Silent Information Regulator 2 Homolog 1 Counters Cerebral Hypoperfusion Injury by Deacetylating Endothelial Nitric Oxide Synthase

Yorito Hattori, MD; Yoko Okamoto, MD, PhD; Takakuni Maki, MD, PhD; Yumi Yamamoto, PhD; Naoya Oishi, MD, PhD; Kenichi Yamahara, MD, PhD; Kazuyuki Nagatsu, MD, PhD; Ryosuke Takahashi, MD, PhD; Raj N. Kalaria, MD, PhD; Hidenao Fukuyama, MD, PhD; Makoto Kinoshita, MD, PhD; Masafumi Ihara, MD, PhD, FACP

Background and Purpose—Silent information regulator 2 homolog 1 (SIRT1) is a protein deacetylase that has been reported to suppress neurodegenerative and cardiovascular diseases in model organisms. We hypothesized that neurovascular protection is one of the diverse actions of SIRT1. This study was designed to determine whether SIRT1 protects against the consequences of cerebral hypoperfusion in vivo.

Methods—Sirt1-overexpressing (Sirt1-Tg) mice driven by a prion promoter and their wild-type littermates were subjected to bilateral common carotid artery stenosis using external microcoils. Using Sirt1-Tg mice, we assessed the effect of SIRT1 on cerebral blood flow, cerebral angioarchitecture, histological and ultrastructural changes, and spatial working memory at several time points. We also evaluated the effects of preadministration of SIRT1 inhibitors or endothelial nitric oxide synthase inhibitors on cerebral blood flow after bilateral common carotid artery stenosis in Sirt1-Tg mice. Levels of acetylated and nonacetylated endothelial nitric oxide synthase were measured semiquantitatively with immunoblotting.

Results—Cerebral hypoperfusion induced by bilateral common carotid artery stenosis caused memory impairment and histological changes in wild-type littersmates. However, these phenotypes were rescued in Sirt1-Tg mice, where cerebral blood flow was maintained even poststenosis. Electron microscopic analyses showed irregularities in the vascular endothelia, such as tight junction openings in wild-type mice, which were absent in Sirt1-Tg littersmates. Brain endothelial nitric oxide synthase was acetylated after cerebral hypoperfusion in wild-type littersmates but remained unacetylated in Sirt1-Tg mice. Moreover, treatment with SIRT1 inhibitors and endothelial nitric oxide synthase inhibitors abolished the vasculoprotective effects of SIRT1.

Conclusions—Our results indicate that neurovascular endothelial SIRT1 potentiation upregulates the nitric oxide system and counters cerebral hypoperfusion injury. This novel cerebral blood flow–preserving mechanism offers potential molecular targets for future therapeutic intervention. (Stroke. 2014;45:00:00.)

Key Words: carotid artery stenosis ■ cerebral ischemia ■ dementia ■ endothelial nitric oxide synthase ■ mouse ■ SIRT1

Silent information regulator 2 homolog 1 (SIRT1), a yeast ortholog of silent information regulator 2,1 is a member of the class III histone deacetylase family termed sirtuins. SIRT1 also deacetylates and modulates the activities of various nonhistone substrates such as p53, peroxisome proliferator-activated receptor γ coactivator-1α, and nuclear factor κ light chain enhancer of activated B cells in a nicotinamide adenine dinucleotide–dependent manner.2,3 In diverse organisms, the activity of silent information regulator 2/ SIRT1 homologs has positive effects on the lifespan and tolerance against various genetic defects and environmental insults.4–7 In mammalian nervous systems, the overexpression of SIRT1, or pharmacological potentiation of SIRT1 by resveratrol or other small molecules, exerts protective effects on cellular and animal models of Alzheimer disease,8–11 Parkinson disease,12 and amyotrophic lateral sclerosis.13,14
SIRT1 activator–dependent neuroprotective activity has also been demonstrated in noncell autonomous neurological paradigms such as a global cerebral ischemia model in the rat\textsuperscript{15} and a permanent middle cerebral artery occlusion model in the mouse.\textsuperscript{16} Because vascular lesions are the major problems that often accompany and aggravate neurodegenerative diseases,\textsuperscript{17,18} molecular mechanism underlying SIRT1-mediated neuroprotection against cerebral hypoperfusion/ischemia is an important open question.

To address this, we assess the effects of transgenic overexpression and pharmacological inhibition of SIRT1 in a mouse model of chronic cerebral hypoperfusion. We previously demonstrated that this model mimics vascular aging by augmenting arterial stiffness, endothelial changes, and blood–brain barrier dysfunction, which jeopardizes brain environment and neuronal survival.\textsuperscript{19,20} We found drastic effects of SIRT1 expression on consequences of chronic cerebral hypoperfusion, implicating a potential application of SIRT1 activation for cerebrovascular diseases.

### Methods

#### Animals

Four groups of male mice with a C57BL/6j background were used for this study: (1) wild-type sham surgery group (n=30), (2) Sirt1-Tg sham surgery group (n=30), (3) wild-type bilateral common carotid artery stenosis (BCAS)–operated group (n=35), and (4) Sirt1-Tg BCAS-operated group (n=44). Detailed information of Sirt1-Tg mice can be found elsewhere.\textsuperscript{21} All mice survived the operation in this study.

#### Generation and Establishment of a Transgenic Mouse Line That Stably Expresses Mouse SIRT1 in the Brain

We constructed a transcription unit by inserting the coding region of the mouse Sirt1 CDNA into the mouse prion promoter–polyA cassette which drives pan-neural gene expression. See online-only Data Supplements for details.

#### Surgical Procedure of BCAS Operation

Through a midline cervical incision, both common carotid arteries were exposed under anesthesia. Microcots with an internal diameter of 0.18 mm were applied to the bilateral common carotid arteries (Figure 1 in the online-only Data Supplement). See online-only Data Supplements for details.

#### Histological Evaluation

The mouse brains were analyzed for demyelinating change with Klüver–Barrera staining and immunostained for silent information regulator 2 (Sigma-Aldrich), CD31 (a marker of vascular endothelial cell, BD Biosciences), glial fibrillary acidic protein (a marker of astrocyte, DAKO), Iba1 (a marker of microglia, Wako), and glutathione S-transferase-π (a marker of oligodendrocyte, Millipore). See online-only Data Supplements for details.

#### Western Blot Analysis

Cerebral protein levels of silent information regulator 2 (Sigma-Aldrich), endothelial nitric oxide synthase (eNOS; BD Biosciences), Ser1177 phospho-eNOS (Cell Signaling Technology), glyceraldehyde-3-phosphate dehydrogenase (Cell Signaling Technology), and β-actin (Sigma-Aldrich) were assessed by Western blot analysis. See online-only Data Supplements for details.

### Immunoprecipitation

Brain homogenates before and at 2 hours after BCAS were immunoprecipitated by antiacetylated-lysine antibody (Cell Signaling Technology) using Immunoprecipitation Kit-Dynabeads Protein A (Life technologies). See online-only Data Supplements for details.

### Results

#### SIRT1 Is Expressed in Neurons and Vascular Endothelial Cells in the Wild-Type and Sirt1-Tg Transgenic Mouse Brain

The amount of SIRT1 was significantly increased in Sirt1-overexpressing (Sirt1-Tg) mouse brain because of the transgenic expression of prion promoter-driven SIRT1. Immunoblots for the full-length, active form of SIRT1 (110 kDa)\textsuperscript{22} indicated that SIRT1 levels in Sirt1-Tg mice were approximately 1.6× in the cerebral cortex, 3× in the caudoputamen, 2.5× in the midbrain, and 5× in the brain stem and the spinal cord compared with wild-type littermates (Figure 1A and 1B). Intriguingly, SIRT1 was overexpressed not only in neurons but also in CD31-positive vascular endothelial cells of capillaries, arterioles, and leptomeningeal arteries (Figure 1C and 1D). These findings agree with the facts that cerebrovascular endothelial cells express prion protein (for transcerebral translocation of amyloid-β40 and other processes).\textsuperscript{23–25}

#### Sirt1-Tg Mice Retain Cognitive Integrity After Chronic Cerebral Hypoperfusion

Consistent with our previous observations,\textsuperscript{20} BCAS operation (~50% common carotid artery stenosis) induced spatial working memory impairment in wild-type littermates at 28 days after surgery. BCAS-operated Sirt1-Tg mice, however, showed significantly decreased number of revisiting errors in the 8-arm radial arm maze test (F(1,33)=13.884; P<0.001), whereas sham-operated control groups did not differ (Figure 2A). These data indicate that the excess SIRT1 partially rescues the impairment of spatial working memory after chronic cerebral hypoperfusion.

#### Sirt1-Tg Mice Retain Histological Integrity After Chronic Cerebral Hypoperfusion

Histopathologic analysis of the corpus callosum revealed that white matter rarefaction (judged with Klüver–Barrera staining) and glial activations (judged by the presence of glial fibrillary acidic protein–positive astrocytes and Iba1–positive microglia and the loss of glutathione S-transferase-π–positive oligodendrocytes) were comparable between Sirt1-Tg mice and wild-type littermates at 28 days after sham operation but significantly milder in Sirt1-Tg mice than in wild-type littermates at 28 days after BCAS (Figure 2B and 2C). When compared between sham- and BCAS-operated Sirt-Tg mice, the white matter rarefaction and astrocytic activation were significantly stronger after BCAS but microglial activation and oligodendroglial loss were comparable between the 2 groups. Consistent with the previous study,\textsuperscript{19,20} there were no neuronal changes in the cerebral cortex or hippocampus on light microscopy at 28 postoperative day (data not shown). In particular, hippocampus is
critical for memory formation but mainly supplied by BCAS-independent posterior circulation. Therefore, the above findings indicate that the excess SIRT1 rescues the white matter deteriorations, the major pathological changes responsible for the cognitive impairment after the chronic cerebral hypoperfusion.

**Sirt1-Tg Mice Show Less Neuronal and Vascular Changes After BCAS**

To explore the acute pathological changes in the blood–brain barrier components including the vascular endothelial cells, we compared the ultrastructure of wild-type and Sirt1-Tg mice at 2 hours after sham or BCAS operation by transmission electron microscopy. Wild-type mice after BCAS exhibited irregularities in the vascular endothelia that included tight junction openings and cuboidal cells (Figure 3A). Continuity of the endothelial cell surface was partly disrupted by numerous microvilli (Figure 3A). By contrast, similar endothelial disruptions were not found in Sirt1-Tg mice after BCAS (Figure 3B). The electron microscopic findings of BCAS-operated Sirt1-Tg mice were comparable to those observed in sham-operated wild-type and Sirt1-Tg mice (Figure 3C and 3D). Of note, neuronal shrinkage that was commonly found in the cerebral cortex of BCAS-operated wild-type mice was rare in BCAS-operated Sirt1-Tg mice (Figure II in the online-only Data Supplement). The latter finding was comparable to those of sham-operated wild-type and Sirt1-Tg mice (Figure II in the online-only Data Supplement).
Figure 2. Silent information regulator 2 homolog 1 (SIRT1) rescued memory impairment and histological changes after bilateral common carotid artery stenosis (BCAS). A, Number of revisiting errors in the 8-arm radial arm maze test was significantly fewer in Sirt1-overexpressing (Sirt1-Tg) mice (n=18) compared with wild-type littermates (n=17) at 28 days after BCAS, and number of revisiting errors was no fewer in Sirt1-Tg mice (n=15) compared with wild-type littermates (n=15) without BCAS. Data were analyzed by 2-way repeated measures ANOVA. B, Klüver–Barrera staining (KB) and immunohistochemistry for glial fibrillary acidic protein (GFAP), Iba1, and glutathione S-transferase-pi (GST-π) in the paramedian parts of the corpus callosum of wild-type littermates (left) and Sirt1-Tg mice (right) at 28 days after sham or BCAS operation. Insets indicate enlarged images of GFAP-positive astrocytes and Iba1-positive microglia. Scale bars indicate 100 μm and 20 μm (insets). C, Histograms showing the grading of the white matter lesions and the density of GFAP-positive astrocytes, Iba1-positive microglia, and GST-π-positive oligodendrocytes of median and paramedian parts of corpus callosum of BCAS-operated, wild-type (n=10) or Sirt1-Tg mice (n=7) and sham-operated, wild-type (n=5) or Sirt1-Tg mice (n=5). The severity of the white matter lesions was graded as normal (grade 0), disarrangement of the nerve fibers (grade 1), the formation of marked vacuoles (grade 2), and the disappearance of myelinated fibers (grade 3) in the corpus callosum. P<0.05: #vs BCAS-operated Sirt1-Tg mice, *vs sham-operated wild-type mice, and +vs sham-operated Sirt1-Tg mice. P<0.01: ##vs BCAS-operated Sirt1-Tg mice, ***vs sham-operated wild-type mice, and ++vs sham-operated Sirt1-Tg mice.
Sirt1-Tg Mice Show Preserved Cerebral Blood Flow After BCAS

To assess the vascular origin of the tolerance against BCAS found in Sirt1-Tg mice, we measured the cerebral blood flow (CBF) by laser speckle flowmetry. The mean baseline CBF of Sirt1-Tg mice showed 96.2% of the baseline level of wild-type littermates without significant intergroup differences. Relative CBF at each time point to baseline was not significantly different between wild-type and Sirt1-Tg mice after sham operation. However, at 2 hours and 1 day after BCAS operation, CBF of wild-type mice reduced to 74.3±3.2% and 72.4±1.3% of the baseline level while that of Sirt1-Tg mice reduced to 91.9±2.5% and 94.1±2.8%, respectively (Figure 3). These observations prompted us to compare their cerebrovascular architecture. Postmortem latex perfusion method indicated that the diameter of the basal arteries did not differ (Figure III in the online-only Data Supplement). Thus, the significant retention of CBF in Sirt1-Tg mice after BCAS is not attributed to the development of the collateral vasculature bypassing the anterior and posterior brain circulations.

Pharmacological Inhibition of SIRT1 Abolishes the CBF Retention in Sirt1-Tg Mice After BCAS

To confirm whether SIRT1 was responsible for the CBF preservation after BCAS, we monitored CBF after BCAS in Sirt1-Tg mice that were pretreated with intravenous sirtinol (1 mg/kg), a cell-permeable 2-hydroxy-1-naphthaldehyde derivative which acts as a specific and direct inhibitor of the sirtuin...
Stroke November 2014

Sirt1-Tg Mice Exhibit a Higher Cerebral Vasodilatory Response to an eNOS Agonist

A previous in vitro study suggested that the vasodilating activity of eNOS is potentiated by SIRT1.26 To test the possible involvement of eNOS in the phenotype of Sirt1-Tg mouse, we measured CBF before and after the exposure with a classical eNOS agonist acetylcholine. CBF of Sirt1-Tg mice showed significantly larger response to acetylcholine than that of wild-type littermates (Sirt1-Tg, 18.8±3.3% versus wild-type, 8.0±1.8%; Figure 5A and 5C). In vivo vessel imaging with fluorescein isothiocyanate-dextran corroborated the results with vasodilatory responses to acetylcholine (Sirt1-Tg, 7.01±0.83% versus wild-type, 3.65±0.44%; Figure 5B and 5D). We also used S-Nitroso-N-acetyl-dl-penicillamine, a nitric oxide (NO) donor, instead of acetylcholine, and determined whether the whole NOS machinery can be bypassed to raise CBF in both genotypes. Wild-type and Sirt1-Tg mice showed similar increase in CBF in response to S-Nitroso-N-acetyl-dl-penicillamine, which was relatively greater than the response to acetylcholine (Figure V in the online-only Data Supplement).

Excess SIRT1 Suppresses eNOS Acetylation After BCAS

To explore the molecular pathway involved in our in vivo findings, we conducted biochemical analyses. The levels of the total eNOS in the brain homogenates did not differ between wild-type and Sirt1-Tg littermates, both before and at 2 hours after BCAS. The cerebral protein level of Ser1177-phosphorylated eNOS increased at 2 hours after BCAS in wild-type mice, but not in Sirt1-Tg mice, reflecting lack of vascular endothelial growth factor–mediated compensatory response after CBF reduction27 in Sirt1-Tg mice. To detect deacetylated eNOS specifically, brain homogenates before

Figure 5. Sirt1-overexpressing (Sirt1-Tg) mice exhibited increased vasodilative response to acetylcholine (ACh). Temporal changes in cerebral blood flow (CBF) assessed by laser speckle flowmetry (A) and in diameters of leptomeningeal arteries (B) before (left) and after (right) perfusion of ACh on brain surface in wild-type and Sirt1-Tg mice. Scale bars indicate 1 mm (A) and 50 μm (B). Histograms showing % increase of CBF assessed by laser speckle flowmetry (C) and % increase of vascular diameter (D) in response to ACh in wild-type (n=6) and Sirt1-Tg mice (n=6).
Endothelial nitric oxide synthase (eNOS) was completely deacetylated in Sirt1-overexpressing (Sirt1-Tg) mice at 2 hours after bilateral common carotid artery stenosis (BCAS). A, Immunoblots of total eNOS, phosphorylated eNOS, acetylated eNOS, and nonacetylated eNOS and β-actin of wild-type and Sirt1-Tg mice before and at 2 hours after BCAS. The results of 2 different animals for each genotype are shown. Similar results were obtained in 2 other animals. B, Histogram showing the ratio of eNOS, phosphorylated eNOS, acetylated eNOS, and nonacetylated eNOS to β-actin. C, Cerebral blood flow of vehicle-treated Sirt1-Tg mice (n=7) and cavtratin-treated Sirt1-Tg mice (n=6) before and at 2 hours after BCAS. *P<0.01 vs vehicle.

and at 2 hours after operation were immunoprecipitated with antibodies against acetylated lysine, and the immunoprecipitates were probed with antibodies against eNOS to detect acetylated eNOS. Intriguingly, acetylated eNOS was undetectable in the both groups before BCAS, whereas it became detectable only in wild-type mice after BCAS. In the pooled elution fractions that are expected to contain nonacetylated eNOS, brain tissues from Sirt1-Tg mice contained greater amounts of nonacetylated eNOS compared with those from wild-type littermates after BCAS, but the amounts of nonacetylated eNOS before BCAS in both groups were similar (Figure 6A and 6B).

**CBF Retention in Sirt1-Tg Mice After BCAS Is Abolished by eNOS Inactivation**

To verify the requirement of eNOS activation for the CBF retention in Sirt1-Tg mice after BCAS, we treated them with an eNOS inhibitor, cavtratin (caveolin-1 scaffolding domain peptide, 10 mg/kg per day 3× days). Cavtratin significantly reduced CBF of Sirt1-Tg mice at 2 hours after
BCAS (cavitratin, 77.7±4.1% versus vehicle, 97.3±3.9%; each normalized with the baseline CBF; Figure 6C), offsetting the increment of CBF by the overexpression of SIRT1 (cf. Figures 4A and 6C). Sham operation did not alter CBF of cavitratin-treated Sirt1-Tg mice (Figure VI in the online-only Data Supplement). These results suggest that the SIRT1-mediated deacetylation activates eNOS, which counters the perfusion failure after BCAS.

**Discussion**

Using Sirt1-overexpressing Tg mice, we found that endothelial SIRT1 deacetylates and activates eNOS and thus normalizes CBF after BCAS. These effects were abolished by treatment with inhibitors of SIRT1 or eNOS in Sirt1-Tg mice, suggesting that SIRT1–eNOS–NO system is responsible for the CBF-preserving effect after cerebral hypoperfusion. SIRT1 overexpression also significantly attenuated BCAS-induced blood–brain barrier disruption, glial activation, myelin loss, and working memory impairment. Furthermore, SIRT1 overexpression suppressed alterations in the vascular microarchitecture and any neuronal derangement. These results firstly show that SIRT1 has a robust role in suppressing the consequence of cerebral hypoperfusion by activating a cerebral NO-dependent mechanism.

Our results are congruent with a previous in vitro study showing that SIRT1 and eNOS colocalize and coprecipitate in endothelial cells and that SIRT1 can deacetylate eNOS. Therefore, an interaction of SIRT1 with eNOS may mediate cerebrovascular protection by facilitating NO-dependent vascular relaxation, which has been firstly demonstrated in vivo in the current study. Oxidative stress is known to trigger acetylation of eNOS in endothelial cells, leading to inactivation of eNOS activity. Based on the current finding that the fall in CBF after BCAS is prevented in Sirt1-Tg mice and our previous finding that the gradual CBF recovery after BCAS was markedly inhibited with NOS inhibitor Nω-nitro-L-arginine methyl ester, cerebral SIRT1–eNOS–NO system may regulate physiological energy stability in the brain.

Accumulating evidence suggests that SIRT1 plays an important role in neurodegenerative diseases through different pathways. Alzheimer disease is one of neurodegenerative diseases, but vascular pathology contributes to Alzheimer disease change to variable degrees. There is an emerging concept of protein elimination failure arteriopathy where waste products such as amyloid-β accumulate in the brain as a result of cerebral perfusion failure and evoke disparate brain disorders because perivascular drainage of waste products are driven by arterial pulsation. Because reduced amyloid-β clearance from the brain seems to be mainly responsible for the pathogenesis of sporadic Alzheimer disease, the drainage of extracellular amyloid-β along the arteries seems to be a significant strategy for removal of amyloid-β from the brain. The CBF-preserving effect of SIRT1 may provide a unified scheme for treatment of broad spectrum of brain diseases involving both cerebrovascular and neurodegenerative mechanisms.

In conclusion, our study provides strong evidence for the role of SIRT1 in protection of the brain after cerebral hypoperfusion and ischemic injury by activating the eNOS–NO system. The robust effects of SIRT1 overexpression on restoration of cerebrovascular reserve in mice may explain the positive effects of SIRT1 reported in animal models of ischemic injury and neurodegeneration. However, it remains to be determined whether sirtuin-activating compounds such as resveratrol can be used as proof of concept agents for novel strategies for the treatment of disparate brain disorders.

**Acknowledgments**

We acknowledge the gift of the mouse Sirt1 cDNA and the thoughtful comments from Dr Shin-ichiro Imai and thank Dr Ahmad Khundakar for editing the article. We are indebted to Takako Kawada for her excellent technical assistance in staining and tissue sections.

**Sources of Funding**

We gratefully acknowledge grant support from the Ministry of Health, Labour, and Welfare (Dr Ihara, no. 0605-1), the Ministry of Education, Culture, Sports, Science, and Technology (Dr Ihara, Grant-in-Aid for Scientific Research (B), no. 23390233; Dr Hattori, Grant-in-Aid for Research Activity Start-up, no. 25893301; Dr Kinoshita, Grant-in-Aid for Scientific Research on Innovative Areas, no. 2311531 and 25116514), the Takeda Science Foundation (Dr Ihara), Japan Science Technology Corporation (Dr Takahashi), and Alzheimer’s Research UK (Dr Kalaria).

**Disclosures**

None.

**References**


Silent Information Regulator 2 Homolog 1 Counts Cerebral Hypoperfusion Injury by Deacetylating Endothelial Nitric Oxide Synthase
Yorito Hattori, Yoko Okamoto, Takakuni Maki, Yumi Yamamoto, Naoya Oishi, Kenichi Yamahara, Kazuyuki Nagatsuka, Ryosuke Takahashi, Raj N. Kalaria, Hidenao Fukuyama, Makoto Kinoshita and Masafumi Ihara

Stroke. published online September 11, 2014;
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/early/2014/09/11/STROKEAHA.114.006265

Data Supplement (unedited) at:
http://stroke.ahajournals.org/content/suppl/2014/09/11/STROKEAHA.114.006265.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org/subscriptions/
SUPPLEMENTAL MATERIAL

SIRT1 counters cerebral hypoperfusion injury by deacetylating eNOS

Yorito Hattori, Yoko Okamoto, Takakuni Maki, Yumi Yamamoto, Naoya Oishi, Kenichi Yamahara, Kazuyuki Nagatsuka, Ryosuke Takahashi, Raj N. Kalaria, Hidenao Fukuyama, Makoto Kinoshita, Masafumi Ihara

Corresponding author: Masafumi Ihara, MD, PhD, FACP; Department of Stroke and Cerebrovascular Diseases, National Cerebral and Cardiovascular Center; 5-7-1 Fujishiro-dai, Suita, Osaka 565-8565, Japan; Telephone, (+81)-6-68335012; FAX, (+81)-6-68355137; E-mail, ihara@ncvc.go.jp
Supplemental Methods

Generation and Establishment of a Transgenic Mouse Line That Stably Expresses Mouse SIRT1 in the Brain
To generate a genetic model that chronically overexpress silent information regulator 2 homolog 1 (SIRT1) in the mouse, we constructed a transcription unit by inserting the coding region of the mouse Sirt1 cDNA into the mouse prion gene promoter-polyA cassette which drives pan-neural gene expression. We obtained transgenic mice by injecting the linearized transcription unit into the oocytes of C57BL/6J mice and selected a founder that transmitted the transgene in Mendelian manner. We backcrossed the founder and the offspring with wild-type C57BL/6J mice for more than ten generations and established a transgenic line that gave consistent, pan-neural expression of exogenous SIRT1 in addition to the endogenous gene products. In this study we consistently analyzed male mice heterozygous for the transgene (Sirt1-Tg) with their wild-type (non-transgenic) male littermates at the age of 5–9 months old. Sirt1-Tg mice and wild-type littermates did not show recognizable differences in physical constitution and brain morphology (data not shown). All mice were maintained in the C57BL/6J background, were housed in a room with a 12-hour light/dark cycle (lights on at 7:00 a.m.) and were given access to food and water ad libitum. All procedures were performed in accordance with the guidelines for animal experimentation from the ethical committee of Kyoto University, and National Cerebral and Cardiovascular Center.

Surgical Procedure of Bilateral Common Carotid Artery Stenosis (BCAS) Operation
Through a midline cervical incision, both common carotid arteries were exposed. Microcoils with an internal diameter of 0.18 mm (Samini) were applied to the bilateral common carotid arteries (Supplemental Figure I). Sham-operated mice underwent the same surgical procedure without using microcoils. Anesthesia was induced with 2% isoflurane and maintained with 1.5% isoflurane in 80% nitrous oxide and 20% oxygen. Rectal temperature was maintained between 36.5°C and 37.5°C.

Eight-arm Radial Maze Test
As described previously, after 28 days post-operation, the eight-arm radial maze test was performed to examine whether spatial working memory was impaired. Each arm (8×35 cm) radiated from an octagonal central starting platform. Identical food wells were placed at the distal end of each arm. From one week before pretraining, mice were deprived of food until their body weight was reduced to 75–85% of the initial level. As the initial pretraining after deprivation of food, each mouse was placed in the central platform and allowed to explore and to consume food pellets scattered on the whole maze for a 5-min period (one session per mouse). Subsequently, these mice received another pretraining to take a pellet from each food
well after being placed at the distal end of each arm. This was repeated 8 times, using 8 different arms, for each mouse.

After these pretraining trials, actual maze acquisition trials were performed. All eight arms were baited with food pellets. Mice were placed on the central platform and allowed to get all eight pellets within 25 min. A trial was terminated immediately after all 8 pellets were consumed or 25 min had elapsed. For each trial, the number of revisiting error was recorded.

**Histological Evaluation of Sirt1-Tg Mice and BCAS-operated Mice**

To examine where SIRT1 was expressed in the brain, 6-μm-thick paraffin-embedded coronal sections were subjected to immunohistochemistry for silent information regulator 2 (Sir2; 1:50; Sigma-Aldrich), and cryosections (20-μm thick) were subjected to immunofluorescence for Sir2 (1:100; Sigma-Aldrich) and double immunofluorescence for Sir2 and CD31 (vascular endothelial cell, 1:500; BD Biosciences).

The brains of Sirt1-Tg mice and their littermates were dissected out at 28 days after sham or BCAS operation. Six-μm thick paraffin-embedded coronal sections were subjected to Klüver-Barrera staining. The severity of the white matter lesions was graded as normal (Grade 0), disarrangement of the nerve fibers (Grade 1), the formation of marked vacuoles (Grade 2), and the disappearance of myelinated fibers (Grade 3) in the corpus callosum. For immunohistochemistry, we used, as first antibodies, a rabbit antiglial fibrillary acidic protein antibody (a marker of astrocyte, 1:2000; DAKO), a rabbit anti-Iba1 antibody (a marker of microglia, 1:200; Wako), and a rabbit antiglutathione S-transferase-pi antibody (a marker of oligodendrocyte, 1:100; Millipore). We counted the numerical density of the glial cell nuclei with immunopositive perikarya (1/0.125 mm²) in the white matter.

**Transmission Electron Microscopy**

Wild-type and Sirt1-Tg mice at 2 h after sham or BCAS operation were examined by transmission electron microscopy. After mice were perfused transcardially with 0.9% saline followed by 4% paraformaldehyde and 2% glutaraldehyde in 0.1 mol/L phosphate buffer, brains were removed and consecutively sectioned at the bregma level at a thickness of 1 mm using a brain tissue matrix. Then, brain tissues were fixed by immersion in 4% paraformaldehyde and 2% glutaraldehyde in 0.1 mol/L phosphate buffer for 48 h at 4°C, and washed in 0.1 mol/L phosphate buffer (5×10 min). Two pieces of brain tissues (approximately 1.5 mm³) were subsequently resected and postfixed with 1% osmium tetroxide for 2 h. Thereafter, the fixed tissue samples were dehydrated, infiltrated, and embedded in epoxy-resin (Luveak 812; Nakalai Tesque) for transmission electron microscope study. Ultrathin sections (80 nm) of selected areas were prepared on an ultramicrotome (EM UC6; Leica) and collected on 200-mesh cooper grids. These sections were counterstained with 2% uranyl acetate and lead citrate solution. The vessels were examined throughout the
Measurement of Cerebral Blood Flow (CBF)
Relative CBF was recorded by laser speckle flowmetry (Omegazone; Omegawave) which obtains high-resolution, two-dimensional imaging and has a linear relationship with absolute CBF values\(^3\). In the day prior to the first CBF measurement, anesthesia was induced with 2% isoflurane and maintained with 1.5% isoflurane in 80% nitrous oxide and 20% oxygen, and the scalp was removed by a midline incision so that the skull was exposed throughout the experiment. During the measurement of CBF, the skull surface was illuminated by 780 nm of laser light. The scattered light was filtered and detected by a CCD camera positioned over the head. The filter detected only scattered light that had a perpendicular polarization to the incident laser light. The raw speckle images were used to compute speckle contrast, which corresponds to the measured velocity of moving red blood cells, approximating CBF. Signal processing was performed by the algorithm developed by Forrester et al.\(^4\). Color-coded blood flow images were obtained in high-resolution mode (639 × 480 pixels; 1 image/sec) and the sample frequency was 60 Hz. One blood flow image was generated by averaging numbers obtained from 20 consecutive raw speckle images. The recordings were initiated after the examiner confirmed that CBF did not change over 1 min, and the five recordings of blood flow image were averaged. In order to prevent the fluctuation of CBF and blood pressure during the measurement of CBF, anesthesia was induced as stated above. During the measurement of CBF, mice were held in a small plastic holder on a warming pad and thermostatically controlled at 36.5°C to 37.5°C in rectal temperature. Blood pressure was measured by the tail cuff method and confirmed to be kept constant.

Evaluation of Vascular Response to Acetylcholine (ACh) and S-Nitroso-N-acetyl-DL-penicillamine (SNAP)
To evaluate vascular responses to vasodilatory stimuli, a cranial window preparation was performed as previously reported with modification\(^5,6\). In brief, a 3 mm × 3 mm diameter craniotomy was performed with dental drill in the right parietal bone and the dura mater was removed. The endothelium-dependent vasodilator ACh (100 μmol/L; Sigma-Aldrich), endothelial nitric oxide synthase (eNOS)-agonist, or S-Nitroso-N-acetyl-DL-penicillamine (SNAP; 500 μmol/L; Sigma-Aldrich), nitric oxide donor, were infused into the cranial window at a rate of 100 μL/min for 5 min. The CBF and the vasodilative changes in response to ACh or SNAP were evaluated at 5 min after infusion. CBF increase during the 5 min after infusing was taken as response amplitude using laser speckle flowmetry. The rate of CBF increase after infusing was calculated as the CBF increase (%) divided by the baseline CBF. Next, we assessed the vasodilatative responses. For real-time in vivo imaging of the cerebral vessels, we used fibered fluorescence microscopy (MVX10; Olympus). After intravenous tail
vein injection of fluorescein isothiocyanate-dextran (2×10^6 molecular weight, 200 μL of 20 mg/mL; Sigma-Aldrich), the leptomeningeal vessels were visualized. Averaged vessel diameters across a 25 μm longitudinal segment (5 consecutive segments per mouse) of the dorsal middle cerebral arteries were analyzed as previously described. Peak vessel diameter increase during the 5 min was taken as response amplitude. Data were calculated as %vasodilation vs. baseline vessel diameter. We distinguished penetrating arteries from bridging (collecting) veins by identifying the location and also by following the direction of flow from the pial surface.

**Western Blot Analysis**

The brains of BCAS-operated mice were dissected out before and at 2 h after BCAS and cut coronally into 5-mm thick slices (bregma -3 to +2 mm) and homogenized in radioimmunoprecipitation assay buffer containing a protease and phosphatase inhibitor mixture (Nakarai Tesque). The amount of proteins applied to each well was 80 μg for acetylated eNOS, non-acetylated eNOS, 40 μg for eNOS and phosphorylated eNOS, and 20 μg for β-actin. Samples were electrophoresed on SDS-polyacrylamide minigels and the proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad). Membranes were incubated with primary antibodies against eNOS (1:2500; BD Biosciences), phospho-eNOS (Ser1177) (1:1000; Cell Signaling Technology) and β-actin (1:5000; Sigma-Aldrich). The signal was visualized using horseradish peroxidase-conjugated secondary antibodies with Luminata Forte Western HRP substrate (Millipore). Immunoblot membranes were developed using the LAS-4000 Imaging System (Fujifilm). The densitometric measurement of immunoblots was performed using Image-J (NIH).

Similarly, brain homogenates of cerebral cortex, caudoputamen, midbrain, brainstem, cerebellum, and spinal cord were immunoblotted using anti-Sir2 antibody (1:500; Sigma-Aldrich) and anti-GAPDH antibody (1:1000; Cell Signaling Technology).

**Analysis of eNOS Acetylation/Non-acetylation**

To determine acetylation or non-acetylation status of eNOS, brain homogenates before and at 2 h after BCAS were first immunoprecipitated by anti-acetylated-lysine antibody (1:100; Cell Signaling Technology) using Immunoprecipitation Kit-Dynabeads Protein A (Life Technologies). As a result, the brain homogenates were separated into bound and unbound fractions to the anti-acetylated-lysine antibodies. The bound fraction was eluted with elution buffer as a fraction that contains total acetylated proteins. The both fractions were then immunoblotted with eNOS to detect acetylated and non-acetylated eNOS, respectively, using the immunoblotting method as stated above.

**Exogenous Administration of Sirtinol**
To examine whether sirtinol (Sigma-Aldrich), SIRT1 inhibitor, abolished CBF-preserving effect of SIRT1, sirtinol was intravenously injected into 10-week-old Sirt1-Tg mice with the dose of 1 mg/kg (dimethyl sulfoxide 50 µL + saline 100 µL) immediately before sham or BCAS operation. The CBF estimate was carried out using laser speckle flowmetry as described above. CBF was expressed as a percentage of baseline flow.

**Exogenous Administration of Cavtratin**
Caveolin-1 scaffolding domain peptide (ENZO Life Sciences), eNOS inhibitor called cavtratin, was intraperitoneally injected into 10-week-old Sirt1-Tg mice with the dose of 10 mg/kg/day (dimethyl sulfoxide 60 µL + saline 90 µL) for 3 consecutive days prior to sham or BCAS operation. Last injection was carried out at 24 h prior to each surgery. The CBF estimate was carried out using laser speckle flowmetry as described above. CBF was expressed as a percentage of baseline flow.

**Visualization of Cerebral Angioarchitecture**
The cerebral angioarchitecture was studied by the postmortem latex perfusion technique. The root of the ascending aorta was cannulated with flexible plastic tubing (0.65 mm external diameter). The tubing was connected to a 5 mL syringe, the cannulated aorta, and a mercury manometer, establishing a closed circuit to monitor perfusion pressure. Immediately after 2 mL saline injection, 4mL white latex compound (Chicago Latex Products) mixed with 50 µL/mL carbon black (Bokusai) diluted 2:1 with saline was injected at a perfusion pressure of 150 mmHg over a 5-min period. After the initiation of infusion, the right atrium of the heart was incised to allow for venous outflow. In order to harden the latex completely for the brain removal procedure, the dead animal was soaked in ice-cold water 20 min after the end of infusion, and the brain was subsequently removed 20 min later. Photographs of dorsal and ventral surface of the brain were taken using a digital microscope (DinoLite; AnMo Electronics Corp.) at ×80 magnification. The vessel diameter of the circle of Willis was measured using image analysis software (DinoCapture; AnMo Electronics Corp.). The diameters of the internal carotid artery, the anterior cerebral artery, the middle cerebral artery and the posterior communicating artery were averaged across both sides. The diameters of the internal carotid artery and middle cerebral artery were measured just proximally and distally to the terminal bifurcation of the internal carotid artery, respectively. The diameter of the anterior cerebral artery was measured just proximally to the origin of the olfactory artery. The diameter of the posterior communicating artery was measured at its origin from the internal carotid artery.

**Statistical Analysis**
Statistical analysis was conducted using StatView (SAS Institute). All values are expressed as
means ± standard error of the mean in the figures. Data were analyzed by unpaired t-test unless noted otherwise. Differences with $p<0.05$ were considered statistically significant in all analyses.
Supplemental References


Supplemental Figure I: Representative image showing surgical implantation of a microcoil on the common carotid artery.
A microcoil is placed on the left common carotid artery after the vagus nerve is separated from the carotid artery.
Supplemental Figure II: SIRT1 rescued neuronal injuries after BCAS
Transmission electron microscopic images of BCAS-operated, wild-type (A) or Sirt1-Tg mice (B), and sham-operated, wild-type (C) or Sirt1-Tg mice (D) at 2 h after each surgery. (A) Neurons of a wild-type mouse undergo shrinkage of nuclei and cytoplasm. (B–D) Neurons appear normal. Scale bars indicate 10 μm (A), and 20 μm (B–D).
Supplemental Figure III: Similar angioarchitecture of wild-type littermates and Sirt1-Tg mice

(A) Representative images of the dorsal and ventral cerebral angioarchitecture by postmortem latex perfusion method of the wild-type and Sirt1-Tg mouse. Abbreviations: ACA, anterior cerebral artery; MCA, middle cerebral artery; ICA, internal carotid artery; PcomA, posterior communicating artery. (B) Histograms showing the mean diameters of ACA, MCA, ICA and PcomA of wild-type (n = 6) and Sirt1-Tg mice (n = 5).
Supplemental Figure IV: Sham operation did not alter CBF of sirtinol-treated Sirt1-Tg mice. Temporal profiles of CBF of vehicle-treated Sirt1-Tg mice (n = 3) and sirtinol-treated Sirt1-Tg mice (n = 3) before and at 2 h after sham operation.
Supplemental Figure V: Temporal changes in CBF before and after perfusion of SNAP on brain surface in wild-type (n = 3) and Sirt1-Tg mice (n = 3). (A) Representative CBF images assessed by laser speckle flowmetry before (left) and after (right) perfusion of SNAP. * indicates an inlet of SNAP. (B) Histogram showing %increase of CBF assessed by laser speckle flowmetry.
Supplemental Figure VI: Sham operation did not alter CBF of cavtratin-treated Sirt1-Tg mice. Temporal profiles of CBF of vehicle-treated Sirt1-Tg mice (n = 3) and cavtratin-treated Sirt1-Tg mice (n = 3) before and at 2 h after sham operation.