# Silent Information Regulator 1 Protects the Brain Against Cerebral Ischemic Damage

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# Silent Information Regulator 1 Protects the Brain Against Cerebral Ischemic Damage

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***Background and Purpose***—Sirtuin 1 (SIRT1) is a member of NAD$^+$-dependent protein deacetylases implicated in a wide range of cellular functions and has beneficial properties in pathologies including ischemia/reperfusion processes and neurodegeneration. However, no direct evidence has been reported on the direct implication of SIRT1 in ischemic stroke. The aim of this study was to establish the role of SIRT1 in stroke using an experimental model in mice.

***Methods***—Wild-type and *Sirt1*$^{-/-}$ mice were subjected to permanent focal ischemia by permanent ligature. In another set of experiments, wild-type mice were treated intraperitoneally with vehicle, activator 3 (SIRT1 activator, 10 mg/kg), or sirtinol (SIRT1 inhibitor, 10 mg/kg) for 10 minutes, 24 hours, and 40 hours after ischemia. Brains were removed 48 hours after ischemia for determining the infarct volume. Neurological outcome was evaluated using the modified neurological severity score.

***Results***—Exposure to middle cerebral artery occlusion increased SIRT1 expression in neurons of the ipsilesional mouse brain cortex. Treatment of mice with activator 3 reduced infarct volume, whereas sirtinol increased ischemic injury. *Sirt1*$^{-/-}$ mice displayed larger infarct volumes after ischemia than their wild-type counterparts. In addition, SIRT1 inhibition/deletion was concomitant with increased acetylation of p53 and nuclear factor κB (p65).

***Conclusions***—These results support the idea that SIRT1 plays an important role in neuroprotection against brain ischemia by deacetylation and subsequent inhibition of p53-induced and nuclear factor κB-induced inflammatory and apoptotic pathways. **(*Stroke*. 2013;44:2333-2337.)**

**Key Words:** neuroprotection ■ SIRT1 ■ stroke

Stroke is one of the main causes of death and is a major cause of long-term disability in developed countries (the third leading cause of death in the United States). The World Health Organization has established that 15 million people experience stroke worldwide each year. Of these, 5 million die and another 5 million are permanently disabled. Furthermore, the overall rate of stroke remains high because of aging of the population. In recent years, stroke has been classified as a medical emergency, and several studies have focused on the development of new drugs with brain-protective effects in experimental models. However, none has led to effective therapies. For this reason, it is necessary to identify new and therapeutic targets involved in the pathophysiology of stroke.

Sirtuin 1 (SIRT1) is a member of NAD$^+$-dependent protein deacetylases implicated in a wide range of cellular functions.[1] Many studies have suggested a role of SIRT1 in aging and diseases[2] that include ischemia/reperfusion processes[3] and neurodegeneration.[4] For instance, Hsu et al[5] demonstrated that cardiac-specific knockout SIRT1 mice exhibit a significant increase in the size of myocardial infarction area at risk. However, no evidence of the direct implication of SIRT1 in stroke has been shown so far. Therefore, the aim of this study was to investigate the role of SIRT1 in experimental ischemic stroke.

## Material and Methods

### Animals

For the generation of *Sirt1* homozygous mice, we used a SIRT knockout allele originally carried on the 129/Sv background,[6] from which we generated knockout mice by intercrossing first with the outbred background ICR (CD-1) and then intercrossing the heterozygotes. Approximately half of the homozygous *Sirt1*$^{-/-}$ mice

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**The online-only Data Supplement is available with this article at http://stroke.ahajournals.org/lookup/suppl/doi:10.1161/STROKEAHA.113.001715/-/DC1.**

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survive to adulthood. We obtained gene-deficient (*Sirt1*$^{-/-}$) and wild-type (WT) animals from the same littermates. Genotyping was performed as described in the online-only Data Supplement. Mice were housed in ventilated cages on a 12-hour light/dark cycle at 22°C and 35% humidity with ad libitum access to food and water. All testing was performed during the light phase of the cycle.

All procedures were performed in accordance with the European Communities Council Directive (86/609/EEC) and reviewed by the Ethics Committees on Animal Welfare of University Complutense. A special effort was made to reduce the number of animals used in the study and to provide them with the most comfortable conditions possible.

### Permanent Cerebral Ischemia

Induction of permanent cerebral ischemia was performed as described in the online-only Data Supplement.

### Determination of Infarct Outcome

Two days after permanent middle cerebral artery occlusion (pMCAO), infarct size and neurological deficit were assessed by investigators blinded to the different treatments and as described in the online-only Data Supplement.

### MCAO Experimental Groups

All the groups were performed and quantified in a randomized fashion by investigators blinded to the treatment groups. The set of experiments aimed for the effect of the pharmacological modulation of SIRT1 on infarct volume was performed in WT mice (n=8/group). Animals were allocated by randomization (coin toss) to 2 different groups: (1) activator 3 (A3; activator of SIRT1; Santa Cruz) and (2) sirtinol (SIRT1 inhibitor; Sigma). Treatments were administered 10 minutes, 24 hours, and 40 hours after ischemia at 2 different doses after preliminary experiments in which we failed to find a significant effect when sirtinol was administered only 10 minutes after ischemia. In addition, a second set of experiments for the study of the effect of the genetic deletion of *Sirt1* on infarct volume was performed in WT and *Sirt1*$^{-/-}$ mice (n=7/group).

### Western Blotting

Brain tissue was collected from peri-infarct area of mice (n=3–6 for each group) killed 18, 24, and 48 hours and 7 days after pMCAO for SIRT1 determination. For other proteins, animals were killed 18 hours after pMCAO (n=4 for each group). Immunodetection of SIRT1 and SIRT1 substrates was performed as described in the online-only Data Supplement.

### Double Immunofluorescence Staining

To examine the cellular location of SIRT1, free-floating coronal brain slices (30 μm) were processed 24 and 48 hours after pMCAO (n=3 in each group). Immunofluorescence and confocal microscopy were performed as described in the online-only Data Supplement.

### Determination of Histone Acetylation

To determine the histone acetylation, a commercial colorimetric kit (EpiQuik Total Histone H3 or H4 Acetylation Detection Fast Kit; Epigentek) was used according to the manufacturer's instructions (n=4 for each group).

### Statistical Analysis

Results are expressed as mean±SD for the indicated number of experiments. Prism4 (GraphPad Software, La Jolla, CA) was used for statistical analysis. Unpaired Student *t* test was used to compare 2 groups. One-way or 2-way ANOVA was used to compare >2 groups or parameters with the Tukey and Bonferroni post hoc tests, respectively. $P<0.05$ was considered statistically significant.

## Results

### SIRT1 Is Upregulated After Experimental Ischemia in Neurons of the Peri-infarct Area

First, Western blot analysis showed an increase in SIRT1 protein expression in mouse brain homogenates from peri-infarct regions of ipsilesional cortices of WT mice exposed at different times or pMCAO (Figure 1A). The expression of SIRT1 also was analyzed by immunofluorescence and confocal microscopy examination. The expression of SIRT1 in the peri-infarct area (infarct border of neocortex corresponding to III–V layers) was significantly increased after pMCAO when compared with sham-operated animals at all times studied (Figure 1A and 1B). To analyze its cellular location, double-immunofluorescence staining was used in equivalent sections. Our data show that SIRT1 is located in neurons (neuronal nuclei-positive) but not in astrocytes (glial fibrillary acidic protein-positive) in both sham and pMCAO-exposed animals (Figure 1B). SIRT1 location was mainly nuclear in sham animals; however, our data also show an increase in cytoplasmic SIRT1 after pMCAO. Immunostaining was specific, as shown by the lack of labeling in sections obtained from *Sirt1*$^{-/-}$ mice (Figure I in the online-only Data Supplement).

### Pharmacological Modulation of SIRT1 Activity Affects Infarct Volume

To ascertain the role of SIRT1 in this setting, WT mice subjected to pMCAO were treated with either an activator or an inhibitor of SIRT1, and the infarct outcome was assessed. At 48 hours after pMCAO, WT mice treated with A3 (10 mg/kg) showed a significant decrease in the infarct volume when compared with WT-vehicle animals (n=8; $P<0.05$; Figure 2A). On the contrary, treatment with the SIRT1 inhibitor, sirtinol (10 mg/kg), caused a significant increase in the infarct volume when compared with the WT-vehicle animals (n=8; $P<0.05$; Figure 2B).

### Genetic Deletion of SIRT1 Worsens Stroke Outcome

To confirm the role of endogenous SIRT1 in cerebral ischemia, *Sirt1*$^{-/-}$ and WT mice were subjected to pMCAO and euthanized 48 hours later. Our data show that the size of the ischemic lesion was significantly higher in *Sirt1*$^{-/-}$ than in WT mice (n=7; $P<0.05$; Figure 2C), suggesting that endogenous SIRT1 plays a protective role during cerebral ischemia.

Before euthanization, we also assessed the neurological functions in these animals by using the modified neurological score scale, calculated based on a series of motor, reflex, and balance tests performed 48 hours after pMCAO in WT and *Sirt1*$^{-/-}$ mice. As shown in Figure 2D, the score of WT mice was significantly lower than that in *Sirt1*$^{-/-}$ animals (n=7; $P<0.05$).

No significant differences were found between WT and *Sirt1*$^{-/-}$ animals in physiological parameters measured in arterial blood 10 minutes before and 30 minutes after pMCAO (n=3; $P>0.05$; Table I in the online-only Data Supplement).

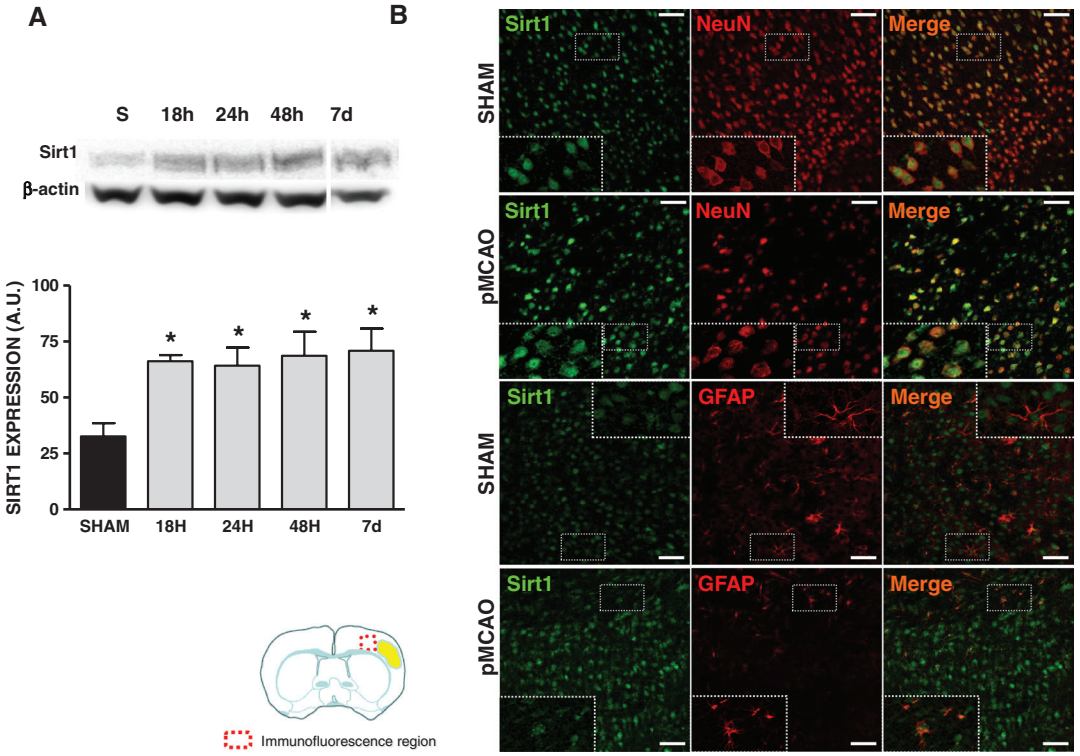


**Figure 1.** Sirtuin 1 (SIRT1) is upregulated after experimental stroke in wild-type mice. **A**, Quantification of SIRT1 protein levels 18, 24, and 48 hours and 7 days after ischemia in peri-infarct region. **B**, Representative SIRT1-NeuN or SIRT1-GFAP immunofluorescence staining in brains from sham or permanent middle cerebral artery occlusion (pMCAO)-exposed mice 48 hours after ischemia. Data are mean±SD (n=3–6; *$P$<0.05).

### Levels of Acetylated Nuclear Factor κB (p65) and p53, but not Histones, Are Modified After Inhibition/Deletion of SIRT1

SIRT1 is a member of NAD$^+$-dependent protein deacetylases, with a vast list of substrate proteins including the nuclear factors (NFs) p53 and NFκB (p65), and histones 3 and 4. To determine their involvement in SIRT1-induced neuroprotection, we investigated the effect of pharmacological modulation and genetic deletion of SIRT1 on the levels of acetylation of these proteins using WT (treated with A3 and sirtinol, 10 mg/kg each) and *Sirt1*$^{-/-}$ animals subjected to pMCAO.

Levels of acetylated histones (H3 and H4) were measured by Western blot (Figure 3A and 3B) and by a colorimetric commercial kit (Figure IIA and IIB in the online-only Data Supplement). With both techniques, our data did not show any significant differences in acetylation of histones 3 and 4 at the time studied among sham-operated, A3-treated, sirtinol-treated, and *Sirt1*$^{-/-}$ animals (n=4; $P$>0.05).

Western blotting also was performed to establish the acetylation levels of p53 and NFκB (p65); both of them are critical modulators of pathological processes after ischemia. As shown in Figure 3C and 3D, acetylation of both proteins was significantly increased after ischemia in sirtinol-treated and *Sirt1*$^{-/-}$ animals (n=4; $P$<0.05). The total levels of these substrates in the different groups were not significantly different at the time studied (data not shown).

## Discussion

In recent years, SIRT1 has become a target for drug development because of its many different effects in processes such as inflammation, cancer, cardiovascular disease, diabetes mellitus, and neurodegeneration.[2,7,8] In brain, several authors demonstrated its implication in normal cognitive function and synaptic plasticity,[9,10] differentiation of stem cells,[11] and neurodegenerative disorders.[1] In addition, SIRT1-protective properties have been described in heart ischemia–reperfusion[5] and ischemic preconditioning.[12] Currently, using gain-of-function and loss-of-function studies in mice, we demonstrate, for the first time to our knowledge, that stroke is an additional target for SIRT1-induced beneficial effects.

First, we found that exposure to pMCAO increases SIRT1 protein expression in homogenates and sections obtained from mouse brain, in agreement with data from our laboratory obtained in rat[13], thus suggesting that SIRT1 might play an important role in ischemic stroke. To ascertain this issue, we first performed pharmacological gain-of-function and loss-of-function experiments using molecules that increase or decrease SIRT1 activity. Our results show that the activation or inhibition of its enzymatic activity cause, respectively, an increase or a decrease in the infarct volume. These results strongly support an important role of SIRT1 in cerebral ischemia. To confirm these data, we used *Sirt1*$^{-/-}$ mice. We show that *Sirt1*$^{-/-}$ animals have larger infarct volumes and worse neurological outcome than their WT littermates. Altogether, these results confirm the neuroprotective role of SIRT1 in cerebral ischemia.

In the present study, we also show that SIRT1 is located in neurons. As a stress response, the increased expression of SIRT1 after exposure to pMCAO is likely to represent an endogenous defense mechanism. Other authors have described

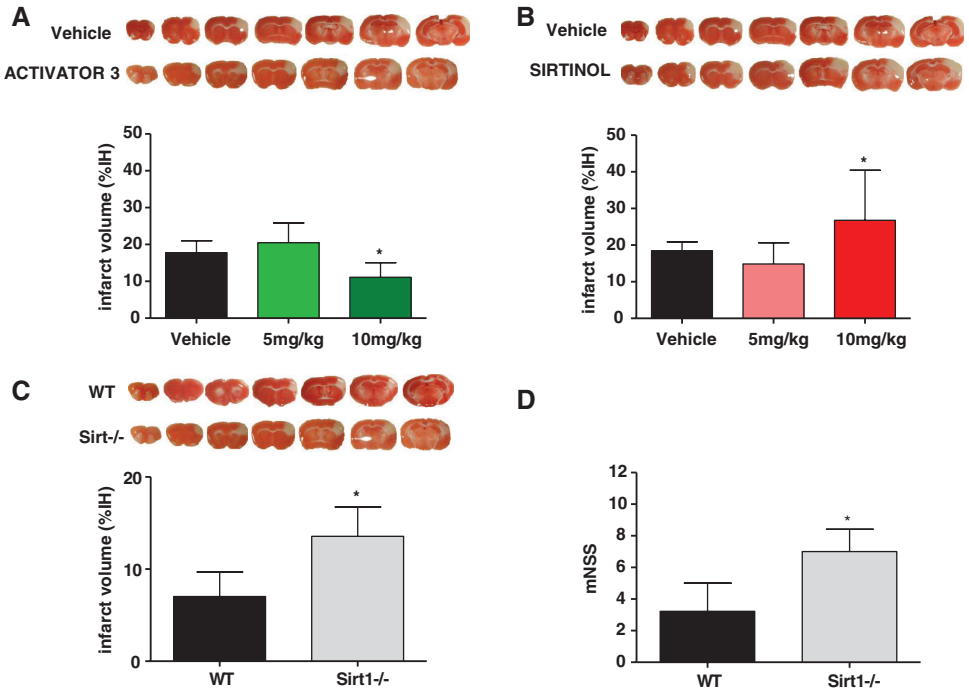


**Figure 2.** Effect of pharmacological modulation or genetic deletion of sirtuin 1 (SIRT1) on the infarct volume after stroke. Pharmacological modulation of SIRT1: quantification of 2,3,5-triphenyltetrazolium chloride-stained brain slices from wild-type (WT) mice treated with SIRT1 activator A3 (5–10 mg/kg, **A**) or SIRT1 inhibitor sirtinol (5–10 mg/kg, **B**). Drugs were administered by intraperitoneal route at 10 minutes and 24 and 40 hours after ischemia. Data are mean±SD (n=8; *$P$<0.05). Genetic deletion of *Sirt1*: quantification of infarct volume (**C**) and neurological outcome (**D**) in WT and SIRT1-deficient (*Sirt1*$^{-/-}$) mice. Neurological outcome was assessed using the modified neurological score scale. Data are mean±SD (n=7; *$P$<0.05).

that SIRT1 activity is regulated by shuttling from the nucleus to the cytoplasm;[14] in this context, our model displays an increase in the expression of cytoplasmic SIRT1, but this seems to be attributable to an increased total expression of SIRT1 rather than a shuttling between intracellular compartments, although further studies are needed to unravel this issue.

Regarding the mechanism involved in SIRT1-induced neuroprotection, our results show a significant increase in p53 and NFκB (p65) acetylation after inhibition/deletion of SIRT1. Given the crucial role of both proteins in stroke pathophysiology,[15,16] the increased acetylation might well explain the concomitant increased ischemic injury. In agreement with

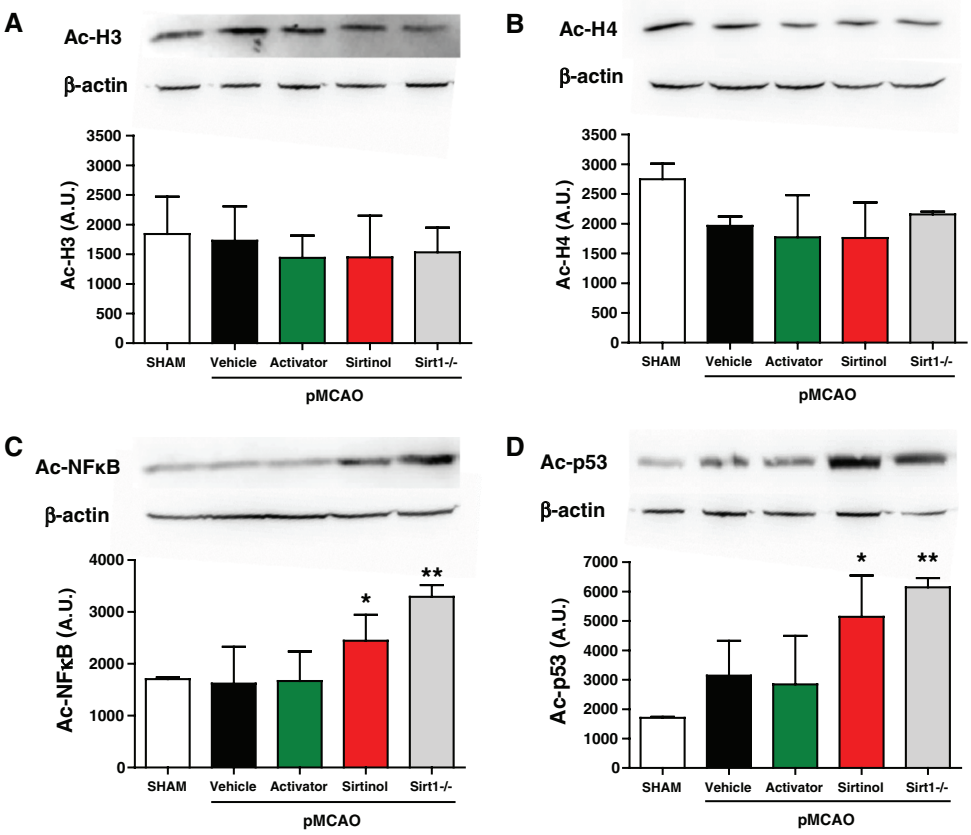


**Figure 3.** Levels of acetylated histones H3 and H4, p53, and nuclear factor (NF) κB after pharmacological modulation or genetic deletion of sirtuin 1 (SIRT1) in ischemia. Quantification of acetylated histones H3 (**A**) and H4 (**B**), and of acetylated NFκB (p65, **C**) and p53 (**D**). Samples were taken 18 hours after ischemia from the peri-infarct area. Data are mean±SD (n=4; *$P$<0.05).

this, deacetylation of p65 and p53 has been proposed to inhibit their action,[17,18] even in ischemic settings.[19,20] In contrast, there seems to be no changes in the acetylation of the histones studied after SIRT1 inhibition. Although the yeast sir2 protein is the founding member of this family and is known to deacetylate histones H4 and H3, a recent study on the acetylome changes in *Sirt1*$^{-/-}$ mouse embryonic fibroblasts failed to identify histone as one of the differentially affected proteins,[21] in agreement with our results.

At the time of study, ischemia per se did not affect the levels of acetylated substrates. This could be explained by assuming that SIRT1 functions by maintaining physiological levels of acetylation of its substrates, despite the presence of pathological stressors. However, the neuroprotective actions of the SIRT1 activator were not associated with increased deacetylation. Further studies at different time points are required to ascertain whether this is attributable to the time selected for the study, and whether this is attributable to the involvement of other SIRT1 targets in neuroprotection.

In conclusion, we have demonstrated that SIRT1 plays an important role in endogenous neuroprotection because its inhibition exacerbates ischemic injury concomitant with increased acetylation of p53 and NFκB (p65), important mediators of inflammatory and apoptotic pathways leading to brain damage in this setting. Interestingly, we recently have demonstrated that the neuroprotector citicoline[22,23] increases the expression of SIRT1 concomitantly with neuroprotection.[13] Our results open a new line of investigation aimed to design therapeutic strategies that increase SIRT1 expression or activity for the treatment of stroke and also of other pathologies in which SIRT1 has demonstrated beneficial properties.

## Acknowledgment

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## Disclosures

None.

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**SUPPLEMENTARY DATA**

## Supplementary Materials and Methods

### *Genotyping of Animals*

Genotyping was performed by PCR of tail clip DNA. Primers TTCACATTGCATGTGTGTGG and TAGCCTGCGTAGTGTTGGTG amplify a 423-bp fragment from the normal *sirt1* allele whereas a 526-bp fragment from the null allele is amplified from the first primer.[1]

### *Induction of permanent cerebral ischemia*

All experiments have been performed and quantified in a randomized manner by investigators blinded to treatment groups for the prevention of bias. Mice were anesthetized with 3% isoflurane (in 70% $N_2O$, 30% $O_2$) for induction and with 1.5% isoflurane for maintenance. Rectal temperature was maintained at 36.5°C with a heating pad. Common carotid (CCA) and middle cerebral artery (MCA) were exposed and occluded permanently by ligation as previously described[2] with some modifications. Due to the anatomical differences between *Sirt1*$^{-/-}$ and WT mice (the trunk of the MCA was not easily accessible for ligation in *Sirt1*$^{-/-}$ animals), MCA was occluded at its rostral branch in both WT and knockout mice in the set of experiments aimed to study the effect of the genetic deletion of *Sirt1* on infarct volume. In contrast, in the set of experiments to ascertain the effect of the pharmacological modulation of SIRT1 on infarct volume, the artery was occluded in its trunk. Finally, in a third set of experiments, aimed to the effect of pharmacological treatments and *Sirt1* deletion on the levels of acetylated SIRT1 substrates, the ligature was performed at the branch of the MCA to compare all the experimental groups.

Complete interruption of the blood flow was confirmed under an operating microscope. Sham-operated animals were subjected to anesthesia and the surgical procedure but the occlusion of the arteries was omitted. Following surgery, subjects were returned to their cages and allowed free access to water and food. The survival rate of the animals until the end of the experiment was 90%.

### *Determination of physiological parameters*

Mice were placed on a homeothermic blanket (Harvard Apparatus) throughout the duration of the measurement to prevent anesthesia-induced hypothermia. A polyethylene catheter was inserted into the right femoral artery. All parameters were measured 10 minutes before surgery and 30 minutes after ischemia. Mean arterial pressure was recorded through a Lab-Trax-4/24T (World Precision Instruments). Physiological parameters were tested using iStat System blood analyzer (Ven-Bios ES).

### *Neurological deficit evaluation*

Two days after surgical procedure, neurological deficit was assessed by two independent researchers blinded to experimental conditions. Neurological assessment was performed by using a modified neurological severity score (mNSS).[3] mNSS is graded on a scale of 0 to 16, with a higher score indicating more severe sensory-motor deficits, the test is sensitive to unilateral cortical injury because it reflects multiple asymmetries, including postural, sensory, and forelimb and hind limb use asymmetries.

***Determination of infarct size***

Two days after permanent MCAO (pMCAO), animals were killed by cervical dislocation to assess infarct outcome. Brain was removed and cut into 1mm thick coronal slices and stained with 2,3,5-triphenyltetrazolium chloride (1% TTC in 0.1M phosphate buffer). Infarct size was determined as follows: infarct volumes were measured by sampling stained sections with a digital camera (Nikon Coolpix 990, Nikon Corporation, Tokyo, Japan), and the image of each section was analyzed using ImageJ 1.44l (NIH, Bethesda, MD, USA). The digitalized image was displayed on a video monitor. With the observer masked to the experimental conditions, the areas of the infarcted tissue (InfArea), the whole ipsilesional hemisphere (IpsArea) and the whole contralesional hemisphere (ContrArea) are delineated for each slice. Then, infarct volume, expressed as % of the hemisphere that is infarcted (%IH), is calculated using the formula:

$$\%IH = InfVol/ContrVol*100$$

where

InfVol (Infarcted Tissue Volume) = $\sum InfArea_i/SwellingIndex_i$,

ContrVol (Contralesional Hemisphere Volume) = $\sum ContrArea_i$

and $SwellingIndex_i = IpsArea_i/ContrArea_i$.

***Protein determination by western blot***

Protein concentration was determined in tissue homogenates with the Bradford protein assay. Equal amounts of total protein (35 µg) were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Immunodetection was performed by standard procedures. The membranes were blocked with 5% nonfat milk in TBS-T (0.05% Tween 20 in TBS) and probed with specific primary

antibodies against Sirt1 (Cell Signaling; 1:500 dilution), acetyl-histone H3 (Millipore; 1:500), acetyl-histone H4 (Millipore; 1:500), p53 (acetyl K381) (Abcam; 1:1000), NFκB (acetyl K310) (Abcam; 1:1000) and mouse anti-β-actin (Sigma; 1:10000) was included to ensure equal protein loading. Specific signals were quantified using densitometry analysis software (ImageJ).

***Inmunofluorescence and confocal microscopy***

Free-floating coronal brain slices (30 μm) were processed as described previously.[4] In brief, brain sections were blocked with 5% goat serum and incubated with mouse anti-neuronal nuclei (NeuN) and anti-glial fibrillary acidic protein (GFAP), and rabbit anti-Sirt1 (Santa Cruz) 1:100 at 4ºC overnight followed by the appropriate rabbit secondary antibody Alexa 488 (Invitrogen A-11008), anti-mouse Cy3 (Jackson Immuno Research; 715-165-151) 2 h at room temperature. All immunofluorescence images were obtained in a blinded manner from seven correlative slices of each brain. Image acquisition was performed with a laser-scanning confocal imaging system (Zeiss LSM710) and image analysis was performed with the ZEN 2009 software (Zeiss). All co-localization images shown were confirmed by orthogonal projection of z-stack files.

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