Angiogenic and Vasoprotective Effects of Adrenomedullin on Prevention of Cognitive Decline After Chronic Cerebral Hypoperfusion in Mice

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Background and Purpose—Although subcortical vascular dementia, the major subtype of vascular dementia, is caused by a disruption in white matter integrity after cerebrovascular insufficiency, no therapy has been discovered that will restore cerebral perfusion or functional cerebral vessels. Because adrenomedullin (AM) has been shown to be angiogenic and vasoprotective, the purpose of the study was to investigate whether AM may be used as a putative treatment for subcortical vascular dementia.

Methods—A model of subcortical vascular dementia was reproduced in mice by placing microcoils bilaterally on the common carotid arteries. Using mice overexpressing circulating AM, we assessed the effect of AM on cerebral perfusion, cerebral angiogenesis, oxidative stress, white matter change, cognitive function, and brain levels of cAMP, vascular endothelial growth factor, and basic fibroblast growth factor.

Results—After bilateral common carotid artery stenosis, mice overexpressing circulating AM showed significantly faster cerebral perfusion recovery due to substantial growth of the capillaries, the circle of Willis, and the leptomeningeal anastomoses and reduced oxidative damage in vascular endothelial cells compared with wild-type mice. Vascular changes were preceded by upregulation of cAMP, vascular endothelial growth factor, and basic fibroblast growth factor. White matter damage and working memory deficits induced by bilateral common carotid artery stenosis were subsequently restored in mice overexpressing circulating AM.

Conclusions—These data indicate that AM promotes arteriogenesis and angiogenesis, inhibits oxidative stress, preserves white matter integrity, and prevents cognitive decline after chronic cerebral hypoperfusion. Thus, AM may serve as a strategy to tackle subcortical vascular dementia. (Stroke. 2011;42:1125-1131.)

Key Words: angiogenesis arteriogenesis adrenomedullin vascular dementia

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spheric white matter (WM) lesions, which are most likely caused by cerebrovascular insufficiency after atherosclerosis and/or arteriosclerosis, are an established marker of risk for cognitive deterioration. Thus, therapeutic vascular growth and vasoprotection, resulting in the preservation of WM integrity, may serve to maintain cognitive function in subjects at risk of developing dementia.

Adrenomedullin (AM) has a variety of effects on the vasculature that include vasodilation, regulation of permeability, inhibition of endothelial cell apoptosis and oxidative stress, regulation of smooth muscle cell proliferation, and promotion of angiogenesis.2,3 Thus, the purpose of this study was to investigate the mechanisms and therapeutic potential of AM-induced neovascularization and/or vasoprotection after chronic cerebral hypoperfusion in a mouse model of subcortical vascular dementia.1,5

Materials and Methods
An expanded Methods section is available in the Online Data Supplement (http://stroke.ahajournals.org).

Results
Adrenomedullin Facilitates Recovery of Cerebral Blood Flow After Placing Microcoils Bilaterally on the Common Carotid Arteries
Immediately after bilateral common carotid artery stenosis (BCAS), cerebral blood flow (CBF) decreased to the lowest...
values but thereafter began to recover in all groups. On Days 1 and 3 post-BCAS, there was a slight, but not significant, increase in CBF (average ±SEM) in mice overexpressing circulating AM (AM-Tg) compared with wild-type (WT) mice. On Day 7 post-BCAS, AM-Tg mice showed significantly faster CBF recovery: CBF was significantly higher in AM-Tg mice (93% ±2%) compared with WT mice (79% ±2%) and hydralazine-treated WT (H-WT; n=8 on Days 0 to 14, n=4 on Day 28) mice, in which the pre-BCAS value of CBF is adjusted to 100. Values are expressed as means ±SEM. *P<0.05, **P<0.01 in AM-Tg vs WT; #P<0.05, ##P<0.01 in WT vs H-WT. D, Temporal profile of CBF after BCAS in AM-treated WT mice (n=11) and vehicle-treated WT mice (n=10). Values are expressed as means ±SEM. *P<0.05, **P<0.01. CBF indicates cerebral blood flow; AM-Tg, mice overexpressing circulating AM.

Figure 1. Adrenomedullin (AM) facilitates recovery of cerebral blood flow after the bilateral common carotid artery stenosis (BCAS). A–B, Representative images of CBF in wild-type or AM-Tg mouse subjected to BCAS operation (WT BCAS or AM BCAS) on Day 7. C, Temporal profile of CBF after BCAS in wild-type (WT; n=16 on Days 0 to 14, n=8 on Day 28), AM-Tg (n=16 on Days 0 to 14, n=8 on Day 28), and hydralazine-treated WT (H-WT; n=8 on Days 0 to 14, n=4 on Day 28) mice, in which the pre-BCAS value of CBF is adjusted to 100. Values are expressed as means ±SEM. *P<0.05, **P<0.01 in AM-Tg vs WT; #P<0.05, ##P<0.01 in WT vs H-WT. D, Temporal profile of CBF after BCAS in AM-treated WT mice (n=11) and vehicle-treated WT mice (n=10). Values are expressed as means ±SEM. *P<0.05, **P<0.01. CBF indicates cerebral blood flow; AM-Tg, mice overexpressing circulating AM.

Adrenomedullin Enhances Arteriogenesis After BCAS
At the dorsal surface of the brain, a significant increase in diameter of the leptomeningeal anastomoses was found in AM-Tg mice (28.7±1.6 μm) compared with WT mice (22.4±1.3 μm) on Day 7 post-BCAS (Figure 2A–C; Supplemental Figure IB, a–d). The number of leptomeningeal anastomoses was not different among the 4 groups. The diameter of the internal carotid artery, anterior cerebral artery, middle cerebral artery, and posterior communicating artery was significantly enlarged at the level of the circle of Willis in AM-Tg mice compared with WT mice (AM-Tg versus WT; anterior cerebral artery, 193±26 versus 161±24 μm; middle cerebral artery, 184±24 versus 153±13 μm; internal carotid artery, 206±30 versus 175±25 μm; posterior communicating artery, 191±16 versus 150±15 μm) on Day 7 post-BCAS (Figure 2D; Supplemental Figure IB, e–h).

To evaluate monocyte recruitments and proliferation of smooth muscle cells, both of which are essential in arteriogenesis, the immunofluorescent analysis of Ki-67 and F4/80, together with α-smooth muscle actin, was performed. BCAS-operated AM-Tg mice showed a significant increase in Ki-67-positive vascular smooth muscle cells compared with BCAS-operated WT mice (Supplemental Figure IC). In addition, a significant increase in vascular smooth muscle cells surrounded by F4/80-positive monocyte/macrophages was found in BCAS-operated AM-Tg mice compared with sham-operated WT mice (Supplemental Figure ID).

Adrenomedullin Enhances Angiogenesis After BCAS
A significant increase in platelet-endothelial cell adhesion molecule-1-positive capillary density of the cortex, corpus cal-
Lossum, and caudoputamen was found in AM-Tg mice compared with WT mice (AM-Tg versus WT; cortex, 540±55/mm² versus 473±38/mm²; corpus callosum, 273±7/mm² versus 213±18/mm²; caudoputamen, 499±36/mm² versus 455±26/mm²) on Day 7 post-BCAS. There was no significant difference in capillary density between AM-Tg and WT mice after sham operation (Figure 3A–C; Supplemental Figure IE).

Taken together, these results suggest that both chronic ischemic stress and AM overexpression are required to induce arteriogenesis and angiogenesis in the brain.

### Adrenomedullin Attenuates Oxidative Damage in Cerebral Microvessels After BCAS

To evaluate oxidative damage in cerebral microvessels, double immunofluorescence staining for platelet-endothelial cell adhesion molecule-1 and 8-hydroxy-deoxyguanosine was performed on Day 3 post-BCAS. A significant decrease in oxidative damage in the cerebral microvessels was found in BCAS-operated AM-Tg mice compared with BCAS-operated WT mice (Figure 3D; Supplemental Figure IF).

### Adrenomedullin Preserves WM Integrity After BCAS

BCAS-operated WT mice showed an increased density of glial fibrillary acidic protein-positive astrocytes and ionized calcium binding adapter molecule 1-positive microglia and a decreased density of glutathione-S-transferase-pi-immunoreactive mature oligodendrocytes in the corpus callosum and the anterior commissure compared with sham-operated WT or AM-Tg
mice on Day 28 (Figure 4A–C; Supplemental Figure IIA, a–c, e–g, and i–k). In BCAS-operated AM-Tg mice, by contrast, the density of astrocytes and microglia significantly decreased and that of mature oligodendrocytes significantly increased compared with BCAS-operated WT mice (Figure 4A–C; Supplemental Figure IIA, d, h, and l). Similarly, BCAS-operated AM-Tg mice on Day 7 showed significantly decreased density of microglia and increased density of mature oligodendrocytes but no difference in the density of astrocytes compared with BCAS-operated WT mice (Supplemental Figure IIB–D).

Klüver–Barrera staining on Day 28 revealed that BCAS-induced WM lesions were predominant in the corpus callosum and the caudoputamen in WT mice (Supplemental Figure IIA, m–o). In AM-Tg mice, such WM lesions became far less severe (Supplemental Figure IIAp). In the hydralazine-treated WT mice, BCAS-induced WM lesions were as severe as those in WT mice, suggesting that such positive effects of AM may be independent of the hypotensive effect of AM (Figure 4D).

Thus, BCAS-induced WM lesions were restored in the AM-Tg mice in parallel with inhibition of glial activation and preservation of mature oligodendrocytes.

### Adrenomedullin Prevents Working Memory Deficits After BCAS

To evaluate working memory, we examined a Y maze test and an 8-arm radial maze test. The Y maze test was performed 1 month after the surgery. Alternations of entries in the arms of the Y maze were significantly increased in BCAS-operated AM-Tg mice (64.6%±7.5%) compared with BCAS-operated WT mice (58.2%±8.0%), although alternations of entries were not significantly different between WT and AM-Tg mice after sham operation (Figure 5A). Spontaneous activity was not significantly different among the 4 groups of mice (Figure 5B). In another set of mice, the 8-arm

![Figure 4. Adrenomedullin (AM) restores white matter integrity after BCAS. Histograms showing the density of cells immunoreactive for GFAP (A), Iba-1 (B), or GST-pi (C) in the medial and paramedial portions of the corpus callosum (CC) and the anterior commissure (AC) of a wild-type (WT) or AM-Tg mouse subjected to sham or BCAS operation on Day 28 (WT sham, n=4; AM sham, n=4; WT BCAS, n=8; AM BCAS, n=8). Error bars indicate SD. **P<0.01 vs WT BCAS; #P<0.05 vs WT sham. D, Histograms showing grading score of the CC of WT BCAS (n=14), AM BCAS (n=8), and hydralazine-treated WT BCAS (H-WT BCAS; n=7) on Day 28 post-BCAS. Error bars indicate SD. **P<0.01 vs WT BCAS and H-WT BCAS. BCAS indicates bilateral common carotid artery stenosis; GFAP, glial fibrillary acidic protein; Iba-1, ionized calcium binding adapter molecule 1; GST-pi, glutathione-S-transferase-pi; AM-Tg, mice overexpressing circulating AM.

![Figure 5. Adrenomedullin (AM) prevents working memory deficits after BCAS. A, Histogram showing spontaneous alternation in the Y maze test of wild-type (WT) or AM-Tg mice at 1 month after sham or BCAS operation (WT sham, n=17; AM sham, n=10; WT BCAS, n=26; AM BCAS, n=17). Error bars indicate SD. **P<0.01 vs WT BCAS; #P<0.01 vs WT sham. B, Histogram showing spontaneous activity in the Y maze test. Error bars indicate SD. C, The number of revisiting errors in the 8-arm radial maze test at 1 month after BCAS of WT or AM-Tg mouse (WT BCAS, n=19; AM BCAS, n=17). Values are expressed as means±SEM. BCAS indicates bilateral common carotid artery stenosis; AM-Tg, mice overexpressing circulating AM; AM, adrenomedullin.](http://stroke.ahajournals.org/)

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radial maze test was started 1 month after BCAS. BCAS-operated AM-Tg mice showed a significant reduction in the number of revisiting errors compared with BCAS-operated WT mice (Figure 5C). We have found a significant correlation of the averaged number of revisiting errors 1 month after BCAS with CBF on Days 7, 14, and 28, but not with CBF immediately after BCAS, or on Days 1 and 3 (Supplemental Figure III).

Taken together, these results suggest that AM restores working memory deficits induced by BCAS.

Adrenomedullin Increases cAMP Level in the Forebrain After BCAS

The restorative effect of AM described led to the investigation of the underlying mechanisms behind angio-/arteriogenesis and antioxidative activity. The brain level of cAMP, a second messenger known to associate with AM, was therefore measured. A significant increase in the brain level of cAMP was found in AM-Tg mice after BCAS (AM BCAS, $1.6\pm0.2$ pmol/mg wet tissue) compared with WT mice after BCAS (WT BCAS, $1.3\pm0.2$ pmol/mg wet tissue) on Day 5, although the level of cAMP was not different between WT and AM-Tg mice after sham operation (Figure 6A).

These results suggest that chronic ischemic stress induced AM-mediated elevation of cAMP in the brain.

Adrenomedullin Increases mRNA and Protein Levels of Vascular Endothelial Growth Factor and Basic Fibroblast Growth Factor in the Forebrain After BCAS

The reasons behind the apparent AM-initiated signaling pathway-led arteriogenesis and angiogenesis were next examined. Brain levels of vascular growth factors, including vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), were therefore measured. The mRNA and protein levels of brain VEGF and bFGF were significantly increased in AM-Tg mice on Days 1 and 5 post-BCAS compared with sham-operated WT mice (Figure 6B–C; Supplemental Figure IV).

Chronic Ischemic Insult Upregulates Brain mRNA Level of Adrenomedullin and Abolishes Receptor Activity-Modifying Protein-2 Suppression Induced by Adrenomedullin

The status of mouse AM, high-affinity AM receptors, calcitonin receptor-like receptors, and Subtypes 2 and 3 of a family of receptor activity-modifying proteins (RAMP2 or 3) were then measured on Day 5. The brain mRNA level of mouse AM was significantly increased (3.1-fold) in BCAS-operated WT mice compared with sham-operated WT mice. In addition, brain RAMP2 mRNA level was significantly lower (0.6-fold) in sham-operated AM-Tg mice compared with sham-operated WT mice. Such downregulation of RAMP2 mRNA levels was significantly lower (0.6-fold) in sham-operated AM-Tg mice compared with sham-operated WT mice.
was abolished after the AM mice were subjected to BCAS operation, suggesting that feedback inhibition is a plausible cause for the downregulation (Figure 6D).

**Discussion**

Three major conclusions may be drawn from the present study. First, it was demonstrated that increased levels of circulating AM restored cerebral hemodynamics, promoted angiogenesis as well as angiogenesis, alleviated oxidative damage in the cerebral microvessels, and preserved WM integrity; this subsequently attenuated working memory deficits in a mouse model of chronic cerebral hypoperfusion. Second, AM selectively upregulated brain levels of cAMP, VEGF, and bFGF in the hypoperfused brain but not in the normoperfused brain. Finally, it was found that such proangiogenic/arteriogenic changes did not occur in sham-operated AM-Tg mice in which the expression of AM receptor component RAMP2 was significantly suppressed, possibly through feedback inhibition.

We have found a significant correlation in the averaged number of revisiting errors at 1 month after BCAS with CBF on Days 7, 14, and 28, but not with CBF immediately after BCAS or on Days 1 and 3. Therefore, the recovery of CBF is one of the substrates for the functional improvements, whereas several phenomena other than CBF recovery may play roles in the pathophysiology of this BCAS model. In fact, we demonstrated that AM induced not only CBF recovery as a result of arteriogenesis, but also angiogenesis (not associated with CBF recovery), antioxidative activity in the microvessels, and attenuation of microglial inflammatory responses. The other effects of AM, including antiapoptotic effects and regulation of endothelial permeability or the blood–brain barrier, need further investigation. Positive effects of AM may be mediated by multiple pathways.

Previous reports showed that the AM/cAMP/PKA cascade blocks oxidative damage in ischemic injury and promotes angiogenic effects of the endothelial cells in vitro. We found that chronic ischemic insult and circulating AM are both required to raise cAMP levels in the brain; this may be associated with alleviating oxidative damage and promoting angiogenesis.

The elevation of VEGF is consistent with the previous report that AM administration upregulates the expression of VEGF in both in vitro and in vivo hindlimb ischemia models. Although no previous studies have reported that AM enhances the expression of bFGF after ischemia, we demonstrated the AM-induced upregulation of bFGF after BCAS in vivo. AM was also found to upregulate bFGF as well as VEGF in the cultured endothelial cells (unpublished data). Previous reports have demonstrated that combined gene delivery of VEGF and bFGF produces additive or synergistic effects on angiogenesis or collateral development, probably due to the protective effects of bFGF against VEGF-induced fluid leakage. Thus, AM-induced elevation of bFGF may be associated with the development of functional vessels.

AM acts through 2 subtypes of receptor (AM1 and AM2), which derive from the interaction of the calcitonin receptor-like receptors with RAMP2 or 3. Interestingly, RAMP2 mRNA level in sham-operated AM-Tg mice was significantly decreased compared with sham-operated WT mice but nearly reached normalization after BCAS. This may explain why arteriogenesis and angiogenesis were significantly promoted in AM-Tg mice only after ischemic insult. Shindo et al have reported that RAMP2 rather than RAMP3 is a key determinant of the effects of AM on the vasculature and is essential for angiogenesis and vascular integrity. These results suggest that the AM-initiated signaling pathway is suppressed by downregulation of RAMP2 in the normoperfused brain but that such suppression is abolished by chronic ischemic stress, leading to AM-induced arteriogenesis and angiogenesis. Such tissue selectivity could be an advantage for clinical application of AM in patients with subcortical vascular dementia.

Recently, the concept of an "oligovascular niche" has been proposed, in which crosstalk between endothelial cells and oligodendrocytes, mediated by an exchange of soluble signals such as fibroblast growth factor, are thought to play an important role in sustaining oligodendrocyte homeostasis and WM integrity. Because cerebral endothelial cells contribute to numerous signaling cascades that help regulate brain homeostasis and function, angio-/arteriogenesis and inhibition of oxidative damage in the cerebral endothelial cells induced by AM might lead to oligovascular protection—namely, successful vascular growth and vasoprotection and preservation of white matter/oligodendrocyte integrity—and prevention of cognitive decline after chronic cerebral hypoperfusion in mice.

In conclusion, this study demonstrates that circulating AM is a highly potent and effective modality for restoring perfusion, promoting arteriogenesis and angiogenesis in the chronically ischemic brain, inhibiting oxidative damage in the cerebral microvessels, preserving ischemic WM integrity, and attenuating working memory deficits in a mouse model of subcortical vascular dementia. Future clinical studies are required to evaluate and confirm the efficacy of AM in chronic cerebral vascular diseases, especially subcortical vascular dementia.

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**Disclosures**

None.
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Supplemental Methods

Mice

A generation of transgenic mice overexpressing human adrenomedullin (AM-Tg mice) has been described in a previous study.1 Because the product is secreted from the liver, it mimics intravenous administration of the agent. All procedures were performed according to the guidelines of the Animal Use and Care Committee of Kyoto University. The adrenomedullin (AM) gene contains coding regions for not only AM but also proadrenomedullin N-terminal 20 peptide (PAMP), a different vasodilating peptide. Amidation at their carboxyl terminals after their synthesis is needed for both AM and PAMP to exert their biological activity. The bioactive amidated forms are known as mature AM and mature PAMP, respectively. To identify the specific effects of AM, we generated a transgene construct with a point mutation on the PAMP amidation signal in the full-length AM gene cDNA. Guanine was substituted for cytosine on the 3’ end of the PAMP coding region so that glycine on the C terminal of the PAMP product was replaced with alanine. In this way, amidation and maturation of PAMP by peptidylglycine α-hydroxylase and α-hydroxyglycine N-C lyase were inhibited. The mutant AM gene cDNA was then inserted into a plasmid containing the human serum amyloid P component promoter, which is widely used to target gene expression specific to the liver. When the product is secreted from the liver, it mimics intravenous administration of the agent. The HindIII-XhoI fragment of the plasmid was microinjected into the pronucleus of fertilized C57BL/6J mice eggs. Comparisons were then performed between AM-Tg mice and wild-type (WT) littermates. Plasma concentrations of human total AM were measured with a commercially available immunoradiometric assay (Shionogi). Blood pressure (BP) was measured by the tail cuff method (Softron). The mice were housed in a room with a 12-hour light/dark cycle (lights on at 7:00 a.m.) with access to food and water ad libitum.
**Induction of chronic cerebral hypoperfusion**

Adult C57BL/6J male mice (10–12 weeks old, 22–29 g; Shizuoka Laboratory Animal Center) were subjected to bilateral common carotid artery stenosis (BCAS) using microcoils with an internal diameter of 0.18 mm, as previously described.\(^2\)–\(^4\) Sham-operated mice underwent the same surgical procedure without using microcoils. Anesthesia was induced with 4% halothane and maintained with 1.5% halothane in 80% nitrous oxide and 20% oxygen. Rectal temperature was maintained between 36.5°C and 37.5°C.

**Exogenous administration of hydralazine**

To evaluate the possibility that the lower BP observed in AM-Tg mice caused beneficial effects on BCAS, we further analyzed BP-matched mice by administration of low-dose hydralazine (0.1 mmol/L) in drinking water.\(^1\)

**Exogenous administration of AM**

Recombinant human mature AM dissolved in 0.9% saline was exogenously administrated to C57BL/6J WT mice by means of osmotic pumps (Alzet Model 1002) as previously described.\(^1\),\(^5\) The AM groups received a continuous intraperitoneal injection of recombinant human AM at a rate of 50 ng/h for 2 weeks beginning on day 1 post-BCAS. The vehicle groups received 0.9% saline only.

**Measurement of cerebral blood flow**

The regions of interest corresponded with the regions around Heubner’s anastomoses connecting the dorsal branches of the anterior cerebral artery (ACA) and the middle cerebral artery (MCA). The baseline cerebral blood flow (CBF) recordings were obtained immediately before and after the operation, as well as 1, 3, 7, 14, and 28 days after surgery. The CBF values were averaged between bilateral sides and expressed as a percentage of the baseline value. Changes in cerebral surface blood
flow were monitored by the use of a laser speckle blood flow imaging system (Omegazone, Omegawave), as previously reported, but with some modifications. In the day prior to the first day of CBF measurement anesthesia was induced with 4% halothane and maintained with 1.5% halothane in 80% nitrous oxide and 20% oxygen, the skull was exposed by a midline scalp incision and the scalp reflected laterally; the scalp was kept open throughout the experiment. During the measurement of CBF, the intact skull surface was diffusely illuminated by 780 nm 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 number and velocity of moving red blood cells, approximating CBF. Signal processing was performed by the algorithm developed by Forrester et al. Color-coded blood flow images were obtained in high-resolution mode (639 × 480 pixels; 1 image/sec). 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 minute, 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 with 1.5% halothane and maintained with the same concentration of halothane in 80% nitrous oxide and 20% oxygen. During the measurement of CBF, blood pressure was measured by the tail cuff method and confirmed to be kept constant. Rectal temperature was maintained between 36.5°C and 37.5°C. In addition, the stability of the level of anesthesia was checked by testing corneal reflexes and motor responses to tail pinch.

**Visualization of cerebral angioarchitecture**

The cerebral angioarchitecture was studied by a modification of the postmortem latex perfusion technique on day 7 after the surgery. 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. A lethal dose of papaverine hydrochloride (40 to 50 mg/kg) was injected to produce
maximal vasodilation. Immediately after 2 mL saline injection, 4 mL white latex compound (Chicago
Latex Products) mixed with 50 µL/mL carbon black (Fueki) diluted 2:1 with saline was injected at a
perfusion pressure of 150 mm Hg over a 5-minute period. After the initiation of infusion, the right
atrium of the heart was incised to allow for venous outflow. The injected volume of latex, as well as
the time taken to harden the latex sufficiently, was much greater than those of previous reports (0.4–2
mL, 2 minutes).9, 10, 13 In order to harden the latex completely for the brain removal procedure, the
dead animal was soaked in ice-cold water 20 minutes after the end of infusion, and the brain was
subsequently removed 20 minutes later. Photographs of the dorsal and ventral surface of the brain
were taken using a digital microscope (DinoLite, AnMo Electronics Corp.) at ×80 magnification. The
vessel diameters of the leptomeningeal anastomoses and the circle of Willis were measured using
image analysis software (DinoCapture, AnMo Electronics Corp.). The distal MCA was identified from
its branch angle and distinguished from the distal ACA or posterior cerebral artery (PCA). The
maximal diameter of the leptomeningeal anastomoses was measured at the point of confluence
between the distal MCA and the distal ACA (Heubner’s anastomoses) or between the distal MCA and
the distal PCA. The point of confluence was defined as the narrowest part of the vessel or half way
between the nearest branching points of the ACA, the MCA, and the PCA branches, respectively. The
diameter of the internal carotid artery (ICA), ACA, MCA and the posterior communicating artery
(Pcom) were averaged across both sides. The diameters of the ICA and MCA were measured just
proximally and distally to the terminal bifurcation of the ICA, respectively. The diameter of the ACA
was measured just proximally to the origin of the olfactory artery. The diameter of the Pcom was
measured at its origin from the ICA.
Histologic and immunohistochemical evaluation

Mice were euthanized 7 and 28 days after the surgery. The harvested brains were subjected to histologic examination using a standard procedure described elsewhere. Klüver–Barrera (KB) staining was used to observe any histological changes. The severity of the white matter (WM) lesions was semiquantitatively graded into four levels by an investigator blind to the experimental condition.

Primary antibodies used for immunohistochemistry included those against: the astrocyte marker, glial fibrillary acidic protein (GFAP) (1:10000, Dako Cytomation); the microglia marker, ionized calcium binding adapter molecule 1 (Iba-1) (1:500, Wako Pure Chemical Industries); the mature oligodendrocyte marker, glutathione-S-transferase-pi (GST-pi) (1:10000, Stressgen); the oxidative DNA damage marker, 8-hydroxy-deoxyguanosine (8-OHdG) (1:100, Japan Institute For the Control of Aging); and the endothelial marker, platelet endothelial cell adhesion molecule (PECAM)-1 (CD31) (1:100, Pharmingen). In the sections immunostained for GFAP, Iba-1, and GST-pi, we counted the number of immunopositive cells in six fields (cells/0.25 mm²) in the median and the paramedian part of the corpus callosum and the anterior commissure at 0 to 0.5 mm anterior from the bregma.

Capillary density was quantified by the number of PECAM-1-positive cells (cells/mm²). Vessels with a diameter between 3 and 8 μm were counted. The vessel counts were performed in ten fields in the cerebral cortex, the corpus callosum, and the caudoputamen of the coronally-cut sections at 0 to 0.5 mm anterior from the bregma.

Analysis of oxidative damage in cerebral vessels

To evaluate oxidative damage in cerebral microvessels, double immunofluorescence staining for PECAM-1 and 8-OHdG was performed on day 3 post-BCAS. The acquired images were processed using Adobe Photoshop (version 7, Adobe System). RGB images were converted to 8-bit grayscale images. Based on an analysis of pixel fluorescence intensities, which ranged from 0 to 255, specific staining was distinguished from background by using a threshold value of 45–50 for 8-OHdG and
30–35 for PECAM-1. Area densities of structures stained with 8-OHdG or PECAM-1 were calculated as the proportion of pixels having a fluorescence intensity value equal to or greater than the threshold. Four fields in the corpus callosum of the coronally-cut sections at 0 to 0.5 mm anterior from the bregma were examined and the number of pixels with 8-OHdG positivity per the number of pixels with PECAM-1 positivity was calculated.

Analysis of monocyte recruitments and proliferation of smooth muscle cells

On day 7 post-BCAS, mice were euthanized by intracardiac perfusion with 4% paraformaldehyde in 0.1 mol/L phosphate buffer (PB, pH 7.4). The brains were post-fixed in 4% paraformaldehyde in 0.1 mol/L PB, and were stored in 20% sucrose in 0.1 mol/L PB (pH 7.4). The brains were embedded in paraffin and sliced into 6 μm-thick coronal sections and then subjected to the immunofluorescent analysis for Ki-67 and F4/80 together with α-smooth muscle actin (α-SMA). Afterdeparaffinization with xylene and rehydration with ethanol,15 antigen retrieval for the Ki-67 antigen was performed using 0.01 mol/L citrate buffer (pH 6.0) at 95°C for 20 minutes. Sections were rinsed briefly with 0.1 mol/L PBS, pH 7.4, and then blocked for 30 minutes at room temperature in a solution of PBS containing 5% preimmune serum and 0.3% Triton-X100 (PBS+). Sections were subsequently incubated at 4°C for 24 hours with primary antibodies diluted in PBS+ then washed thoroughly with PBS at room temperature. Sections were incubated with secondary fluorochrome-conjugated antibodies in PBS for 90 minutes at room temperature then washed thoroughly. Stained tissue sections were evaluated by using a confocal laser-scanning device (FV1000, Olympus). The density of the Ki-67-immunoreactive smooth muscle cells (SMCs) per total SMCs was analyzed on the dorsal surface of the brain in the coronally-cut sections at 0.5 to 1 mm posterior from the bregma. In addition, the density of SMCs surrounded by F4/80-immunoreactive monocyte/macrophages was calculated per total SMCs on the same area of another section. Primary antibodies used for immunofluorescent
analysis were as follows: goat antibody to Ki-67 (1:100, Santa Cruz); rat antibody to F4/80 (10 μg/mL, Abcam); mouse antibody to α-SMA (1:100, Neomarkers). Secondary antibodies used were as follows: FITC-conjugated chicken antibody to goat IgG (1:100, Santa Cruz); FITC-conjugated chicken antibody to rat IgG (1:100, Santa Cruz); TRITC-conjugated rabbit antibody to mouse IgG (1:100, Dako Cytomation).

Y maze test
The Y maze test was performed at 1 month after the surgery in a manner similar to that described previously.16 The Y maze is a three-arm maze with equal angles between all arms that is used to evaluate working memory. Mice were initially placed within one arm and allowed to move in the maze freely. The sequence and number of arm entries were recorded for each mouse over an 8-minute period. The percentage of triads in which all three arms were represented (ABC, CAB, or BCA but not BAB) was recorded as an alternation to estimate short-term memory of the last arms entered. The total number of possible alternations is the number of arm entries minus two. Furthermore, the number of arm entries serves as an indicator of spontaneous activity.

Eight-arm radial maze test
The 8-arm radial maze test was started at 1 month after BCAS. The test was conducted in a manner similar to that described previously.17 Each arm (8×35 cm) radiated from an octagonal central starting platform (perimeter 10×8 cm). Identical food wells were used (1 cm deep and 2.1 cm in diameter). One week before pretraining, animals were deprived of food until their body weight was reduced to 75–85% of the initial level. Pretraining started on the eighth day. Each mouse was placed in the central starting platform and allowed to explore and to consume food pellets scattered on the whole maze for a 5-minute period (one session per mouse). After completion of the initial pretraining, mice received another pretraining to take a pellet from each food well after being placed at the distal end of each arm.
A trial was finished after the subject consumed the pellet. This was repeated eight times, using eight 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 obtain all eight pellets within 25 minutes. A trial was terminated immediately after all eight pellets were consumed or 25 minutes had elapsed. An "arm visit" was defined as traveling for >5 cm from the central platform.

**cAMP assay**

cAMP levels in the forebrain were determined by the radioimmunoassay method as previously described. Mice were euthanized 5 days after surgery, the brain removed and frozen with liquid nitrogen, then preserved at –80°C. About 100 mg of frozen forebrain tissue was homogenized and extracted twice with 1 mL of ice-cold 0.1 N HCl solution. After centrifugation at 3000 rpm for 15 minutes at 4°C, the amounts of cAMP in the mixed supernatant was determined by radioimmunoassay using Yamasa cAMP assay kits (Yamasa).

**Analysis of VEGF and bFGF protein expression in the forebrain**

Western blot analysis of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) was performed using protein extracts from the forebrain tissue of sham- and BCAS-operated mice on days 1 and 5. Forebrain tissue was homogenized in RIPA buffer (1X PBS, 1% Igepal, 0.5% sodium deoxycholate and 0.1% SDS with protease and phosphatase inhibitors (Sigma)) Homogenates were centrifuged at 10,000 x g for 15 minutes at 4°C to remove insoluble proteins and fibrous tissue, and protein content for each sample was determined using bicinchoninic acid (BCA protein assay; Pierce-Rockford). Samples were separated by SDS-PAGE on 10% gels then transferred to PVDF membranes. Membranes were blocked in 5% non-fat dry milk for 30 minutes at room temperature then incubated with anti-VEGF antibody (0.5 μg/mL, R&D systems) or anti-bFGF antibody (1:200,
Santa Cruz), and with **anti-β-tubulin antibody (1:1000, Sigma)**, which served as an internal control, at 4°C overnight. Immunoblots were then incubated with horseradish peroxidase-conjugated secondary antibody (1:10,000, Santa Cruz) for 1 hour at room temperature. Immunoblots were detected using Enhanced Chemiluminescence (ECL) Western Blotting Detection reagent (Amersham). ECL blots were digitally captured with a LAS-3000 imaging system (Fujifilm). The LAS-3000 imaging system has a CCD camera which is equipped with shading correction without automatic gain control, and has an ample and linear dynamic range (from 0 to 4.0 OD values). Images were processed using Multi Gauge v3.1 software (Fujifilm). The intensity of each band was determined by measuring the AUC-BG/mm² (AUC: area under the curve, BG: background). Anti-VEGF antibody detected only one band at about 30 kDa, anti-FGF antibody detected only one band at about 18 kDa, and **anti-β-tubulin antibody detected only one band at about 50 kDa**.

**Quantitative real-time RT-PCR analysis of the forebrain**

Total RNA was extracted from the forebrain tissue obtained from sham and BCAS-operated mice on days 1 and 5 using QIAzol Reagent (QIAGEN), following standard protocol. Quantitative real-time RT-PCR analysis was carried out using an ABI PRISM 7300 Sequence Detection System (Applied Biosystems) with SYBR Green (Toyobo) or TaqMan probe, and values were normalized to 18S rRNA (TaqMan Ribosomal RNA Control Reagents VIC Probe; Applied Biosystems) to evaluate the levels of **AM**, calcitonin receptor-like receptor (CRLR), receptor activity-modifying protein (RAMP) 2, RAMP3, VEGF, and bFGF. The primers and probes used for quantitative real-time RT-PCR Analysis were as follows: **mouse AM forward, 5’-TACACGGGGACCTACAATGCT-3’; mouse AM reverse, 5’-TCGCACAGCTTGGTACGCTT-3’; mouse AM probe, 5’; mouse CRLR forward, 5’-GCTGGAA TGACGTTGCAGC-3’; mouse CRLR reverse, 5’ –GCCTTCACAGATCCACT-3’; mouse RAMP2 forward, 5’-CCGGAGTCCCAGATCAATCT-3’; mouse RAMP2 reverse, 5’ –CCAGTTGCACCAGTCCTTGA-3’; mouse RAMP3 forward, 5’-ACCTGTAGAGGTGCATCG-3’;
mouse RAMP3 reverse, 5’-ATCAGTGCTTGCTGCG-3’; mouse VEGF forward, 5’-CCCACGTCAGAGCAACATC-3’; mouse VEGF reverse, 5’-CTTTCTTTTGTTCTGCTACATC-3’; mouse bFGF forward, 5’-AGCGGCTCTACTGCAAGAAC-3’; mouse bFGF reverse, 5’-GCCGTCCATCTTCTCCTCATA-3’.

**Statistical analysis**

All values are expressed as means ± SD, unless stated otherwise. Group differences for hemodynamic, morphological, and general physiological measurements were analyzed for statistical significance by Student’s t test or ANOVA. Statistical analysis for the 8-arm radial maze test was conducted using StatView (SAS Institute) and data were analyzed by two-way repeated measures ANOVA. Correlations between variables were analyzed with Pearson’s coefficient. Differences with a probability value of \(P<0.05\) were considered to be statistically significant.

**Supporting information**

**High adrenomedullin level in the circulating blood in AM-Tg mice**

The plasma concentrations of human total AM were 355.2±44.5 fmol/mL in AM-Tg mice but not detected in WT mice. The physiological concentration of mouse total AM reportedly ranges from 5 to 20 fmol/mL.\(^{19,20}\) Therefore, the transgenic mice were expected to overproduce AM at about 50 times the magnitude of endogenous AM. The systolic BP (mmHg) was 116.1±7.5 in WT mice (n=16), 105.6±6.5 in AM-Tg mice (n=16), and 102±3.9 in hydralazine-treated WT (H-WT) mice (n=8). There were no apparent differences in overall appearance, behavior, growth, or fertility between wild-type and AM-Tg mice. The body weight (g) at 10–12 weeks old before the operation was 27.4±1.9 in WT mice (n=16), 26.5±1.4 in AM-Tg mice (n=16), and 27.1±1.7 in H-WT mice (n=8). There was no significant difference among the three groups.
Supplemental References


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Supplemental Figure S1.

(A) Representative images showing temporal changes of CBF in wild-type (WT) and adrenomedullin (AM)-Tg mice after BCAS. (see Figure 1C for statistical analyses) (B) Representative images of the dorsal (a–d) and ventral (e–h) cerebral angioarchitecture by postmortem latex perfusion method of WT or AM-Tg mouse that is subjected to sham (WT sham or AM sham) or BCAS operation (WT BCAS or AM BCAS) on day 7. Scale bars, 1 mm (upper panels of a–d), 250 μm (lower panels of a–d), and 500 μm (e–h). (see Figure 2, C and D for statistical analyses) (C) Representative double immunofluorescence images for Ki-67 (green)/α-SMA (red) and their quantitative data of WT sham, WT BCAS, and AM BCAS on day 7. Scale bar, 10 μm (n=4 each). *P<0.05 in AM BCAS vs. WT BCAS; #P<0.05 vs. WT sham. (D) Representative double immunofluorescence images for F4/80 (green)/α-SMA (red) and their quantitative data of WT sham, WT BCAS, and AM BCAS on day 7. Scale bar, 10 μm (n=4 each). #P<0.05 vs. WT sham. (E) Representative images of the PECAM-1-positive capillaries in sections from the cortex (a–d), the corpus callosum (e–h) and the caudoputamen (i–l) of WT sham, AM sham, WT BCAS, or AM BCAS on day 7. Scale bar, 50 μm. (see Figure 3C for statistical analyses) (F) Cerebral microvessels were stained for PECAM-1 and with 8-hydroxy-deoxyguanosine (8-OHdG). Representative images of sections from the corpus callosum of WT BCAS (a, c, e) or AM BCAS (b, d, f) on day 3. Scale bar, 50 μm. (see Figure 3D for statistical analyses)
Supplemental Figure S2.

(A) Representative images of the corpus callosum of the immunohistochemical staining for GFAP (a–d), Iba-1 (e–h) and GST-pi (i–l) and the Klüver-Barrera (KB) staining (m–p) of wild-type or adrenomedullin (AM)-Tg mouse that is subjected to sham (WT sham or AM sham) or BCAS operation (WT BCAS or AM BCAS) on day 28. Scale bars, 50 μm. (see Figure 4 for statistical analyses) (B–D) Histograms showing the density of cells immunoreactive for GFAP (B), Iba-1 (C), or GST-pi (D) in the medial and paramedial portions of the corpus callosum (CC) and the anterior commissure (AC) of WT BCAS or AM BCAS on day 7 (n=7 each). Error bars indicate SD. *P<0.05, **P<0.01 in AM BCAS vs. WT BCAS.
Supplemental Figure S3.

Correlation analysis between the cerebral blood flow (CBF) immediately after BCAS, as well as 1, 3, 7, 14, and 28 days after BCAS and the averaged number of revisiting errors (trial 1 to 26) at 1 month after BCAS (n=24).
Supplemental Figure S4.

Western blotting of VEGF, bFGF, and β-tubulin (internal control) in extracts of the brain of wild-type or adrenomedullin (AM)-Tg mouse that is subjected to sham (WT sham or AM sham) or BCAS operation (WT BCAS or AM BCAS) on days 1 and 5 (n=6–8 each). Error bars indicate SD. *P<0.05 in AM BCAS vs. WT BCAS; #P<0.05 vs. WT sham.