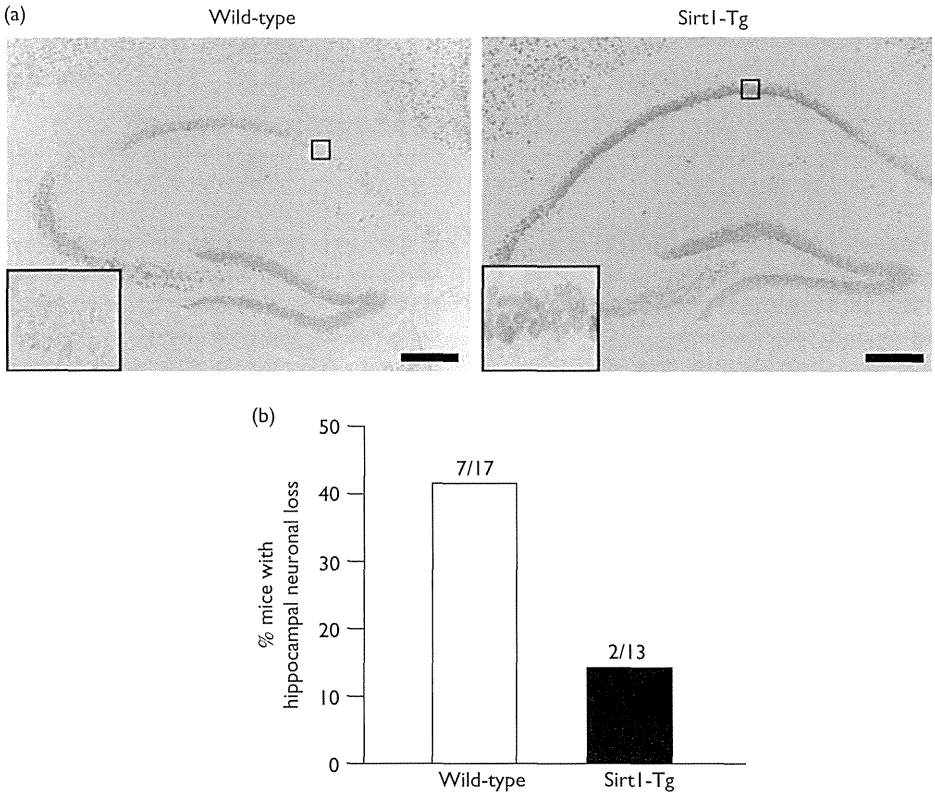
Fig. 1



Silent information regulator 2 homolog 1 (SIRT1) reduced hippocampal neuronal loss after bilateral common carotid artery occlusion (BCAO). (a) Representative immunohistochemical images for NeuN in wild-type and *Sirt1*-overexpressing (Sirt1-Tg) mice 7 days after BCAO for 10 min. The inset shows a magnified image of the indicated area. Scale bars, 200 μm. (b) Histogram showing the percentage of mice that experienced hippocampal neuronal loss. Hippocampal neuronal loss was observed in seven (41.2%) of 17 wild-type mice and two (15.4%) of 13 Sirt1-Tg mice.

Using a *Sirt1*-overexpressing (Sirt1-Tg) mouse line, we recently showed that SIRT1 overexpression preserved CBF after bilateral common carotid artery stenosis (BCAS, ~50% stenosis) by activating endothelial nitric oxide synthase by deacetylation, and this effectively countered cerebral hypoperfusion injury [15]. SIRT1 overexpression considerably maintained CBF after BCAS, whereas CBF decreased to ~70% of baseline in wild-type littermates. To further corroborate the cerebrovascular involvement of SIRT1 in protecting against cerebral ischemia, we subjected wild-type and Sirt1-Tg mice to bilateral common carotid artery occlusion (BCAO) surgery to determine whether Sirt1-Tg mice show preserved CBF during BCAO.

Materials and methods

Animals

All procedures were performed in accordance with the guidelines for animal experimentation from the Ethics Committee of National Cerebral and Cardiovascular Center. Male C57BL/6J mice aged 13–14 weeks (Japan SLC, Hamamatsu, Japan) were used and allowed free access to food and water *ad libitum*. All animals survived 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 mouse *Sirt1* into the mouse prion gene promoter-polyA cassette that drives pan-neural gene expression [see Text document, Supplemental digital content 1 (*http://links.lww.com/WNR/A315*), which indicates the detail of Sirt1-Tg mouse].

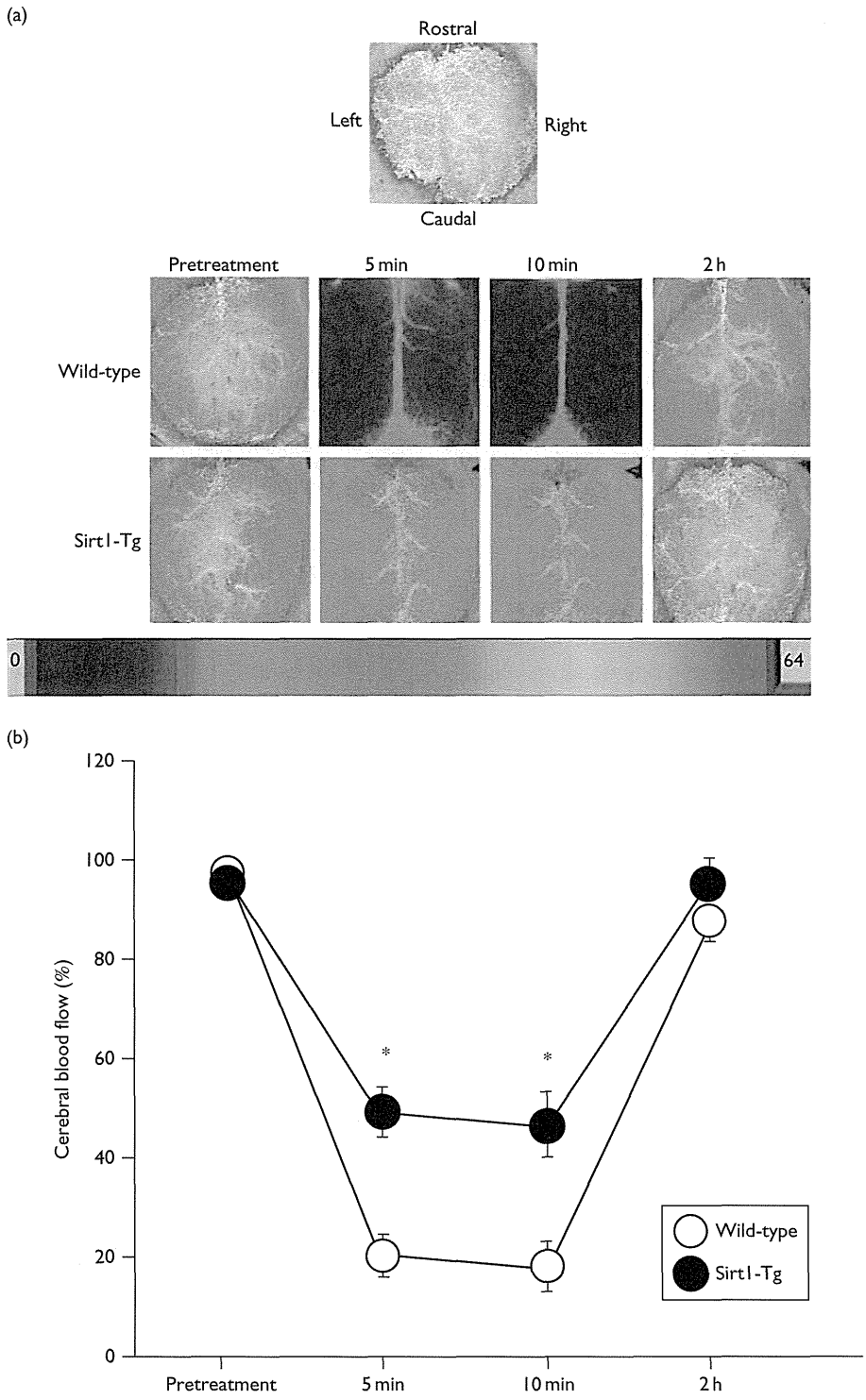
Study design

Two groups of male C57BL/6J mice were used in this study: (a) the wild-type BCAO group ($n=17$) and (b) the Sirt1-Tg BCAO group ($n=13$). Temporal CBF changes were measured by laser speckle flowmetry (OMEGAZONE; OMEGAWAVE Inc., Tokyo, Japan) before and 5, 10 min, and 2 h after BCAO. Histological evaluation of hippocampal changes was performed 7 days after BCAO.

Surgical procedure of transient BCAO

Through a midline cervical incision, the bilateral common carotid arteries were exposed. Microaneurysm clips (stainless-steel microserrefines; Muromachi Kikai, Tokyo, Japan) were applied to the bilateral common

Fig. 2



Silent information regulator 2 homolog 1 (SIRT1) overexpression facilitated the maintenance of cerebral blood flow (CBF) after bilateral common carotid artery occlusion (BCAO). (a) Representative CBF images assessed by laser speckle flowmetry. (b) Temporal profiles of wild-type ($n=5$) and *Sirt1*-overexpressing (Sirt1-Tg) mice ($n=5$) before and after BCAO are shown, which were constructed using data obtained by laser speckle flowmetry. *$P<0.05$ versus the wild-type.

carotid arteries, which were subsequently occluded for 10 min. Anesthesia was induced with 2% isoflurane and maintained with 1.5% isoflurane in 80% nitric oxide and 20% oxygen.

Histological evaluation of hippocampal neuronal changes

To identify ischemic neuronal loss in the hippocampus 7 days after BCAO, mice were deeply anesthetized by an intraperitoneal injection of sodium pentobarbital (40 mg/kg) and perfused transcardially with 0.01 M PBS, followed by a fixative containing 4% paraformaldehyde. The mouse brains were removed and divided coronally at the hippocampus. They were embedded in paraffin and sliced into 6 μm-thick coronal sections. These sections were subjected to immunohistochemistry for NeuN (1 : 100; Millipore, Billerica, Massachusetts, USA). We determined the number of mice that had hippocampal neuronal loss.

Measurement of CBF by laser speckle flowmetry

Relative CBF was determined by laser speckle flowmetry, which provides high-resolution, two-dimensional images and has a linear relationship with absolute CBF values [16]. The detailed methods are described in Supplemental digital content 2 (*http://links.lww.com/WNR/A315*).

Statistical analysis

All values are expressed as mean ± SE in the text and figures. Differences with P value less than 0.05 were considered statistically significant in all analyses, which included Student's t-test.

Results

Hippocampal integrity is preserved in Sirt1-Tg mice after BCAO

The hippocampus is one of the brain regions most vulnerable to ischemia, and hippocampal neuronal loss can be a sensitive indicator of ischemic neuronal damage. In total, seven (41.2%) of 17 wild-type mice showed hippocampal neuronal loss in the CA1 region versus only two (15.4%) of 13 Sirt1-Tg mice (Fig. 1). These results indicate that SIRT1 overexpression confers protection against severe ischemia in the hippocampus.

Sirt1-Tg mice show preserved CBF after BCAO

We performed laser speckle flowmetry to examine CBF before and after BCAO. The mean baseline CBF of Sirt1-Tg mice was 96.2% of the mean baseline CBF level of wild-type littermates, without significant intergroup differences. The CBF of wild-type mice decreased to 25.0 ± 2.2 and 20.7 ± 3.4% of baseline 5 and 10 min after BCAO, respectively, whereas that of Sirt1-Tg mice decreased to 49.3 ± 3.1 and 45.3 ± 7.7%, respectively, at the same time points, indicating the significant CBF-preserving effects of SIRT1 overexpression during BCAO. In both Sirt1-Tg and wild-type mice, CBF 2 h after BCAO recovered to near baseline levels, with no differences between the groups (Fig. 2). We assume that this CBF-preserving effect circumvents hippocampal neuronal loss caused by severe ischemia in Sirt1-Tg mice.

Discussion

Our findings show that SIRT1 overexpression partially preserves CBF and the histological integrity of the hippocampus, one of the regions most susceptible to global cerebral ischemia, after BCAO-induced transient global ischemia for 10 min. We reported previously that SIRT1 overexpression almost normalized CBF, even after BCAS, by activating endothelial nitric oxide synthase, whereas in wild-type littermates, CBF decreased to ~ 70% of that measured at baseline [15]. Taken together, these results suggest that SIRT1 overexpression protects against severe cerebral ischemia by preserving CBF.

The vascular basis of cerebral ischemia consists of Virchow's triad: vascular endothelial injury, hemorheological abnormalities, and reduced flow within vascular beds [17]. The primary target of current treatments and prophylactic medications for acute cerebral infarction is hemorheological abnormalities, which are treated with antiplatelet and/or anticoagulant drugs. Flow reduction within vascular beds, another component of the triad, can be targeted with fasudil, a Rho kinase inhibitor that is used as a vasodilator for cerebral vasospasms that occur after subarachnoid hemorrhage [18]. However, fasudil is not used to treat cerebral ischemia because preclinical studies have shown that the drug failed to protect against damage induced by middle cerebral artery occlusion in a murine model [18]. Other cerebral vasodilators (e.g. acetazolamide) are contraindicated because of the risk of the intracerebral steal phenomenon. The other component of the triad is vascular endothelial injury, for which appropriate interventions have not been developed. In our previous study, endothelial SIRT1 overexpression was found to rescue disrupted microvilli and open tight junctions of the endothelium after BCAS [15]. Therefore, SIRT1 activators (e.g. resveratrol) may represent novel treatments for vascular endothelial injury and flow reduction in ischemic lesions.

Conclusion

Our study provides evidence for the promising role of SIRT1 in protecting against cerebral global ischemia by preserving CBF. The robust effects of SIRT1 overexpression on the restoration of cerebrovascular reserve in mice may explain the positive effects of SIRT1 that have been reported in several animal models of ischemic injury.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

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REVIEW ARTICLE
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New therapeutic approaches for Alzheimer's disease and cerebral amyloid angiopathy

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Accumulating evidence has shown a strong relationship between Alzheimer's disease (AD), cerebral amyloid angiopathy (CAA), and cerebrovascular disease. Cognitive impairment in AD patients can result from cortical microinfarcts associated with CAA, as well as the synaptic and neuronal disturbances caused by cerebral accumulations of β-amyloid (Aβ) and tau proteins. The pathophysiology of AD may lead to a toxic chain of events consisting of Aβ overproduction, impaired Aβ clearance, and brain ischemia. Insufficient removal of Aβ leads to development of CAA and plays a crucial role in sporadic AD cases, implicating promotion of Aβ clearance as an important therapeutic strategy. Aβ is mainly eliminated by three mechanisms: (1) enzymatic/glial degradation, (2) transcytotic delivery, and (3) perivascular drainage (3-"d" mechanisms). Enzymatic degradation may be facilitated by activation of Aβ-degrading enzymes such as neprilysin, angiotensin-converting enzyme, and insulin-degrading enzyme. Transcytotic delivery can be promoted by inhibition of the receptor for advanced glycation end products (RAGE), which mediates transcytotic influx of circulating Aβ into brain. Successful use of the RAGE inhibitor TTP488 in Phase II testing has led to a Phase III clinical trial for AD patients. The perivascular drainage system seems to be driven by motive force generated by cerebral arterial pulsations, suggesting that vasoactive drugs can facilitate Aβ clearance. One of the drugs promoting this system is cilostazol, a selective inhibitor of type 3 phosphodiesterase. The clearance of fluorescent soluble Aβ tracers was significantly enhanced in cilostazol-treated CAA model mice. Given that the balance between Aβ synthesis and clearance determines brain Aβ accumulation, and that Aβ is cleared by several pathways stated above, multi-drugs combination therapy could provide a mainstream cure for sporadic AD.

Keywords: Alzheimer's disease, cerebral amyloid angiopathy, treatment, perivascular drainage, cilostazol

INTRODUCTION

Alzheimer's disease (AD) is the most common cause of dementia in the elderly. AD is pathologically characterized by β-amyloid (Aβ) plaques within the brain parenchyma and Aβ accumulation in blood vessels (cerebral amyloid angiopathy; CAA), as well as by the formation of neurofibrillary tangles and neurodegeneration (Duyckaerts et al., 2009). AD was not previously thought to be closely linked to cerebrovascular disease (CVD), but accumulating lines of evidence have shown a strong relationship between AD and vascular dementia (VaD) (Fotuhi et al., 2009; Kalaria and Ihara, 2013). AD and CVD share common risk factors (Viswanathan et al., 2009; Kalaria et al., 2012), and treatment of vascular risk factors is associated with slower decline in cognitive impairments of AD patients (Deschaintre et al., 2009). The Nun study revealed that CVD plays an important role in determining the presence and severity of the clinical symptoms of AD (Snowdon et al., 1997). Aβ accumulation and other AD changes are also recognized in elderly patients without apparent dementia (Funato et al., 1998; Schneider et al., 2007), which implies a strong relationship between AD neuropathology and the aging processes. Many reports have described that a majority of sporadic dementia patients have a mixture of AD and CVD pathology (Neuropathology Group of Medical Research Council Cognitive Function and Aging Study (MRC CFAS), 2001; Toledo et al., 2013). Hemorrhage, infarctions, and vascular changes are not specific indicators for VaD.

Cerebral amyloid angiopathy often induces lobar hemorrhage and cortical microhemorrhage, which mainly affects the occipital lobe (Charidimou et al., 2012). In addition, imaging technology advances, including 7 T MRI, have identified numerous cortical microinfarcts (CMI), which have been attributed to CAA (Suter et al., 2002; van Veluw et al., 2013; Westover et al., 2013). Cognitive impairment in AD patients may result from hypoperfusion/ischemia and CMIs, as well as synaptic disturbance and neuronal loss caused by Aβ and tau accumulation (Okamoto et al., 2009; Launer et al., 2011; Smith et al., 2012). Small vessel injury is frequent in both AD and VaD. CAA was previously thought to be pathologically different from Binswanger disease, one of the common forms of VaD characterized by arteriolosclerosis and white matter change. However, Binswanger disease and CAA are now often regarded as part of the same spectrum disease; the former labeled type 1 and the latter type 2 small vessel disease (Pantoni, 2010). Both types of arteriopathies

make dementia patients vulnerable to hemodynamic fluctuation through impairments in cerebral autoregulation and vascular reactivity (Tanoi et al., 2000; Pimentel-Coelho and Rivest, 2012). Consequently, hypoperfusion induces Aβ overproduction and elimination failure (Zlokovic, 2011; Carare et al., 2013; Elali et al., 2013). Brain ischemia and hypoxia modulates amyloid precursor protein (APP) cleavage enzymes such as β-secretase and γ-secretase, thereby resulting in increased Aβ production (Sun et al., 2006; Guglielmotto et al., 2009; Kitaguchi et al., 2009; Li et al., 2009). Excess Aβ contributes to the impairment of Aβ clearance and CAA (Joachim et al., 1989; Rovelet-Lecrux et al., 2006; Han et al., 2008). Aβ elimination failure could also result from arteriolosclerosis (Weller et al., 2009). Thus, dementia patients with a single simple etiology are scarcely seen, except for juvenile familial AD cases caused by mutations in the APP or presenilin genes, comprising <1% of AD cases (Campion et al., 1999).

In order to explore novel therapies in AD, we must consider the "AD malignant cycle" (**Figure 1**). In this scheme, cessation of Aβ overproduction is not sufficient to treat patients with sporadic AD, and important components of the cycle, brain ischemia, and CAA should also be noted. Insufficient Aβ clearance seems to be more crucial than Aβ overproduction in sporadic AD patients (Mawuenyega et al., 2010). Even in familial AD cases, the onset of dementia is often delayed until the fifth or sixth decade, suggesting that the aging-associated failure in clearance also plays a part in the pathogenesis of inherited types of the disease (Weller et al., 2008). Therefore, recent work has focused on the failure of Aβ elimination as the most important therapeutic targets and adopted a "neurovascular" approach as a strategy to tackle AD (Vardy et al., 2005; Deane et al., 2008; Carare et al., 2013).

This review mainly focuses on the mechanisms of Aβ elimination and the drug development to facilitate Aβ clearance. The perivascular lymphatic drainage system, one of the Aβ clearance mechanisms, is closely associated with AD and CAA (Carare et al., 2013). In addition, the possibility of drugs enhancing perivascular drainage as well as future strategies for AD and CAA treatment will be reviewed.

Aβ CLEARANCE: 3-d MECHANISM

So far, several mechanisms of eliminating Aβ proteins have been identified, which fall into three main categories (3-"d," **Figure 2**):

- Enzymatic/glial degradation
- Transcytotic delivery
- Perivascular drainage

ENZYMATIC/GLIAL DEGRADATION

Aβ catabolism is regulated by a series of degrading enzymes as well as glial cells, such as astrocytes and microglia, in the brain parenchyma (Vardy et al., 2005). Among them, neprilysin has received much attention (Iwata et al., 2000). Previous reports have described impaired Aβ degradation in neprilysin-deficient mice (Iwata et al., 2001) and amelioration of Aβ pathology in APP-transgenic mice, when injected with viral vector expressing human neprilysin gene (Marr et al., 2003; Iwata et al., 2004, 2013). Levels of neprilysin mRNA were found to be significantly lower in the hippocampus and middle temporal gyrus of AD brains compared with normal control patients (Yasojima et al., 2001). Decreased neprilysin activity was also associated with CAA (Miners et al., 2006). Thus, the up-regulation of cerebral neprilysin

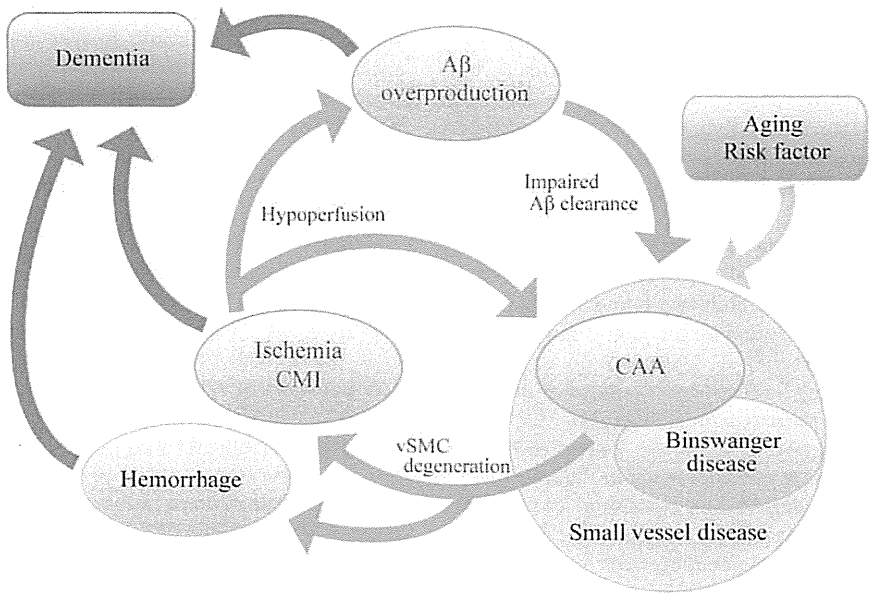

FIGURE 1 | "AD malignant cycle". Aβ overproduction impairs Aβ elimination leading to vascular smooth muscle cells (vSMC) degeneration, cerebral ischemia, and microinfarcts. Ischemia also induces Aβ overproduction. Such vicious circle consists of core pathology in sporadic AD. Note that cessation of Aβ overproduction is not sufficient to sever the cycle.

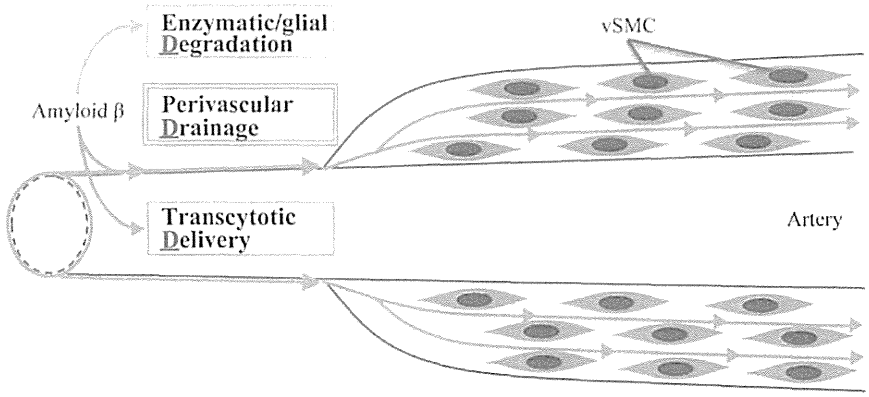

FIGURE 2 | Aβ clearance: 3-d mechanism. Aβ is mainly eliminated by the following mechanisms: (1) enzymatic/glial degradation, (2) transcytotic delivery, and (3) perivascular drainage.

activity could potentially be targeted in the treatment of AD. Indeed, a somatostatin receptor agonist has recently been shown to increase neprilysin activity and decrease Aβ levels in senescence-accelerated mice (Sandoval et al., 2012). However, Meilandt et al. reported that an 11-fold greater neprilysin overexpression failed to reduce pathogenic Aβ oligomers and improve deficits in spatial learning and memory in AD model mice (Meilandt et al., 2009). It was also reported that cerebral Aβ concentration was too low to be degraded by neprilysin (Shibata et al., 2000). The affinity of neprilysin for its physiological substrates (e.g., enkephalins, tachykinins, atrial natriuretic peptide) is within the millimolar range (Hersh and Morihara, 1986), while the levels of Aβ in the brain are normally in the nanomolar range and up to 1 μM/kg even in APP-transgenic mice (Hsiao et al., 1996). Thus, only small concentrations of Aβ will likely bind to neprilysin under physiological and pathological conditions. Many issues should be solved to proceed to drug development of neprilysin activators.

Angiotensin-converting enzyme (ACE) is another Aβ-degrading agent. Captopril, a blood–brain barrier (BBB) penetrating ACE inhibitor, increases Aβ accumulation (Zou et al., 2007), and ACE overexpression in myelomonocytes reduces Aβ deposition in AD model mice (Bernstein et al., 2014). However, brain ACE deficient mice showed no significant alteration in endogenous Aβ levels (Eckman et al., 2006). In addition, two small studies assessing the clinical use of ACE inhibitors, found that they did not deteriorate dementia in AD and amnestic mild cognitive impairment (MCI) patients (Ohrui et al., 2004; Rozzini et al., 2006). Because of such conflicting findings, contributions of ACE to Aβ degradation in the brain *per se* remain ambiguous.

Insulin-degrading enzyme (IDE) is also known to have Aβ-degrading properties, and hyperinsulinemia in diabetes mellitus competitively inhibits Aβ degradation (Craft and Watson, 2004; Qiu and Folstein, 2006). Indeed, IDE deficient mice demonstrate increased cerebral accumulation of endogenous Aβ with hyperinsulinemia and glucose intolerance (Farris et al., 2003), and IDE overexpression ameliorates Aβ pathology (Leissring et al., 2003), suggesting a link between insulin metabolism and Aβ degradation. However, clinical evidence is still lacking and further studies on the association of IDE with AD pathogenesis may uncover potential treatment targets in AD. Some researchers have labeled AD "type 3 diabetes" (de la Monte and Wands, 2008). If hyperinsulinemia is related to resistance of neuronal cells to insulin, impaired insulin signaling in neurons is thought to lead to neuronal disturbances. A clinical trial assessing intranasal insulin therapy in the treatment of AD and amnestic MCI is anticipated to further elaborate on the relationship between AD and insulin signaling (Craft et al., 2012).

TRANSCYTOTIC DELIVERY

The cerebral vasculature originates from large arteries, such as middle cerebral artery and the circle of Willis. These arteries branch into the leptomeningeal arteries, which travel on the surface of the brain in the subarachnoid space. Leptomeningeal arteries further branch into smaller arteries and arterioles consisting of three layers: tunica intima (endothelium), tunica media (smooth muscle cells), and tunica adventitia (mainly collagen fibers). Finally, the terminals of arterioles become capillaries. Capillary lumen and brain parenchyma are separated by the BBB, which prevents the passive exchange of solutes between blood and brain (Iadecola, 2004).

Lipoprotein receptor-related protein-1 (LRP-1), a multifunctional scavenger and signaling receptor, is expressed in neural cells and cerebral microvessels including capillaries, small venules, and arterioles (Wolf et al., 1992; Tooyama et al., 1995; Shibata et al., 2000). LRP-1 has received increasing attention as it mediates transport of Aβ out of the brain across the BBB (Bell and Zlokovic, 2009). Many reports have described the genetic linkage of LRP-1 with AD (Kang et al., 1997; Lambert et al., 1998; Wavrant-DeVrièze et al., 1999) and CAA (Christoforidis et al., 2005). Colocalization of LRP-1 with Aβ was pathologically recognized in senile plaques (Rebeck et al., 1993; Donahue et al., 2006), strengthening the linkage. The relationship is further supported by reduced LRP-1 staining in vessels both in AD patients (Shibata et al., 2000; Donahue et al., 2006) and CAA model mice carrying a vasculotropic Dutch/Iowa mutant form of APP gene (Deane et al., 2004).

Animal experiments have confirmed the importance of transcytosis in the regulation of cerebral Aβ levels. Five hours after microinjection of ^{125}I-labeled $A\beta_{1-40}$ into the caudate nucleus, 73.8% of labeled tracer had been found in blood across the BBB in young wild-type mice, while ^{125}I-labeled $A\beta_{1-40}$ in cerebrospinal fluid (CSF) measured 10.7%, and only 15.6% of the dose remained in the brain parenchyma (Shibata et al., 2000). These findings suggest that endothelial transcytosis by LRP-1 and others is probably one of the most prominent pathways in cerebral Aβ clearance, although this study might underestimate other clearance pathways as all the Aβ peptides found in blood are considered to derive from transcytotic delivery.

LRP-1 binds to Aβ directly (Deane et al., 2004), but also binds indirectly via its ligands including α2-macroglobulin, receptor-associated protein, and apolipoprotein E (ApoE) (Narita et al., 1997; Bu, 2009; Kanekiyo and Bu, 2009). ApoE is the main chaperone of Aβ in central nervous system (Holtzman et al., 2012; Zolezzi et al., 2014). To date, three isoforms of ApoE have been described (ε2, ε3, and ε4), and the ApoE ε4 variant is considered to be one of the most relevant risk factors for AD and CAA (Premkumar et al., 1996; Zolezzi et al., 2014). ApoE immunoreactivity is common in amyloid plaques, suggesting that ApoE interacts with Aβ directly in AD brains and could strongly influence the rate of Aβ removal (Namba et al., 1991; Holtzman et al., 2012). Several authors have proposed ApoE as therapeutic target for Aβ clearance (Cramer et al., 2012; Zolezzi and Inestrosa, 2014). Cramer et al. reported that bexarotene, a retinoid X receptor agonist, stimulated the ApoE-dependent Aβ clearance through the actions of liver X receptors and peroxisome proliferator-activated nuclear receptor gamma in AD model mice (Cramer et al., 2012). As a result, cognitive deficits improved with reduced burden of Aβ plaque. However, some conflicting reports have been also documented (Fitz et al., 2013; Price et al., 2013; Tesseur et al., 2013; Veeraraghavalu et al., 2013). Further analysis and experimentation should be performed.

Receptor for advanced glycation end products (RAGE), an immunoglobulin supergene family member, is also known to be a key molecule in Aβ transcytosis (Yan et al., 2012). Strong staining for RAGE has been reported in the vessels of AD patients (Yan et al., 1996; Donahue et al., 2006) and has been shown to mediate influx of circulating Aβ into brain across the BBB (Deane et al., 2003). In addition, RAGE contributes to Aβ-related synaptic dysfunction and microglial activation (Yan et al., 1996; Origlia et al., 2008, 2010). These findings suggest that RAGE could be a therapeutic target in AD and CAA. Indeed, a RAGE inhibitor ameliorated cerebral Aβ burden and normalized cognitive performance in APP-transgenic mice (Deane et al., 2012). The phase III 18 month clinical trial of the RAGE inhibitor TTP488 is being planned for mild to moderate AD patients (The U.S. National Institutes of Health, 2014); positive results in phase II testing have been reported (Burstein et al., 2014).

PERIVASCULAR DRAINAGE

The central nervous system is devoid of conventional lymphatic vessels, unlike other organs containing networks of lymphatic vessels, which process various substances, such as wastes, fluid, proteins, and cells from tissues to lymph nodes. However, the lymphatic perivascular drainage system in the brain performs the main function assigned to systemic lymphatic vessels. Analysis of the lymphatic perivascular drainage system dates back as far as the nineteenth century, where it was shown that Indian ink injected into cisterna magna drained to the cervical lymph nodes (Schwalbe, 1869; Weller et al., 2010).

The detail of perivascular drainage system has been examined mainly by intracranial injection of various tracers, including ^{125}I-labeled albumin (Bradbury et al., 1981; Szentistványi et al., 1984; Yamada et al., 1991), Indian ink (Zhang et al., 1992), and various fluorescent tracers (Carare et al., 2008). Recently, this drainage pathway was also confirmed by multi-photon imaging (Arbel-Ornath et al., 2013).

Fluorescent tracers, injected to the striatum, spread diffusely through the extracellular spaces of the brain parenchyma and enter the walls of blood vessels almost immediately. Confocal microscopy showed tracers colocalize with laminin in the basement membranes of capillary walls. Injected tracers were cleared from the basement membranes in the walls of capillaries and arteries, while some tracers were taken up by smooth muscle cells and perivascular macrophages (Zhang et al., 1992; Carare et al., 2008). Studies using radiolabeled tracers showed that drainage of interstitial fluid (ISF) and solutes continues along tunica media and the tunica adventitia of leptomeningeal and major cerebral arteries, through the base of the skull to the deep cervical lymph nodes (Szentistványi et al., 1984; Weller et al., 2010). Tissue soluble Aβ was detected by enzyme immunoassay in meningeal arteries and intracranial arteries but not in extracranial vessels (Shinkai et al., 1995). The clearance system leading to cervical lymph nodes was confirmed by subsequent injection into the inferior colliculus (Ball et al., 2010). Theoretical models have indicated that arterial pulsations could be the motive force behind ISF and solutes being driven centrifugally from the brain by reflection waves that follow each cardiac pulse wave (Schley et al., 2006).

This drainage route closely corresponds with the distribution of Aβ in the basement membranes of capillary and artery walls in CAA (Weller et al., 1998), which implies that the congestion of drainage pathway is associated with the pathogenesis of CAA. The fact that CAA was accelerated in the brains of immunized AD patients and that the CSF Aβ concentration was decreased both in AD and CAA patients may result from an impaired perivascular drainage pathway (Nicoll et al., 2004; Patton et al., 2006; Verbeek et al., 2009). Consistent with this, perivascular drainage of solutes is impaired in the aging mouse brain and in the presence of CAA (Hawkes et al., 2011). The fact that cerebral Aβ clearance was delayed after photothrombosis within individual vessels or middle cerebral artery occlusion (Garcia-Alloza et al., 2011), and after bilateral common carotid artery stenosis (Okamoto et al., 2012), further supports the notion that brain ischemia and impaired arterial pulsation could be an exacerbation factor of CAA. Consistent with the experimental data is the clinical finding that arterial stiffness, indicated by pulse wave velocity, has been associated with Aβ deposition in the brains of non-demented elderly adults (Hughes et al., 2013). Therefore, vasoactive drugs could have potential in the improvement of lymphatic congestion and facilitation of Aβ clearance in the brain.

CONVINCING EFFECTS OF PHOSPHODIESTERASE INHIBITOR

Among varieties of vasoactive drugs, cilostazol, a selective inhibitor of type 3 phosphodiesterase (PDE), is likely to be a promising agent for AD and CAA (**Figure 3**). PDE3 can hydrolyze both cAMP and cGMP, while increasing cAMP level is a major pharmacological effect of cilostazol (Ikeda, 1999). PDE3 is widely expressed in central nervous system and up-regulated in Aβ-positive vessels, especially in vascular smooth muscle cells (vSMC) (Maki et al., 2014), suggesting the possibility that PDE3 inhibition could be therapeutic for CAA. Cilostazol possesses multiple effects, such as increasing pulse rate (Shinohara et al., 2010) and arterial elasticity (Han et al., 2013), prolonging pulse duration time (Aruna and Naidu, 2007), and dilating cerebral vessels (Tanaka et al., 1989; Birk et al., 2004a,b); such vasoactive actions may promote efficiency of perivascular drainage. In support of this, clearance of fluorescent soluble Aβ tracers is significantly enhanced in cilostazol-treated CAA model mice, thereby resulting in maintenance of vascular integrity, amelioration of Aβ deposits (**Figure 4**), and prevention of cognitive decline (Maki et al., 2014). Memory-preserving activity of cilostazol has been demonstrated in aged wild-type mice (Yanai et al., 2014) and a rat model of chronic cerebral hypoperfusion (Watanabe et al., 2006), suggesting that cilostazol could be a potential disease modifying therapy of AD and other dementing disorders.

Recently, Nedergaad et al. suggested the "glymphatic pathway," consisting of para-arterial CSF influx route, para-venous ISF efflux route, and convective bulk fluid flux (Iliff and Nedergaard, 2013; Nedergaard, 2013), as another clearance system in central nervous system. Aβ proteins may be cleared through this perivascular pathway, as well as the perivascular drainage system (Iliff et al., 2012), although the relationship to CAA pathogenesis remains to be clarified as Aβ does not accumulate in the venous system. Cerebral arterial pulsation with a vasoactive agent dobutamine drives perivascular CSF–ISF exchange (Iliff et al.,

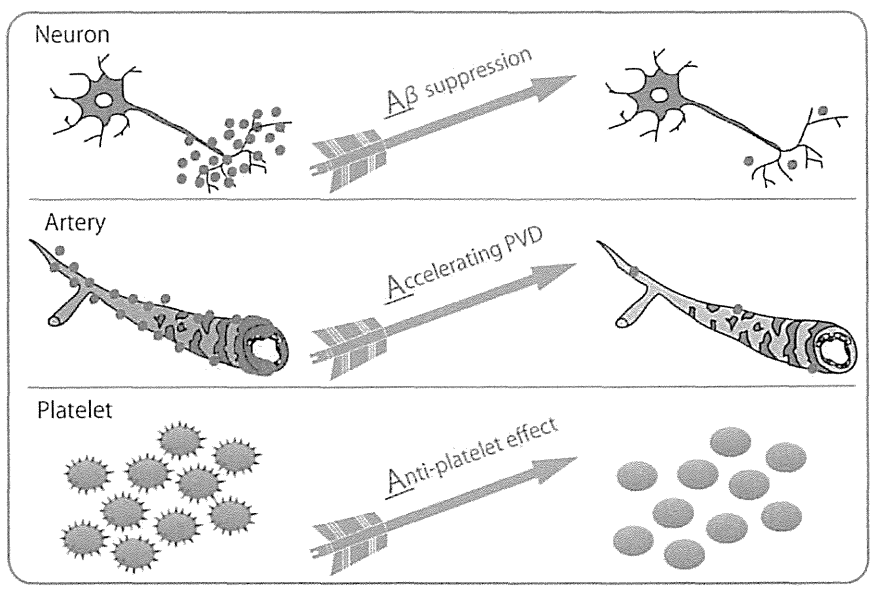

FIGURE 3 | Cilostazol with 3 Arrows: triple effects toward potential resolution of dementia. Cilostazol, a selective inhibitor of PDE3, has pleiotropic capabilities of suppressing Aβ production in neurons, enhancing Aβ clearance through perivascular drainage system, and inhibiting platelet aggregation (anti-platelet effects).

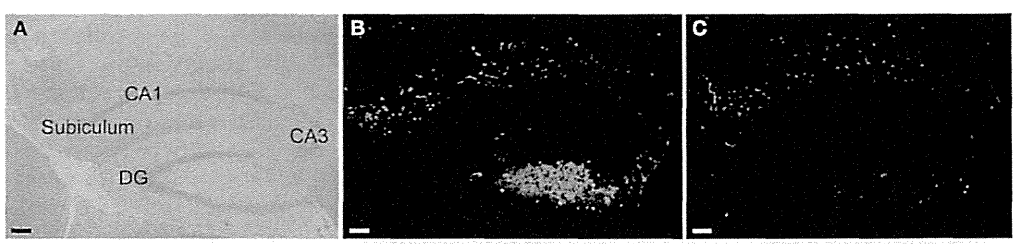

FIGURE 4 | Cilostazol reduced Aβ deposition. Hippocampal images obtained from 17-month-old homozygous Tg-SwDI mice, a model of CAA, treated with vehicle **(A,B)** or cilostazol **(C)** for 15 months show that cilostazol treatment reduced levels of Aβ deposits in the hippocampus compared with vehicle treatment. Scale bars indicate 100 μm. **(A)** HE staining. **(B,C)** Thioflavin-S staining.

2013). Further investigation is required to determine whether other vasoactive drugs such as cilostazol could have a potential to facilitate paravascular clearance.

Many inhibitors of other PDE subtypes have been reported to produce cognitive enhancement (Reneerkens et al., 2009) and have been associated with neuronal cAMP signaling activation. Rolipram, a PDE4 inhibitor, reverses the decrease in cAMP regulatory element-binding protein (CREB) phosphorylation, which results in persistent improvement in synaptic function in AD model mice (Gong et al., 2004). Sildenafil, a PDE5 inhibitor, decreases Aβ levels in extracts of cerebral cortex and improves associative and spatial memory in AD model mice (Puzzo et al., 2009). Caffeine is a non-specific PDE inhibitor (Yoshimura, 2005), and its beneficial effects have been clarified in many clinical AD studies (Eskelinen et al., 2009; Eskelinen and Kivipelto, 2010). Caffeine stimulates cAMP-dependent protein kinase A signaling and increases CREB phosphorylation in AD model mice (Arendash et al., 2006; Zeitlin et al., 2011). Protein kinase A activation then suppresses the expression of Aβ-synthesizing enzymes such as β- and γ-secretase, leading to reduced Aβ production (Arendash et al., 2009). Cilostazol also reduces Aβ production *in vitro* (Lee et al., 2012, 2014; Maki et al., 2014), and suppresses Aβ-induced tauopathy and tau phosphorylation *in vitro* (Lee et al., 2012, 2014). However, as only a minor fraction of cilostazol passes through BBB (Akiyama et al., 1985), it remains to be elucidated whether these positive effects of cilostazol do occur in AD patients.

Cilostazol has a wide variety of pleiotropic effects capable of inducing neurogenesis (Lee et al., 2009; Tanaka et al., 2010), promoting oligodendrocyte precursor cell differentiation (Miyamoto et al., 2013), preventing lipid peroxidation (Hiramatsu et al., 2010; Kurtoglu et al., 2014), enhancing cholesterol efflux from macrophages (Nakaya et al., 2010), ameliorating insulin resistance (Wada et al., 2013), reducing inflammatory burden (Otsuki et al., 2001; Tsai et al., 2008; Hattori et al., 2009), and improving systemic lymphatic function by inducing proliferation and stabilization of lymphatic endothelial cells (Kimura et al., 2014). In a clinical setting, cilostazol is currently used as an anti-platelet drug (Gotoh et al., 2000; Shinohara et al., 2010), and may be used to prevent ischemic events in patients with CAA. Major manifestations of CAA include lobar hemorrhage and cortical microhemorrhage, as well as CMI. As most CAA patients are elderly (Zhang-Nunes et al., 2006), this necessitates the use of anti-platelet drugs with little risk of hemorrhage (Charidimou et al., 2012). The second Cilostazol Stroke Prevention Study (CSPS2) for patients with cerebral infarction showed that the hemorrhagic stroke was significantly less frequent in cilostazol treatment than with aspirin (Shinohara et al., 2010; Uchiyama et al., 2014). The prevention of cerebral hemorrhage may be explained by reproducible experimental evidence showing that cilostazol inhibits expression of matrix metalloproteinase-9 and protects vascular endothelial cells (Ishiguro et al., 2010; Hase et al., 2012; Kasahara et al., 2012). Endothelial protection with cilostazol mediates increase in nitric oxide, which dilates blood vessels (Oyama et al., 2011), leading to increased cerebral blood flow (Mochizuki et al., 2001; Matsumoto et al., 2011; Sakurai et al., 2013). These results suggest that cilostazol could be suitable for patients with both AD and CVD, the most common type of dementia in the elderly.

Favorable effects have already been described in observational clinical studies, which demonstrated the efficacy of cilostazol in patients with MCI (Taguchi et al., 2013), donepezil-treated patients with clinically probable AD (Arai and Takahashi, 2009; Ihara et al., 2014), and AD with CVD (Sakurai et al., 2013). Randomized placebo-controlled clinical trials are being planned for patients with MCI.

FUTURE STRATEGY FOR AD AND CAA TREATMENT

Aging inevitably increases the amount of Aβ burden in the brain, implying a strong relationship between impaired Aβ metabolism and age (Funato et al., 1998). Since heterogeneity and multimorbidity are common in the elderly (Barnett et al., 2012), dementia likely originates from a combination of different pathological substrates. As the population ages, the distribution of AD shifts to older ages in developed countries (Hebert et al., 2013), resulting in an increasing number of demented patients with numerous complicated etiologies. Given that the balance between Aβ synthesis and clearance determines brain Aβ accumulation, and that Aβ is cleared by several pathways stated above, multi-drugs combination therapy would likely be necessary for sporadic AD with complicated etiologies. Combination therapy has already been applied to various diseases, such as hypertension, diabetes mellitus, and malignant tumors. The ultimate goal will be to develop a sovereign remedy of AD, and we hope that the recent rapid advances in drug development will enable us to delay the onset or modify the progression of cognitive impairment with multi-targeting therapies. Further investigation from various viewpoints will thus be essential for the development of novel treatment for AD and CAA.

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

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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 littermates. 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 littermates. Brain endothelial nitric oxide synthase was acetylated after cerebral hypoperfusion in wild-type littermates 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;45:3403-3411.)**

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]

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SIRT1 activator–dependent neuroprotective activity has also been demonstrated in noncell autonomous neurological paradigms such as a global cerebral ischemia model in the rat[15] and a permanent middle cerebral artery occlusion model in the mouse.[16] Because vascular lesions are the major problems that often accompany and aggravate neurodegenerative diseases,[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.[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.[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 gene 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. Microcoils with an internal diameter of 0.18 mm were applied to the bilateral common carotid arteries (Figure I 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. An expanded Methods section is available in the online-only Data Supplements.

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. Immunoblot for the full-length, active form of SIRT1 (110 kDa)[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 transcellular translocation of amyloid-β40 and other processes).[23–25]

Sirt1-Tg Mice Retain Cognitive Integrity After Chronic Cerebral Hypoperfusion

Consistent with our previous observations,[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,[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

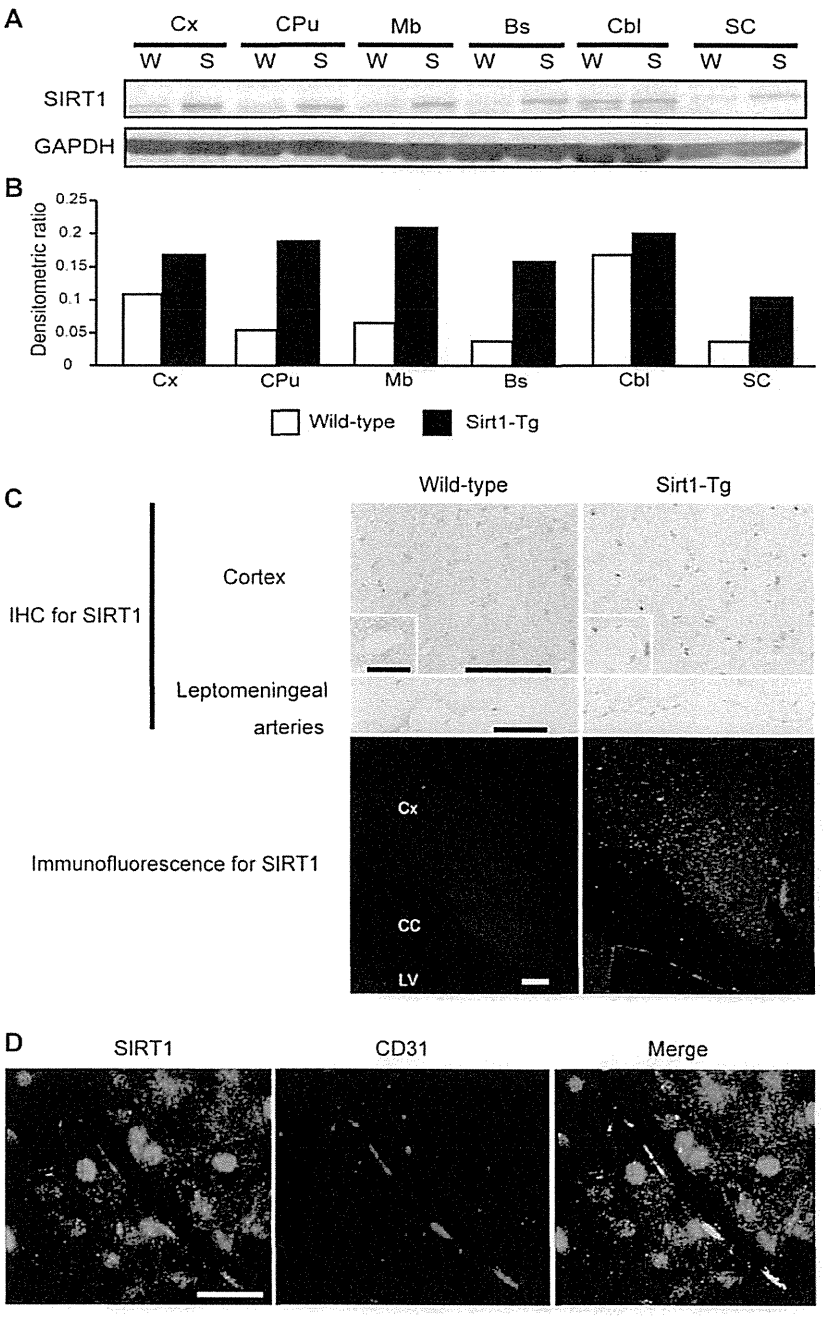

Figure 1. Silent information regulator 2 homolog 1 (SIRT1) expressions in neurons and endothelial cells in the brain. **A**, Representative immunoblots of SIRT1 and glyceraldehyde-3-phosphate dehydrogenase (GADPH) in the cerebral cortex, the caudoputamen, the midbrain, the brain stem, the cerebellum, and the spinal cord of wild-type and *Sirt1*-overexpressing (Sirt1-Tg) mouse. **B**, Histogram showing the ratio of full-length SIRT1 to GAPDH (n=2, each). **C**, Immunohistochemistry (IHC) for SIRT1 at the cerebral cortex with insets indicating enlarged images of the arteriole and leptomeningeal arteries of the wild-type and Sirt1-Tg mouse, and immunofluorescence for SIRT1 at the cortex and the corpus callosum of the wild-type and Sirt1-Tg mouse. Scale bars indicate 100 μm and 30 μm (insets). **D**, Immunofluorescence for SIRT1 (green) and CD31 (red) and merged image in the cerebral cortex of Sirt1-Tg mouse. Scale bar indicates 20 μm. CC indicates corpus callosum; Cbl, cerebellum; CPu, caudoputamen; Cx, cortex; Bs, brain stem; LV, lateral ventricle; Mb, midbrain; S, Sirt1-Tg; SC, spinal cord; and W, wild-type.

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).

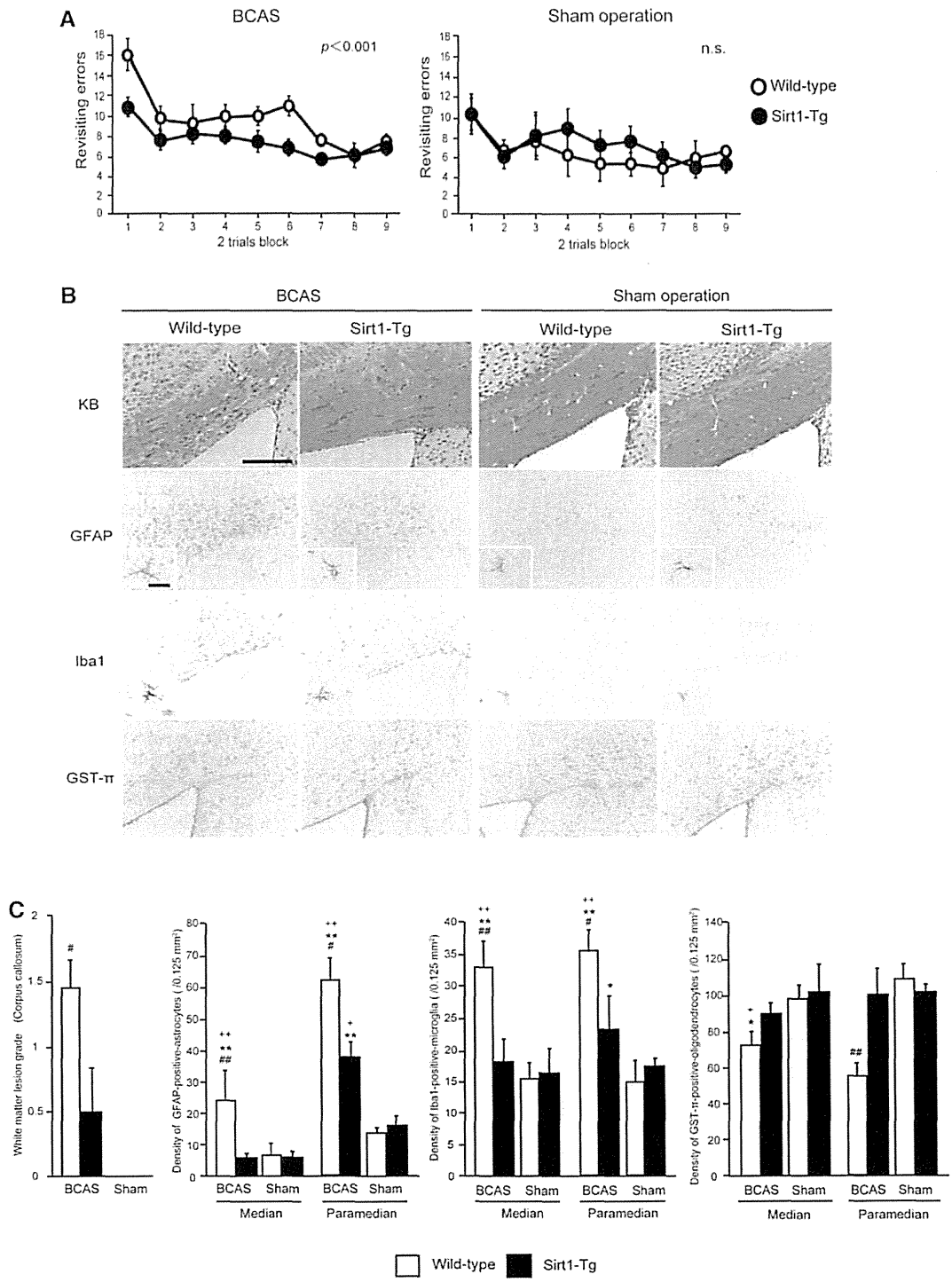

Figure 2. Silent information regulator 2 homolog 1 (SIRT1) rescued memory impairment and histological changes after bilateral common carotid artery stenosis (BCAS). **A**, Number of revisiting errors in the 8-arm radial arm maze test was significantly fewer in *Sirt1*-overexpressing (Sirt1-Tg) mice (n=18) 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.

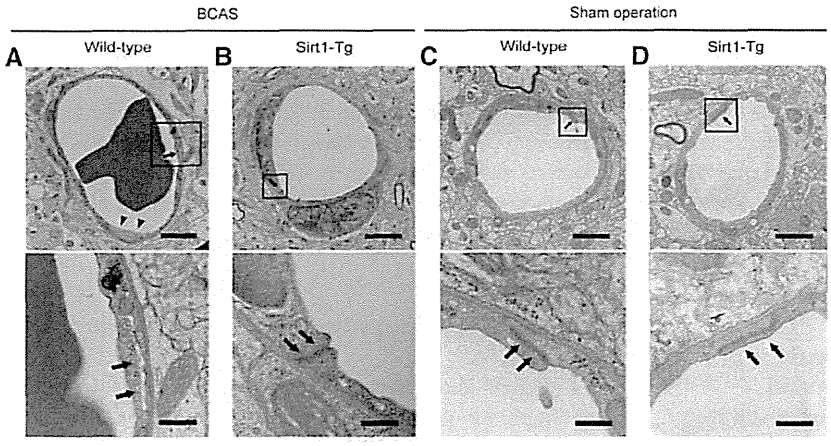

Figure 3. Silent information regulator 2 homolog 1 (SIRT1) rescued endothelial injuries after bilateral common carotid artery stenosis (BCAS). Transmission electron microscopic images of BCAS-operated, wild-type (**A**) or *Sirt1*-overexpressing (Sirt1-Tg; **B**) mice and sham-operated, wild-type (**C**) or Sirt1-Tg (**D**) mice at 2 hours after each operation. **A**, A capillary of wild-type littermate shows disrupted microvilli with loss of continuous surface of the endothelium (arrowheads, **top**) and open tight junction (arrow, **top**). An enlarged image of marked region in the **top** shows tight junction opening (arrows, **bottom**). A normal-appearing capillary (**B–D**, in **top**) showing intact tight junction (**B–D**, arrows in **bottom**). Scale bars indicate 1 μm (**A**, **C**, **D**; **top**), 2 μm (**B**; **top**), and 500 nm (**A–D**; **bottom**).

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 4A). 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

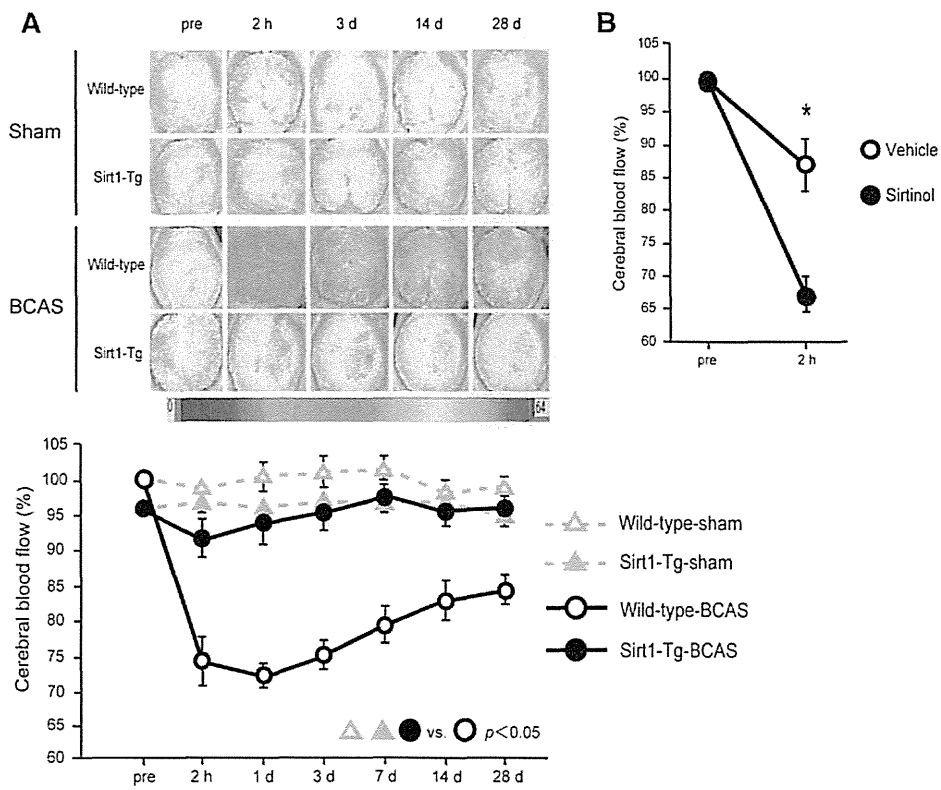

Figure 4. Silent information regulator 2 homolog 1 (SIRT1) had cerebral blood flow (CBF)-preserving effects after bilateral common carotid artery stenosis (BCAS). **A**, Representative CBF images and temporal profiles of CBF of the BCAS-operated, wild-type (n=7) or *Sirt1*-overexpressing (Sirt1-Tg; n=5) mice and sham-operated, wild-type (n=3) or Sirt1-Tg (n=3) mice as assessed by laser speckle flowmetry before and after each surgery. Data were analyzed by 2-way repeated measures ANOVA. **B**, Temporal profiles of CBF of vehicle-treated Sirt1-Tg mice (n=5) and sirtinol-treated Sirt1-Tg mice (n=5) before and at 2 hours after BCAS. **P*<0.01 vs vehicle.