SUPPLEMENTAL MATERIAL

The single-nucleotide polymorphism *309T>G* in the *MDM2* promoter determines functional outcome after stroke

Supplemental Methods

Animals

Animals were maintained in specific-pathogen free facilities at the University of Salamanca, in accordance with Spanish legislation (RD53/2013) under license from the Spanish government and the European Union (2010/63/EU). Protocols were approved by the Bioethics Committee of the Institute of Biomedical Research of Salamanca. All efforts were made to minimize the numbers of animals used and ensure minimal suffering.

Primary cultures of cortical neurons

Neuronal cultures were prepared from C57BL/6J and p53 null ($p53^{-/-}$, B6.129S2, The Jackson Laboratories, Sacramento, CA, USA) mouse embryo (E14.5) cortices. Neurons were seeded at 2×10^5 cells/cm^2 in Neurobasal medium (Invitrogen, Madrid, Spain) supplemented with 2% B27 (Invitrogen) and glutamine 2 mM (Invitrogen), and incubated at 37ºC in a humidified 5% CO_2-containing atmosphere. Half of the culture medium was replaced with fresh medium every 3-4 days [1]. Neurons were used for the experiments on day 9-10 *in vitro*.

Human monocyte primary culture

10 mL sample of venous blood was used to isolate a highly enriched population of monocytes. Mononuclear cells were first isolated from peripheral blood by Ficoll (Ficoll® Paque Plus, GE Healthcare, Madrid, Spain) density gradient centrifugation. Cells were seeded at 2×10^5 cells/cm^2 in RPMI-1640 medium (Sigma, Barcelona, Spain). After 2 hours, non-adherent cells (non-monocyte) were removed while adherent (monocytes) cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) FBS (HyClone™ Fetal Bovine Serum, GE Healthcare) at 37ºC in a humidified 5% CO_2-containing atmosphere. Cells were used for the experiments on day 2-3 *in vitro*.

Oxygen and glucose deprivation (OGD) protocol

After 9-10 days in culture, neurons were subjected to oxygen and glucose deprivation (OGD) by incubating cells at 37ºC in an incubator equipped with an air lock and continuously gassed with 95% N_2/5% CO_2, for 90 min. The incubation medium (buffered Hanks' solution without glucose: 5.26 mM KCl, 0.43 mM KH_2PO_4, 132.4 mM NaCl, 4.09 mM $NaHCO_3$, 0.33 mM Na_2HPO_4, 2 mM $CaCl_2$, and 20 mM HEPES, pH 7.4) was previously gassed with 95% N_2/5% CO_2 for 30 min. In parallel, neurons were incubated in buffered Hanks´solution buffer containing 5 mM glucose (normoxia condition) at 37ºC in a humidified atmosphere of 95% air/5% CO_2. After OGD, neurons were further incubated in Neurobasal medium at 37ºC in a humidified atmosphere of 95% air/5% CO_2 (reoxygenation) [2].

Small interfering RNA

Specific depletion of MDM2 was achieved by using small interfering double-stranded ribonucleotides (siRNA) designed specifically to target the coding sequence of the mouse Mdm2 mRNA. We used the following siRNA (only the forward strand shown): 5'-CCAGGAGAGUGACGACUAU-3' (nucleotides 1455-1473, GenBank accession number

NM_010786). The siRNA against luciferase 5'- CUGACGCGGAAUACUUCGAUU-3' was used as control siRNA (siControl) [3]. Annealed siRNAs were purchased from Ambion®, Invitrogen®, Thermo Fischer Scientific (Offenbach, Germany).

Cell transfections and treatments
Neurons were transfected with siRNAs (1 pmol) using Lipofectamine RNAiMAX (Lipofectamine® RNAiMAX Transfection Protocol, Invitrogen), following the manufacturer instructions. Neurons were further incubated in culture medium for 48 hours before their use. When indicated, the OGD protocol and incubations were performed in the presence of 10 μM MG132 (Sigma) or 1 μM nutlin-3a (Sigma).

Flow cytometric detection of apoptotic cell death
Neurons were carefully detached from the plates using 1mM EDTA (tetrasodium salt) in PBS (pH 7.4) at room temperature. Cells were stained with annexin V-APC and 7-AAD in binding buffer (100 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$), according to the manufacturer's instructions, to determine quantitatively the percentage of apoptotic neurons by flow cytometry. Triplicates obtained from three different cultures ($3X10^5$ cells each) were analyzed on a FACScalibur flow cytometer (15 mW argon ion laser tuned at 488 nm; CellQuest software, BD Biosciences). The annexin V-APC stained cells that were 7AAD negative were considered to be apoptotic [4].

Quantitative reverse transcription-polymerase chain reaction (RT-qPCR) analysis
Total RNA samples were purified from cells using a commercially available kit (Sigma) and RT-qPCR was performed with Power SYBR Green RNA-to-CT TM 1-Step kit (Applied Biosystems, Township, USA). RT was carried out at 48ºC for 30 min, and PCR conditions were 10 min at 95ºC followed by 40 cycles of 15 s at 95ºC and 1 min at 60ºC using the PrimePCR Assay Trp53 (Mmu #10025636, BioRAD Laboratories, CA, USA) and appropriate forward and reverse primers (0.3 μM), respectively (Thermo Scientific): 5'-CAGCAGCGAGTCCACAGAG-3' and 5'-TCCTGATCCAGGCAATCAC-3' (*Mdm2* Mmu); 5'-GCAGTGAATCTACAGGGACGC-3' and 5'-ATCCTGATCCAACCAATCACC-3' (*MDM2* Hsa); 5´- TCAGCAATGCCTCCTGCACCA-3´ and 5´- GCATGGACTGTGGTCATGAG-3´ (*GAPDH*, Hsa/Mmu). The mRNA levels of each transcript were normalized to the *GAPDH* mRNA abundance obtained in the same sample. The relative mRNA levels were calculated using the ΔΔCt method and were expressed as the fold change between sample and calibrator [5].

Western blot analysis
Cells were lysed in buffer containing 1% nonidet NP40, 5 mM EDTA di-K^+, 20 mM Tris-HCl pH 8.0, 137 mM NaCl, 10% glicerol supplemented with phosphatase inhibitors (1mM Na_3VO_4 and 50mM NaF) and protease inhibitors (100 μM phenylmethylsulfonyl fluoride, 50 μg/ml anti-papain, 50 μg/ml pepstatin, 50 μg/ml amastatin, 50 μg/ml leupeptin, 50 μg/ml bestatin and 50 μg/ml soybean trypsin inhibitor), stored on ice for 30 min and boiled for 5min. Protein concentrations were determined with the BCA (bicinchoninic acid) method, using bovine serum albumin as a standard (BCA Protein Assay kit, Thermo Fisher Scientific). Neuronal extracts were subjected to SDS-polyacrylamide gel (MiniProtean; Bio-Rad). The antibodies used were anti-p53 (1:2000; 2524, Cell Signaling Technology), anti-cleaved caspase-3 (1:2000; Asp175, 9661, Cell Signaling), anti-MDM2 (1:500; 2A10, ab16895, Abcam) and anti-GAPDH (1:40000; Ambion, Cambridge, UK) overnight at 4ºC. GAPDH was used as loading control. After incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (Pierce, Thermo Scientific) or goat anti-mouse IgG (Bio-Rad), membranes

were immediately incubated with the enhanced chemiluminescence SuperSignal West Dura (Pierce) for 5 min before exposure to Kodak XAR-5 film for 1 to 5 min, and the autoradiograms were scanned. Band intensities were quantified using ImageJ software [5].

Co-immunoprecipitation assay
Neurons were lysed in ice-cold buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, and 1% NP-40, supplemented with the phosphatase and protease inhibitors cited in Western blot analysis. Neuronal extracts were clarified by centrifugation and supernatants (500 µg protein) were incubated with 2 µg anti-MDM2 for 4 hours at 4°C, followed by the addition of 30 µl of protein A- sepharose (GE Healthcare) for 2 hours at 4°C. Immunoprecipitates were extensively washed with lysis buffer and detected by Western blot analysis [6].

Immunocytochemistry
Neurons grown on glass coverslips were fixed with 4% (w/v, in PBS) paraformaldehyde for 30 min and immunostained with rabbit anti-cleaved caspase-3 (1:300; Cell Signaling Technology), mouse anti-Map2 (1:300; Sigma), mouse anti-p53 (1:200; 554157, BD Biosciences), and rabbit anti-MDM2 (1:500; ab38618, Abcam) antibodies. Immunolabeling was detected using IgG-Cy2 (1:500) or IgG-Cy3 (1:500) secondary antibodies (Jackson ImmunoResearch Inc.). Nuclei were stained with 6-diamidino-2-phenylindole (DAPI, Sigma D9542). Coverslips were washed, mounted with SlowFade light antifade reagent (Invitrogen) and examined under an Olympus IX81 Spinning disk confocal microscope (Olympus®, Tokyo, Japan) [6].

Transient middle cerebral artery occlusion (tMCAO)
Surgical endovascular insertion of a silicon-coated monofilament (602012PK10; Doccol Corporation, Sharon, MA, USA) was performed to induce transient middle cerebral artery occlusion (tMCAO) for 45 minutes of ischemia, followed by filament removal to allow reperfusion [7]. Briefly, 16-week-old C57BL/6J mice were anesthetized with sevoflurane (4% for induction, 3% for maintenance) in a mixture of O_2/N_2O (30/70%). After surgical exposure of the right carotid artery tree, the filament was inserted through the external carotid artery and advanced through the internal carotid artery until it reached the MCA. The regional cerebral blood flow was monitored during surgery with a laser Doppler probe (Moor Instruments, Devon, UK). After 45 minutes of ischemia, the filament was removed to allow reperfusion. Body temperature was maintained at 37 ± 0.5°C using a heating pad connected to a rectal probe (BAT-12 thermometer; Physitemp Instruments Inc., Clifton, NJ, USA). Mice were then sutured an allowed to recover for 24 hours. Sham-operated mice underwent the same surgical procedure without MCA occlusion. Twenty per cent of mice died after tMCAO surgery (1 mice among 5 mice). The infarct size was measured by 2,3,5-triphenyltetrazolium chloride (TTC) staining. Brain tissues from ipsilateral (ipsi) and contralateral (contra) hemispheres were collected for western blot analysis.

Experimental model of intracerebral hemorrahge (ICH)
Experimental ICH was induced in 16-week-old C57BL/6J mice by injecting bacterial collagenase VII from *Clostridium histolyticum* (Sigma) into the basal ganglia [8]. Mice under sevoflurane anesthesia evaporated in an O_2/N_2O mixture (30/70%) were placed in a stereotaxic frame (model 1900; Kopf, Tujunga, CA, USA) with a digital coordinate readout system (Wizard 550 Readouts; Anilam, Schaumburg, IL, USA). Collagenase (0.1 U in 0.5 µl of saline) was injected unilaterally into the striatum using stereotactic coordinates: +0.9 mm anterior and +2.0 mm lateral to bregma, to a depth of 3.5 mm and at a rate of 0.1 µl/min over

5 min. The needle stayed in place for additional 10 min to prevent reflux. During the procedure, rectal temperature was monitored (BAT-12 thermometer; Physitemp) and maintained at 37 ± 0.5°C. After the surgery, the animals were sutured and placed in a warm environment for recovering for 24 hours. Sham-operated mice underwent the same procedure, including needle insertion, but without collagenase injection. All mice survived to the surgery. Brain tissues from ipsilateral and contralateral hemispheres were collected for western blot analysis.

Patients

An observational prospective study was performed on two independent cohorts of consecutive patients with either a first IS (the IS cohort) or non-traumatic ICH (the ICH cohort) at less than 12 hours after the onset of symptoms (or from the start of sleep in those with symptoms upon awakening), previously independent for daily living activities, during 2011-2013. Patients were admitted to the University Hospital of Santiago de Compostela (Galicia, Spain). Baseline demographic and clinical features of IS and ICH patients are shown in **Table I**. In relation to the IS cohort, patients receiving tissue plasminogen activator (tPA) treatment (59 intravenously and 6 intra-arterially), those included in clinical trials (33), and those showing severe systemic disease (28) were excluded. In the ICH cohort, patients with structural brain abnormalities (3 arteriovenous malformations; 13 tumors) and those with severe systemic disease (9) were excluded. Some patients (24) refused to participate in the study, and 36 were lost during the follow-up. Thus, the IS cohort contained 408 patients (male, 59.3%; mean age, 72.8 ± 12.1 years) and 128 patients were included in the ICH cohort (male, 57.8%; mean age, 71.2 ± 12.3 years). In all cases, the research was carried out in accordance with the Declaration of Helsinki of the World Medical Association (2008) and approved by the Ethics Committee of the Servizo Galego de Saúde. Informed consent was obtained from each patient or their relatives after full explanation of the procedures.

Clinical variables

Patients were admitted to the Acute Stroke Unit (University Hospital of Santiago de Compostela, Spain) and treated according to the Guidelines of the Cerebrovascular Disease Study Group of the Spanish Society of Neurology [9, 10]. Past medical history, previous treatment, vascular risk factors, blood and coagulation tests, 12-lead ECG, chest X-ray, and carotid and transcranial ultrasonography were recorded upon admission. IS subtypes were classified according to the TOAST criteria [11]. Etiological diagnosis of ICH was made according the guidelines for the management of spontaneous ICH from the American Heart Association/American Stroke Association stroke council [12]. To evaluate the neurologic deficit, the National Institutes of Health Stroke Scale (NIHSS) was performed upon admission. Functional outcome was evaluated at 3 and 12 months by using the modified Rankin scale (mRS). NIHSS and mRS were evaluated by internationally certified neurologists [13]. mRS score >2 was considered poor functional outcome, as previously done [4, 8].

Neuroimaging studies

IS patients were subjected to computed tomography (CT) scans upon admission and at days 4-7; the infarct volume was calculated in the second CT scan using the formula: 0.5 x a x b x c, where a and b represent the largest perpendicular diameters, and c the slice thickness. Lesion volumes of ICH patients were determined by CT scans using the same formula; the hematoma volume was determined upon admission, and the peri-hematoma hypodensity volume (total volume minus hematoma volume) in a second CT performed after 48-72 h. The residual cavity volume was determined with CT at 3 months ± 15 days. ICH lesion volume reduction was calculated using the following formula: (ICH volume on admission-Residual

cavity volume at 3 months/ICH volume on admission) x100. ICH topography was classified as lobar when it mainly affected the cortical or subcortical white matter of the cerebral lobes, or as deep when it was limited to the internal capsule, the basal ganglia, or thalamus. The presence of ventricular extension of the hematoma was also recorded. All neuroimaging evaluations were performed by neuroradiologists blinded to the patients' clinical and laboratory results.

Outcome variables

The main outcome measure for all patients was functional outcome at 3 months ± 15 days. In the case of the IS cohort, secondary variable was infarct volume. For the ICH cohort, the residual cavity volume was considered as the secondary variable.

Human polymorphism analysis

Genotyping of human *MDM2* polymorphism was performed at the University of Salamanca by authors blinded to the clinical status of patients, using the PCR-RFLP technique. The SNP309 in the promoter region of the *MDM2* gene (SNP309) was determined with forward 5'-GAGGTCTCCGCGGGAGTTC-3' and reverse 5'-CGTGTCTGAACTTGACCAGC-3' primers. The 190 bp amplified-product was digested overnight with MspA11 (New England Biolabs) at 37ºC. Digests were separated on agarose gel (3%) and the Midori Green Advance-stained DNA fragments (Nippon Genetics Europe GmbH, Düren, Germany) were analyzed under a UV source using the Bio-Rad Universal Hood II Gel Imager system (Bio-Rad, CA, USA). The distribution of genotype frequencies between the stroke patients was within the Hardy-Weinberg equilibrium ($p>0.1$ in all cases). When indicated, two groups were considered: *T/T* genotype, denominated as *T* group, and *T/G* + *G/G* genotypes, denominated as *G* group.

Statistical analysis

The results are expressed as percentages for categorical variables and as either the mean ± S.D. or median (25th and 75th percentiles) for the continuous variables, depending on whether or not the data followed a normal distribution, respectively. The Kolmogorov-Smirnov test was used for testing the normality of the distribution. The Student's t-test (normal data) or the Mann-Whitney test (non-normal data) was used to compare continuous variables between two groups. Proportions were compared using the Chi-square test. Spearman analysis was used for bivariate correlations. To exclude a non-random mating population, the allele frequencies for Hardy-Weinberg equilibrium were tested with a goodness-of-fit $\chi2$. The influence of the SNP on functional outcome was assessed by logistic regression analysis, while the influence on volumes was assessed by multiple linear regression models, after adjusting for the main baseline variables related to each main variable in the univariate analysis (enter approach and probability of entry $p<0.05$). The results are expressed as adjusted odds ratios (AOR) with the corresponding 95% confidence intervals (95% CI). Experimental results are expressed as mean ± S.E.M. A one-way ANOVA with a least significant difference post hoc test was used to compare mean values between multiple groups, and a two-tailed, unpaired Student's t-test was used for two-group comparisons. In all instances, $p<0.05$ were considered significant. Statistical analyses were performed using SPSS Statistics 24.0 for Macintosh (IBM).

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Supplemental Table I. Baseline demographic and clinical features of stroke patients

Variables	**Ischemic stroke (n=408)**	**ICH (n=128)**
Age, years	72.8 ± 12.1	71.2 ± 12.3
Males, n (%)	242 (59.3)	74 (57.8)
TOAST:		
- Cardioembolic, n (%)	148 (36.3)	
- Atherotrombotic, n (%)	38 (9.3)	
- Lacunar, n (%)	46 (11.3)	
- Undetermined, n (%)	167 (40.9)	
- Others, n (%)	9 (2.2)	
Etiology:		
- Hipertensive, n (%)		78 (60.9)
- Anticoagulant, n (%)		18 (14.1)
- Amyloid, n (%)		8 (6.2)
- Undetermined, n (%)		24 (18.8)
NIHSS on admission	5 [2, 11]	8 [4, 12]
ASPECTS on admission	10 [8, 10]	
Infarct volume at day 4-7, mL	31.1 ± 58.7	
Early neurological deterioration, n (%)	43 (10.5)	
Lesion volume on admission, mL		20.5 ± 26.9
Ventricular extension, n (%)		34 (26.6)
mRS at 3 months	2 [1, 4]	3 [1, 4]
mRS at 12 months	3 [1, 6]	2 [1, 5]
Poor prognosis at 3 months, n (%)	161 (39.5)	59 (46.1)
Mdm2 SNP309		
- *T/T* genotype, n (%)	225 (55.1)	76 (59.4)
- *T/G* genotype, n (%)	149 (36.5)	44 (34.4)
- *G/G* genotype, n (%)	34 (8.3)	8 (6.2)

Patients were admitted at the University Hospital of Santiago de Compostela (Galicia, Spain). Data are shown as number (%), means ± SD or medians [quartiles]. ICH: intracerebral hemorrhage; mRS: modified Rankin Scale; NIHSS: National Institute of Health Stroke Scale.

Supplemental Table II. Univariate analysis of baseline variables according to functional outcome at 3 months in ischemic stroke patients

	Ischemic stroke		
	Good outcome n=247	**Poor outcome n=161**	***P***
Age, years	70.1 ± 11.7	78.8 ± 9.9	<0.0001
Males, %	68.0	46.0	<0.0001
Hypertension, %	51.1	64.6	0.007
Diabetes, %	27.1	24.8	0.347
Smoking, %	17.4	8.1	0.005
Heavy alcohol intake, %	9.3	7.5	0.321
Hyperlipidemia, %	33.2	28.6	0.191
Coronary disease, %	13.8	14.3	0.496
Atrial fibrillation, %	14.6	24.8	0.007
SBP on admission, mm Hg	148.5 ± 25.9	155.6 ± 28.1	0.053
DBP on admission, mm Hg	81.7 ± 13.9	82.5 ± 14.7	0.708
Maximum SBP at 24 hours, mm Hg	133.1 ± 23.2	145.8 ± 25.4	<0.0001
Maximum DBP at 24 hours, mm Hg	73.8 ± 12.1	76.7 ± 13.1	0.224
Temperature on admission, ºC	36.3 ± 0.5	36.3 ± 0.5	0.296
Maximum temperature at 24 hours, ºC	36.3 ± 0.5	36.6 ± 0.5	<0.0001
Blood glucose on admission, mg/dL	133 ± 60.5	140 ± 60.6	<0.0001
Maximum blood glucose at 24 hours, mg/dL	121.1 ± 43.9	128.5 ± 56.5	0.052
Leukocytes on admission, x10^3/mL	8.2 ± 2.4	9.0 ± 2.8	<0.0001
Platelet number on admission, x10^3/mL	231.1 ± 65.8	255.3 ± 70.0	0.045
International normalized ratio on admission	1.1 ± 0.3	1.1 ± 0.2	0.003
Fibrinogen on admission, mg/dL	452.9 ± 103.8	526.9 ± 130.7	<0.0001
hs-CRP on admission, mg/dL	1.6 ± 2.4	4.6 ± 7.9	<0.0001
NIHSS on admission	3 [1, 5]	11 [6, 17]	<0.0001
Early neurological deterioration, %	3 [1, 5]	11 [6, 17]	<0.0001
Infarct Volume at day 4-7, mL	10.4 ± 16.0	58.8 ± 81.1	<0.0001
ASPECTS	10 [9, 10]	10 [7, 10]	<0.0001
TOAST			0.056
T/T genotype	42.5	74.5	<0.0001

Functional outcome was evaluated at 3 months using the modified Rankin Scale (mRS); mRS score >2 was considered poor prognosis. Data are percentage, means ± SD or medians [quartiles]. Proportions were compared using the Chi-square test. Student's *t* test was used to compare continuous variables with normal distribution between the groups. Continuous variables without normal distribution (NIHSS) were compared using the Mann-Whitney test. SBP, systolic blood pressure; DBP, diastolic blood pressure; hs-CRP, high-sensitivity C-reactive protein; NIHSS, National Institute of Health Stroke Scale.

Supplemental Table III. Univariate analysis of baseline variables according to functional outcome at 3 months in intracerebral hemorrhage patients

	Intracerebral hemorrhage		
	Good outcome n=69	**Poor outcome n=59**	***P***
Age, years	69.3 ± 13.6	76.4 ± 9.5	0.009
Males, %	56.5	59.3	0.445
Hypertension, %	55.1	55.9	0.532
Diabetes, %	10.1	28.8	0.007
Smoking, %	10.1	11.9	0.487
Heavy alcohol intake, %	17.4	11.9	0.267
Coronary disease, %	5.8	10.2	0.278
Atrial fibrillation, %	15.9	10.2	0.244
SBP on admission, mm Hg	154.5 ± 30.2	165.9 ± 28.7	0.097
DBP on admission, mm Hg	88.6 ± 17.8	87.6 ± 16.8	0.745
Temperature on admission, ºC	36.3 ± 0.5	36.4 ± 0.8	0.867
Blood glucose on admission, mg/dL	114.5 ± 28.5	134.8 ± 41.8	0.007
Leukocytes on admission, x10^3/mL	8.3 ± 2.3	9.4 ± 3.3	0.072
Platelet number on admission, x10^3/mL	219.8 ± 52.7	246.6 ± 117.1	0.525
International normalized ratio on admission	1.2 ± 0.5	1.2 ± 0.5	0.270
Fibrinogen on admission, mg/dL	453.8 ± 120.5	504.6 ± 113.7	0.161
hs-CRP on admission, mg/dL	3.7 ± 6.8	5.7 ± 8.9	0.083
Lesion volume on admission, mL	11.2 ± 11.4	27.5 ± 37.8	<0.0001
Hypodensity volume at 48-72 hours, mL	7.5 ± 12.9	28.8 ± 36.0	<0.0001
NIHSS on admission	3 [2, 6]	10 [8, 15]	<0.0001
Ventricular extension, (%)	14.5	40.7	0.001
Topography			0.232
Etiology			0.318
T/T genotype	13.8	86.4	<0.0001

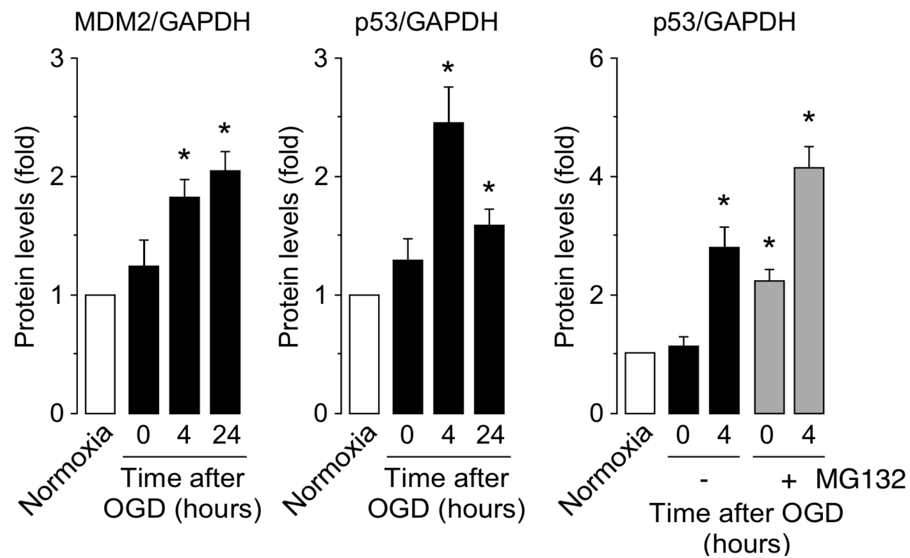
Functional outcome was evaluated at 3 months using the modified Rankin Scale (mRS); mRS score >2 was considered poor prognosis. Data are percentage, means ± SD or medians [quartiles]. Proportions were compared using the Chi-square test. Student's *t* test was used to compare continuous variables with normal distribution between the groups. Continuous variables without normal distribution (NIHSS) were compared using the Mann-Whitney test. SBP, systolic blood pressure; DBP, diastolic blood pressure; ICH, intracerebral hemorrhage; hs-CRP, high-sensitivity C-reactive protein; NIHSS, National Institute of Health Stroke Scale.

Supplemental Table IV. Distribution of *MDM2 SNP309* genotype according to ischemic stroke and intracerebral hemorrhage etiology

	Ischemic stroke			**Intracerebral hemorrhage**		
	G/G	***T/G***	***T/T***	***G/G***	***T/G***	***T/T***
TOAST (p=0.228):						
- Cardioembolic, %	37.0	33.5	38.8			
- Atherotrombotic, %	8.7	9.5	9.3			
- Lacunar, %	15.2	9.5	12.0			
- Undetermined, %	37.0	43.0	39.9			
ICH Etiology (p=0.457):						
- Hipertensive, %				50.0	63.2	57.9
- Anticoagulant, %				7.1	12.3	17.5
- Amyloid, %				7.1	5.3	7.0
- Undetermined, %				35.8	19.3	17.6

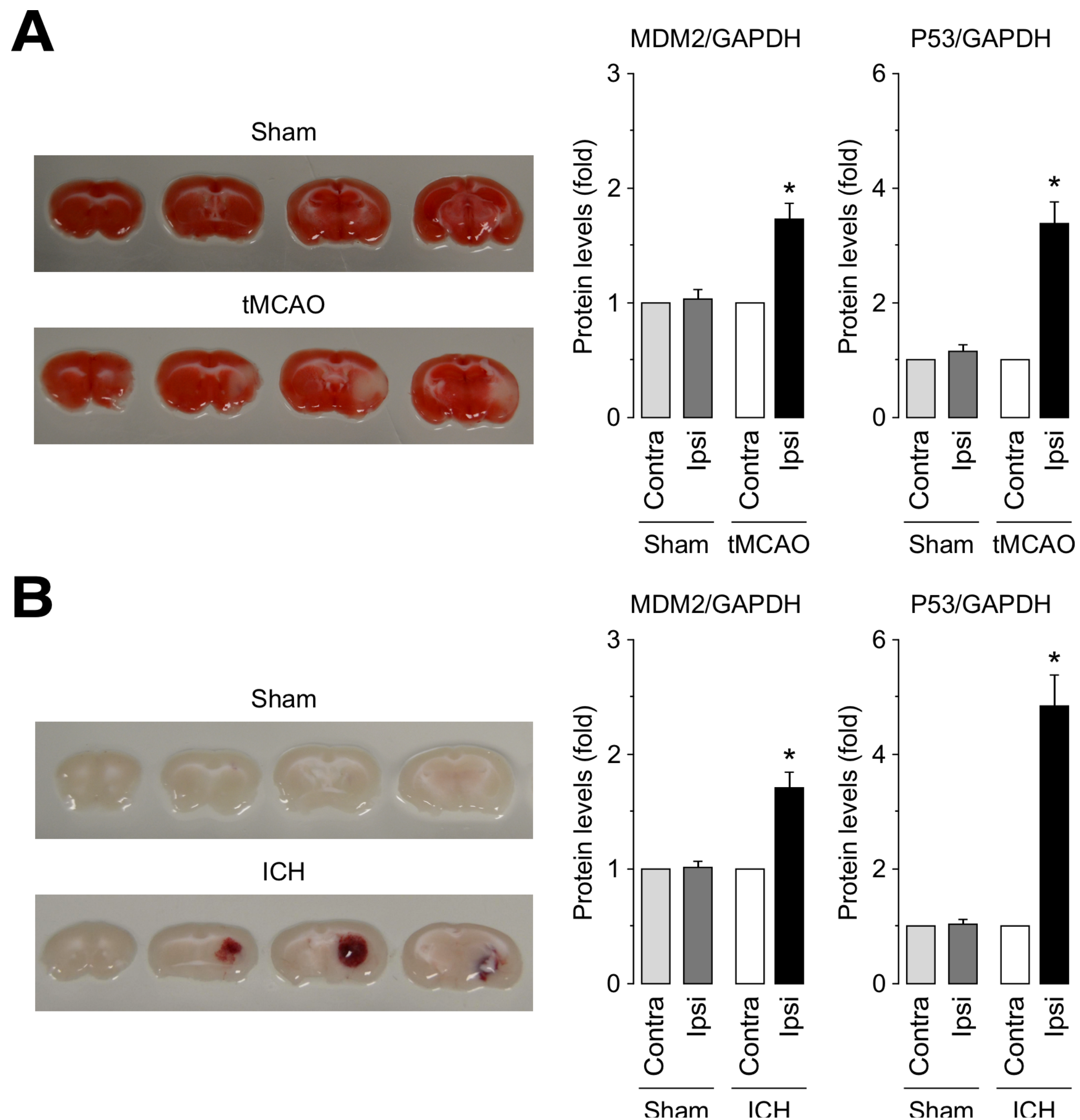
Patients were admitted at the University Hospital of Santiago de Compostela (Galicia, Spain). Data are shown as percentage (%).

Supplemental Figure I



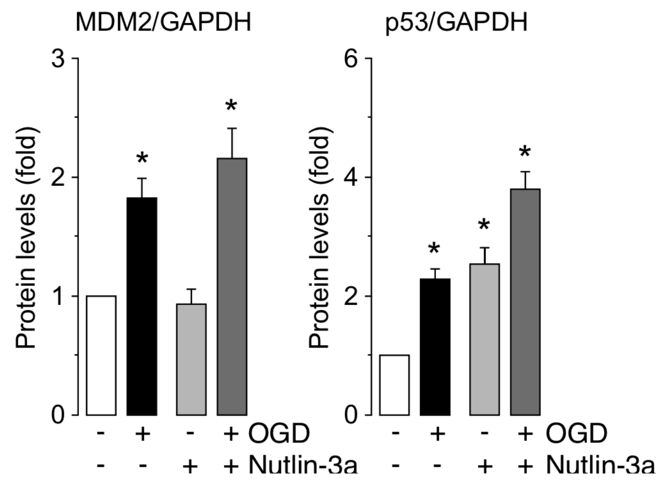
Supplemental Figure I. Results of the protein abundance quantification of proteins shown in Figure 1A and 1C. OGD increased MDM2 and p53 protein levels in neurons. Levels of p53 protein further decreased, while those of MDM2 remained high at 24 h after OGD. Neuronal treatment with the proteasome inhibitor MG132 (10 µM) promoted p53 accumulation. Data are expressed as mean ± SEM from 4 different neuronal cultures. *P<0.05 compared to normoxia.

Supplemental Figure II



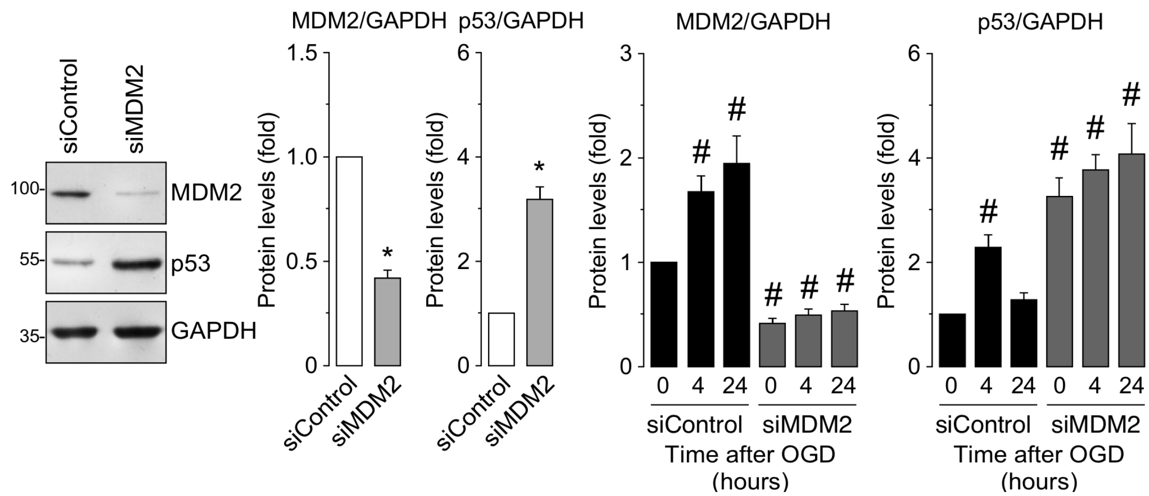
Supplemental Figure II. Mice were subjected to (**A**) ischemic (transient middle artery occlusion, tMCAO) or (**B**) ICH (collagenase injection) stroke models. Results of the protein abundance quantification of proteins shown in Figure 1B. Both tMCAO and ICH increased MDM2 and p53 protein levels in the ipsilateral (ipsi) hemisphere, in comparison with the contralateral (contra) hemisphere, at 24 hours after surgery. Infarct volume at 24 hours: 35.05 ± 8.23 mm^3 (n = 4 mice); hemorrhagic lesion volume at 24 hours: 16.88 ± 3.47 mm3 (n = 4 mice). Data are expressed as mean ± SEM (n = 4 mice). *P<0.05 compared to the corresponding contralateral (contra) hemisphere.

Supplemental Figure III



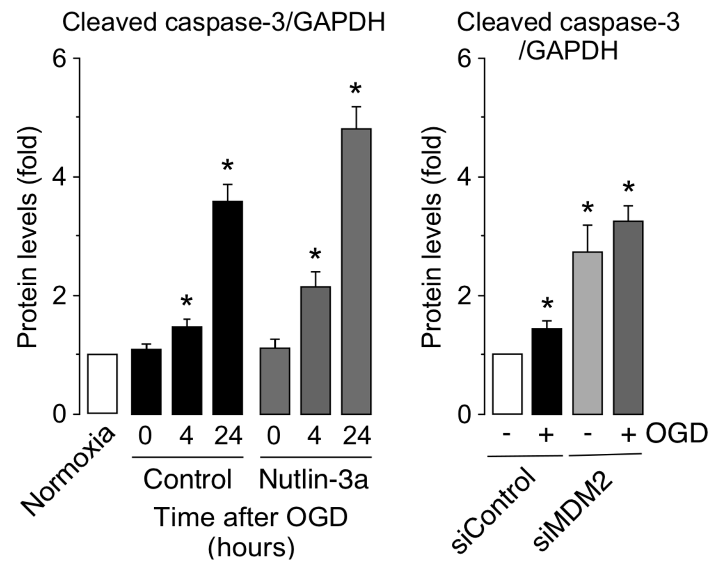
Supplemental Figure III. Results of the protein abundance quantification of proteins shown in Figure 2B, left panel. Nutlin-3a (1 μM) enhanced OGD-induced p53 accumulation in neurons at 4 hours of reoxygenation. Data are expressed as mean ± SEM from 4 different neuronal cultures. **P*<0.05 compared to -OGD and -Nutlin-3a condition.

Supplemental Figure IV



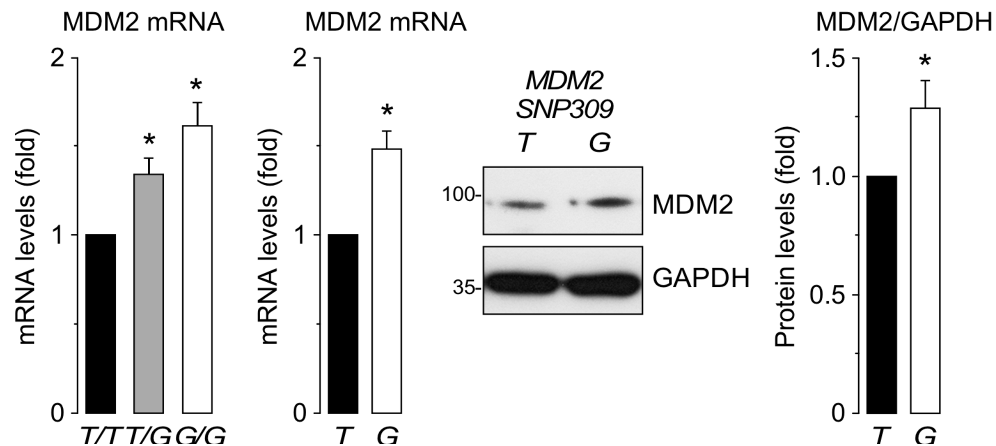
Supplemental Figure IV. MDM2 knockdown by siRNA (siMDM2) transfection for 2 days triggers p53 accumulation in neurons, as revealed by Western blot images and quantitative analysis. Results of the protein abundance quantification of proteins shown in Figure 2B, right panel. Data are expressed as mean ± SEM from 4 different neuronal cultures. **P*<0.05 compared to siControl. #*P*<0.05 compared to siControl neurons at 0 hours after OGD.

Supplemental Figure V



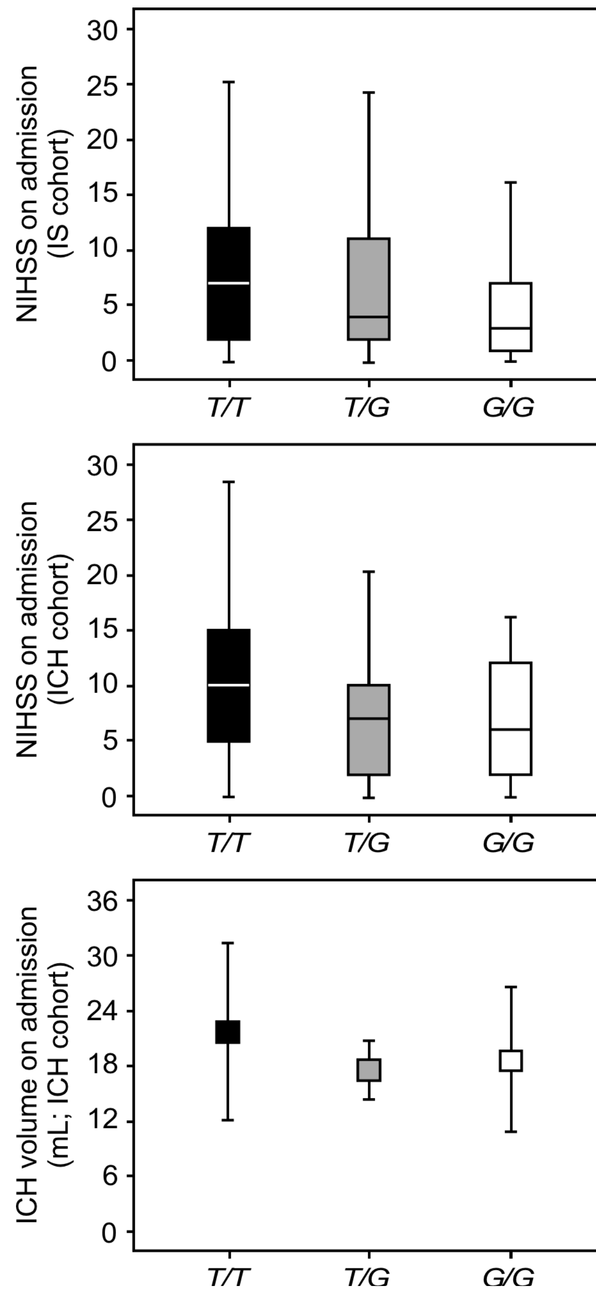
Supplemental Figure V. Results of the protein abundance quantification of proteins shown in Figure 2C. Nutlin-3a (1 μM) increased OGD-induced caspase-3 activation at 4 and 24 hours after OGD. MDM2 knockdown (siMDM2) promoted caspase-3 activation in neurons at 4 hours after OGD. Data are expressed as mean ± SEM from 4 different neuronal cultures. *$P<0.05$ compared to Vehicle at 0 hours after OGD (left) or to siControl -OGD (right).

Supplemental Figure VI



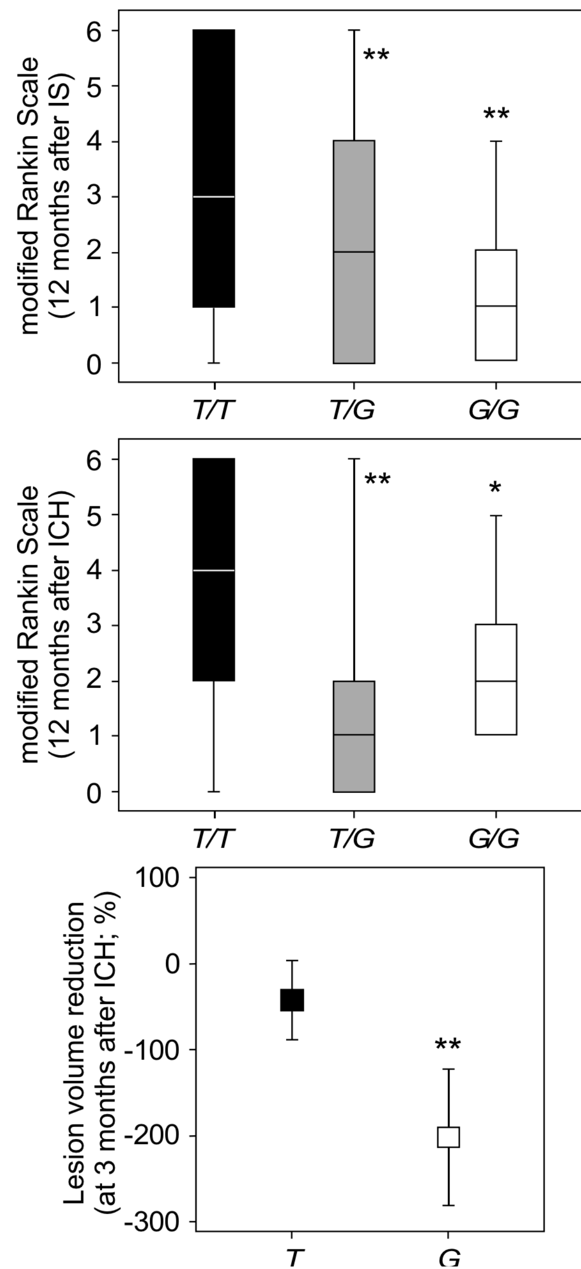
Supplemental Figure VI. RT-qPCR assays reveal higher MDM2 mRNA levels in primary cultured monocytes from healthy individuals harboring the *G* allele in homozygosity (*G/G*) and heterozygosity (*T/G*) (G group), than those with the *T/T* genotype (T group). Western blot images and quantitative analysis showing higher levels of MDM2 protein in primary cultured monocytes from healthy individuals of the G group than those in the T group. Data are expressed as mean ± SEM from 4 different cell cultures. *P<0.05 compared to *T/T* or *T* group.

Supplemental Figure VII



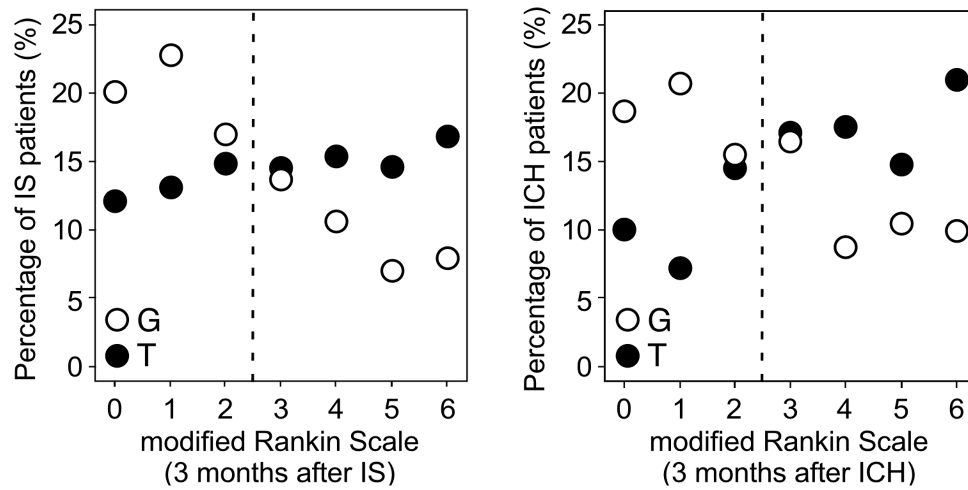
Supplemental Figure VII. The ischemic stroke (IS) cohort included 408 patients and the intracerebral (ICH) cohort included 128 patients. The *MDM2 309SNP* do not affect NIHSS (National Institute of Health Stroke Scale) scores and ICH volume on admission in stroke patients. Boxplots show median values (horizontal line inside the box), quartiles (box boundaries), and the largest and smallest observed values (error bars). Points represent mean values and error bars indicate standard deviation.

Supplemental Figure VIII



Supplemental Figure VIII. The ischemic stroke (IS) cohort included 408 patients (*T/T*: 225; *T/G*: 149; *G/G*: 34). Patients harboring the *T/T* genotype showed higher median of modified Rankin scale (mRS) scores at 12 months after IS, than those with *T/G* and *G/G* genotypes. The intracerebral hemorrhage (ICH) cohort included 128 patients (*T/T*: 76; *T/G*: 44; *G/G*: 8). Patients carrying the *T/T* genotype showed higher median mRS scores at 12 months after ICH, when compared with *T/G* and *G/G* patients. The lesion volume reduction was lower in *T/T* patients (*T* group) than in the *G* group (*T/G* + *G/G*). Boxplots show median values (horizontal line inside the box), quartiles (box boundaries), and the largest and smallest observed values (error bars). Points represent mean values and error bars indicate standard deviation. **P*<0.05; ***P*<0.0001 compared to *T/T* or *T* group.

Supplemental Figure IX



Supplemental Figure IX. Percentage distribution of patients per mRS outcome categories, according to SNP309 genotype, in both the ischemic stroke (IS; 408 patients) and intracerebral hemorrhage (ICH; 128 patients) cohorts. We found that the majority (%) of patients harboring the *G* allele (G group) had mRS scores ≤ 2, while T group (*T/T* genotype) patients mainly have mRS scores > 2, at 3 months after stroke.

*** 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:	N/A

Inclusion and exclusion criteria

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

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:	Yes
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:	N/A

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:	N/A
Details on important adverse events and death of animals during the course of experimentation have been provided, for all experimental arms:	N/A
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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