovine serum, plated in 75 cm^2 TC flasks, and maintained in a CO_2 incubator (5% CO_2) at 37.0±0.5 ºC. Half of the culture medium was changed every 3 days. After a total of 14-21 days in culture, the astrocytes form a confluent layer signifying that the culture is ready for microglia isolation. The loosely adherent microglia were harvested by shaking (220 rpm for 30 min) and then re-plated onto poly-L-lysine coated plates with or without German-glass inserts at a density of 1-4x10^5 cells/ml.

Phagocytosis assay for dead neurons. Assessing phagocytosis of dead neurons (DNs) by mouse microglia was performed as we reported[4], with minor modifications. Briefly, the isolated cortical neuronal cells from E16-18 prenatal pups were seeded on 60 mm culture dishes and cultured *in vitro* for 1-2 days before being subjected to gamma irradiation (32 Gy) to induce apoptosis. After irradiation, the cells were placed back in the CO_2 cell culture incubator for 48 h. After washing in PBS, the DNs were re-suspended in Neurobasal medium at 1-5x10^8 cells/ml, which were then added into the microglia cultures at a ratio of 50:1 (DNs:Microglia). After 1.5 h of incubation, the non-phagocytosed DNs suspended in the culture medium were removed by aspiration. The microglia-captured (phagocytosed) DNs were immunolabled with neuronal-specific class III β-tubulin (Tuj1) antibody. The microglia were visualized using anti-CD68 antibodies. The nuclei were stained with DAPI. The images were captured by a Zeiss Confocal microscopy LSM 800. The numbers of the $Tuj1^+$ neurons in each microglial cell were counted manually, on still images. Fifty microglial cells from each condition were analyzed. The average number of phagocytized DNs within each microglial cell was calculated and used as the phagocytosis index.

Immunohistochemistry. The immunofluorescent labeling for microglia and DNs were conducted as we described[5]. Briefly, at 1.5 h after adding DNs, the microglia cultures were fixed in 2% PFA for 10 min and incubated in rat anti-mouse CD68 (Serotec) and rabbit anti-mouse Tuj1 (PRB-435P, Covance) at 1:1000 and incubated overnight at 4ºC. Goat anti-rat IgG-Alexa Fluor 488 and

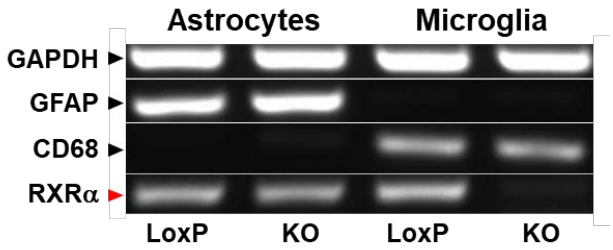
goat anti-rabbit IgG-Alexa Fluor 546 were used to visualize the CD68 and Tuj1 signals, respectively. DAPI was applied to counterstain the cell nuclei.

RNA-sequencing and analysis for microglia. Primary cultured microglia from Mac-RXR$\alpha^{-/-}$ and RXRα^{LoxP} mice were incubated with 0.5 μM BEX or vehicle control (0.1% DMSO) for 24 h before harvesting and isolation of total RNA. Each RNA sample was extracted from $2x10^6$ cells. Three samples were analyzed in each RXRα^{LoxP} group and two samples in each Mac-RXR$\alpha^{-/-}$ group. RNA sequencing, including library construction and data analysis, was performed by Novogene Inc. The libraries were constructed using NEBNext Ultra RNA Library Prep Kit and were quality assessed with Qubit 2.0 before sequencing. After quality control, the clean reads were mapped to the reference genome using STAR software, and the mapping results were visualized with Intergrative Genomics Viewer (IGV). Differential expression analysis was performed using DESeq2 R package; Enrichment analysis, including gene ontology (GO) enrichment and Kyoto encyclopedia of genes and genomes (KEGG) pathway, was done using clusterProfiler software.

Statistical analysis. All data are expressed as mean±SEM unless otherwise stated in the figure legends. All statistical analyses, including correlation analysis, were performed using the GraphPad Prism 7 and InStat programs. Repeated measure two-way analysis of variance (ANOVA) followed by Tukey post-hoc test was used to evaluate differences among groups at different time points in behavioral tests. Two-way ANOVA followed by Tukey post-hoc test was used to analyze data with two grouping variables. Remaining data were analyzed using one-way ANOVA followed by Tukey post-hoc test. Non-paired t-test was used when two groups are compared.

FIGURE I

A



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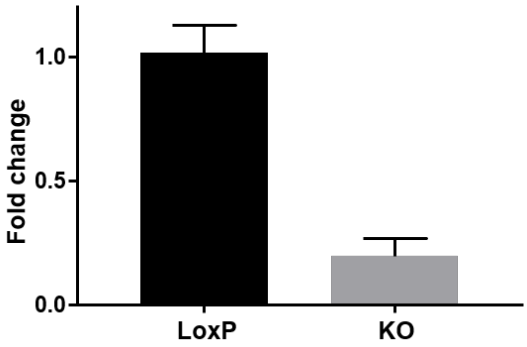

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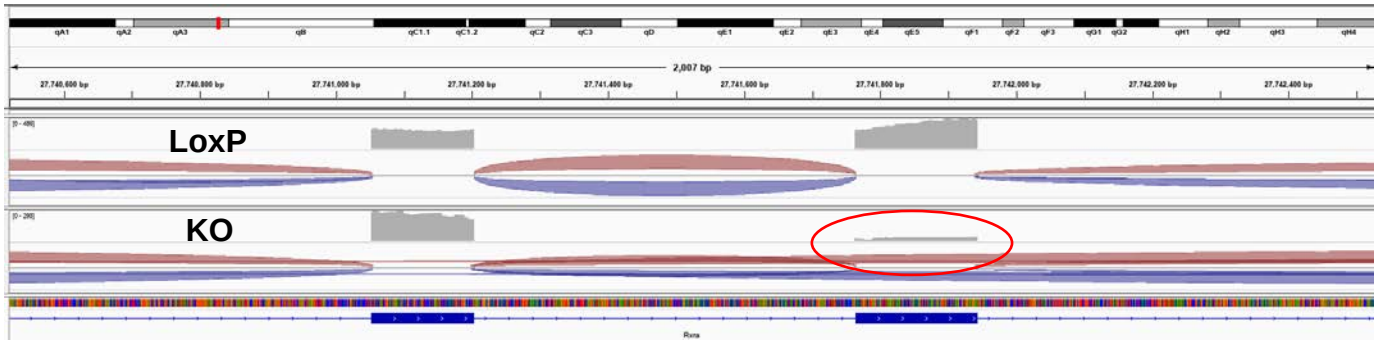


Exon 3 Exon 4

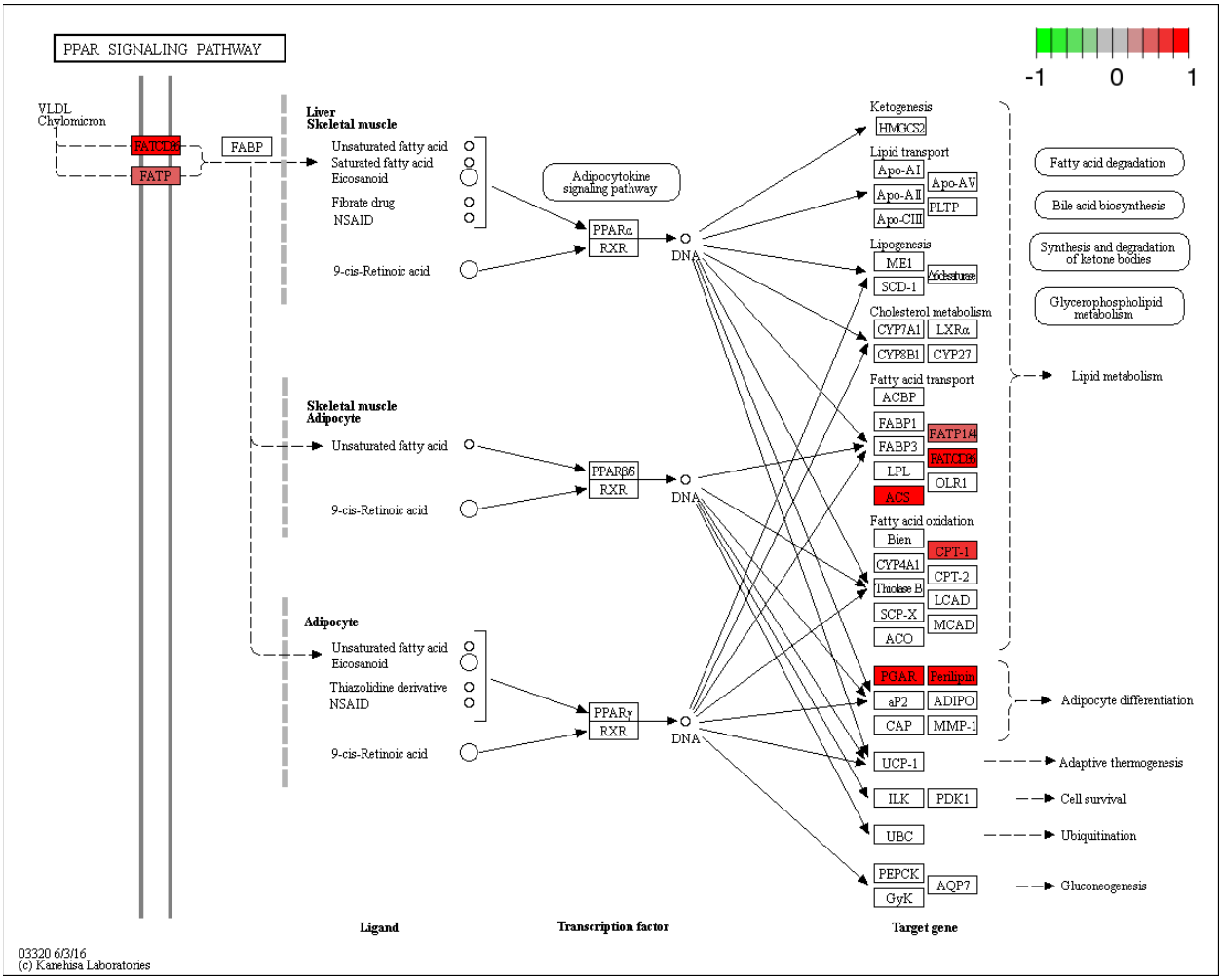
Supplemental Figure I. Three different ways to confirm the efficacy of M Lyz-Cre driven cell-specific RXRα knockout in microglia

(A) Representative RT-PCR gel to establish the RXRα-knockout profile in microglia primary culture and astrocytic primary culture established from RXRα^{LoxP} (LoxP) and Mac-RXRα$^{-/-}$ (KO) pup brain. Expression of CD68 was used as a marker for microglia and GFAP was used as a positive marker for astroglia. GAPDH was used as an internal control. **(B)** Expression of RXRα in microglia primary culture established from RXRα^{LoxP} (LoxP) and Mac-RXRα$^{-/-}$ (KO) pup brain analyzed with real-time PCR. GAPDH was used as the reference gene. The expression fold-change was calculated using the delta-delta Ct method. $P < 0.01$. Three samples were analyzed

in each group. **(C)** Mapping RNA-seq results on genome showed deletion of RXRα exon 4 in primary microglia cultured from Mac-RXR$\alpha^{-/-}$ mice (KO) but not from RXRα^{LoxP} mice (LoxP).

FIGURE II

A


PPAR SIGNALING PATHWAY
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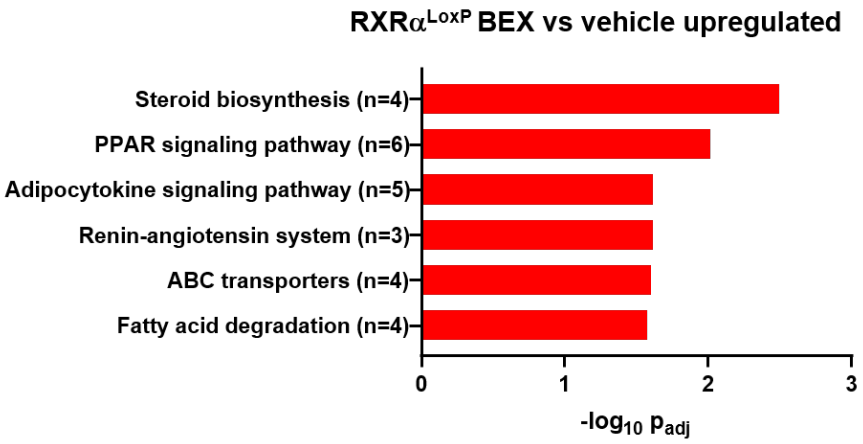
1
VLDL
Chylomicron
FATCD36
FATP
FABP
Liver
Skeletal muscle
Unsaturated fatty acid
Saturated fatty acid
Eicosanoid
Fibrate drug
NSAID
9-cis-Retinoic acid
Adipocytokine signaling pathway
PPARα
RXR
DNA
Skeletal muscle
Adipocyte
Unsaturated fatty acid
9-cis-Retinoic acid
PPARβδ
RXR
DNA
Adipocyte
Unsaturated fatty acid
Eicosanoid
Thiazolidine derivative
NSAID
9-cis-Retinoic acid
PPARγ
RXR
DNA
Ketogenesis
HMGCS2
Lipid transport
Apo-AI
Apo-AII
Apo-CIII
Apo-AV
PLTP
Lipogenesis
ME1
SCD-1
Aldosterase
Cholesterol metabolism
CYP7A1
CYP8B1
LXRα
CYP27
Fatty acid transport
ACBP
FABP1
FABP3
LPL
ACS
FATP1/4
FATCD36
OLR1
Fatty acid oxidation
Bien
CYP4A1
Thiolase B
SCP-X
ACO
CPT-1
CPT-2
LCAD
MCAD
PGAR
aP2
CAP
Perilipin
ADIPO
MMP-1
UCP-1
ILK
PDK1
UBC
PEPCK
GyK
AQP7
Fatty acid degradation
Bile acid biosynthesis
Synthesis and degradation of ketone bodies
Glycerophospholipid metabolism
Lipid metabolism
Adipocyte differentiation
Adaptive thermogenesis
Cell survival
Ubiquitination
Gluconeogenesis
Ligand
Transcription factor
Target gene
03320 6/3/16
(c) Kanehisa Laboratories

B


RXRα^LoxP BEX vs vehicle upregulated
Steroid biosynthesis (n=4)
PPAR signaling pathway (n=6)
Adipocytokine signaling pathway (n=5)
Renin-angiotensin system (n=3)
ABC transporters (n=4)
Fatty acid degradation (n=4)
0
1
2
3
-log10 padj

Supplemental Figure II. RXR agonist enhances PPAR signaling pathway

(A) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis showed increase expression in PPAR signaling pathway between BEX treated RXRα^{LoxP} microglia and vehicle control RXRα^{LoxP} microglia. **(B)** A list of significantly upregulated pathways in KEGG pathway analysis between BEX treated RXRα^{LoxP} microglia and vehicle control RXRα^{LoxP} microglia. Primary cultured microglia from RXRα^{LoxP} mice were incubated with 0.5 μM BEX or vehicle control (0.1% DMSO) for 24 h before isolation of total RNA. Each RNA sample was extracted from 2x10^6 cells. Three samples were analyzed in each group.

1. Nunez V, Alameda D, Rico D, Mota R, Gonzalo P, Cedenilla M, et al. Retinoid x receptor alpha controls innate inflammatory responses through the up-regulation of chemokine expression. *Proceedings of the National Academy of Sciences of the United States of America*. 2010;107:10626-10631
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3. Zhao X, Sun G, Zhang J, Strong R, Song W, Gonzales N, et al. Hematoma resolution as a target for intracerebral hemorrhage treatment: Role for peroxisome proliferator-activated receptor gamma in microglia/macrophages. *Annals of neurology*. 2007;61:352-362
4. Zhao X, Zhang L, Ting SM, Aronowski J. Phagocytosis assay of microglia for dead neurons in primary rat brain cell cultures. *Bio-protocol*. 2016;6
5. Zhao X, Strong R, Zhang J, Sun G, Tsien JZ, Cui Z, et al. Neuronal ppargamma deficiency increases susceptibility to brain damage after cerebral ischemia. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2009;29:6186-6195

*** 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:	Yes
Type and methods of randomization have been described:	Yes
Methods used for allocation concealment have been reported:	Yes

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:	Yes
Blinding procedures have been described with regard to masking of group assignment during outcome assessment:	Yes

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:	No

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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