Characterization of White Matter Injury in a Rat Model of Chronic Cerebral Hypoperfusion

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Background and Purpose—Chronic cerebral hypoperfusion can lead to ischemic white matter injury resulting in vascular dementia. To characterize white matter injury in vascular dementia, we investigated disintegration of diverse white matter components using a rat model of chronic cerebral hypoperfusion.

Methods—Chronic cerebral hypoperfusion was modeled in Wistar rats by permanent occlusion of the bilateral common carotid arteries. We performed cognitive behavioral tests, including the water maze task, odor discrimination task, and novel object test; histological investigation of neuroinflammation, oligodendrocytes, myelin basic protein, and nodal or paranodal proteins at the nodes of Ranvier; and serial diffusion tensor imaging. Cilostazol was administered to protect against white matter injury.

Results—Diverse cognitive impairments were induced by chronic cerebral hypoperfusion. Disintegration of white matter was characterized by neuroinflammation, loss of oligodendrocytes, attenuation of myelin density, structural derangement at the nodes of Ranvier, and disintegration of white matter tracts. Cilostazol protected against cognitive impairments and white matter disintegration.

Conclusions—White matter injury induced by chronic cerebral hypoperfusion can be characterized by disintegration of diverse white matter components. Cilostazol might be a therapeutic strategy against white matter disintegration in patients with vascular dementia. (Stroke. 2016;47:00-00. DOI: 10.1161/STROKEAHA.115.011679)

Key Words: cilostazol ▪ dementia, vascular ▪ models, animal ▪ white matter

Chronic cerebral hypoperfusion induced by long-standing hypertension or severe carotid stenosis can lead to ischemic white matter injury.1-2 Integrity of the white matter, which consists of a complex structural unit, including neuronal axons, surrounding myelin with nodal or paranodal proteins at the nodes of Ranvier, and supportive glial cells, is critical to maintain brain function.3-5 Diverse components of white matter interact with each other, such as axon–glial connection6 or oligovascular signaling with a vascular component.4 Diverse nodal or paranodal proteins at the nodes of Ranvier are essential for the structural and functional stabilization of myelinated axons comprising white matter.1 Diffusion tensor imaging is a recently developed magnetic resonance imaging technique, which is highly sensitive to the directional diffusivities of water, where tissues are oriented according to particular directions of white matter tracts.6

For chronic cerebral hypoperfusion, rats subjected to permanent occlusion of the bilateral common carotid arteries (BCCAo) were used, which has been previously suggested as a possible animal model for vascular dementia.7-8 As time went on after the surgery, cerebral blood flow and metabolism decreased chronically in our previous study7 (Figure I in the online-only Data Supplement). In this study, we investigated cognitive impairment and its association with disintegration of diverse white matter components. Previously, we have shown that cilostazol, a phosphodiesterase III inhibitor, has beneficial effects against memory impairments in a rat model of chronic cerebral hypoperfusion.8 Its protective effect against white matter damage has been reported in other studies using an animal model of chronic cerebral hypoperfusion.9,10

Material and Methods

All experimental procedures were approved by the Animal Experiment Review Board of Laboratory Animal Research Center of Konkuk University, and procedures were in accordance with the Stroke Therapy Academic Industry Roundtable criteria for preclinical...
stroke investigations. Rats were allocated randomly (Figure IIA in the online-only Data Supplement). Diverse behavioral tests and serial brain magnetic resonance imaging were performed (Figure IIB in the online-only Data Supplement). Detailed Experimental Methods are described in the online-only Data Supplement.

**Results**

**Cognitive Impairments**

Sham-operated rats quickly learned to find the hidden platform in the Morris water maze task (Figure 1A). In the last session, it took a longer time and path for the rats with BCCAo to find the hidden platform (P=0.037 for time latency; P=0.039 for path length; and P=0.047 for search error). However, performances improved when rats were treated with cilostazol (P<0.05, compared with rats with BCCAo). Swimming speeds did not differ among the groups (P=0.373). In the second probe trial, the number of crossings still remained low for the rats with BCCAo, indicating poor memory (P=0.020, compared with sham-operated rats); however, the number of crossings increased when rats were treated with cilostazol (Figure 1B).

In the odor discrimination task (Figure 1C), it took more time for rats with BCCAo to retrieve the reward owing to impairments in learning and memorizing the rewarding odorant with a marginal statistical significance among 3 groups (P=0.054). When rats were treated with cilostazol, their performance improved (P=0.041, compared with rats with BCCAo). Percentage of correct trials across all 15 consecutive trials was significantly lower in rats with BCCAo (P=0.039, compared with sham-operated rats). However, performance improved to the level of sham-operated rats when rats were treated with cilostazol (Figure 1C).

In the novel object location test (Figure 1D), compared with 77.4% preference for the new location in the sham-operated rats, 52.6% preference in rats with BCCAo (P<0.001, compared with sham-operated rats) implicates that they chose objects randomly. When rats were treated with cilostazol, preference for the new location was increased to 73.6%, suggesting successful memorization (P=0.002, compared with rats with BCCAo).

**Neuronal Morphology in the Hippocampus**

Thionin staining of the hippocampus demonstrated no significant morphological changes of neurons in the CA1, CA3, or dentate gyrus in any group (Figure III in the online-only Data Supplement).

**Neuroinflammation**

Neuroinflammation was significantly increased in rats with BCCAo (P=0.046, compared with sham-operated rats in the CD11b staining). When rats were treated with cilostazol, neuroinflammation was attenuated to the level of sham-operated rats (Figure 2A).

**Oligodendrocyte and Myelin Basic Protein**

The loss of oligodendrocytes and myelin basic protein (MBP) were prominent in rats with BCCAo (P=0.031 for oligodendrocytes and P=0.008 for MBP, compared with sham-operated rats). Cilostazol treatment did not protect the loss of oligodendrocytes in rats with BCCAo (P=0.044, compared with sham-operated rats); however, MBP levels were similar to those of sham-operated rats when rats were treated with cilostazol (P=0.089, compared with sham-operated rats; Figure 2B). Similar pattern was observed in diaminobenzenidine staining for MBP (Figure IV in the online-only Data Supplement). Although there was a trend for protective effect of cilostazol on MBP attenuation in rats with BCCAo, however, it was not statistically significant compared with vehicle treatment.

**Nodal and Paranodal Integrity**

Ankyrin-G signals at the nodes of Ranvier and contactin-associated protein (Caspr) signals at the paranodal junction were distinct in the sham-operated rats (Figure 2C). However, ankyrin-G signals were widely dispersed along the paranodal area in rats with BCCAo. When rats were treated with cilostazol, ankyrin-G signals were mainly localized in the nodal areas in a similar pattern to the sham-operated rats. Increased colocalization coefficient of ankyrin-G and Caspr in rats with BCCAo (P=0.007, compared with sham-operated rats) was normalized when rats were treated with cilostazol (Figure 2D).

**White Matter Integrity in Serial Diffusion Tensor Imaging**

The optic chiasm, corpus callosum, and both external capsules, where white matter tracts are the most abundant in the rat brain, were selected (Figure 3A). Compared with the baseline, fractional anisotropy in the optic chiasm in rats with BCCAo was significantly reduced 1 week after surgery (P=0.009). When rats were treated with cilostazol, an ongoing reduction of fractional anisotropy (P=0.034) in the optic chiasm and an ongoing elevation of mean diffusivity (P=0.024) with a concomitant elevation of axial diffusivity (P=0.044) and radial diffusivity (P=0.042) in the corpus callosum were halted 5 weeks after surgery (Figure 3B). Representative tractography (Figure 3C) and quantitative analyses (Figure 3D) show more abundant white matter tracts when rats were treated with cilostazol (P=0.002 for fiber length and P=0.001 for fiber density).

**Discussion**

**Cognitive Impairments Induced by Chronic Cerebral Hypoperfusion**

Consistent with previous studies,7 we confirmed the presence of memory impairments using the water maze task and novel object test in a rat model of chronic cerebral hypoperfusion. Poor performance in the odor discrimination task suggests that cognitive functions associated with olfaction were also impaired in a rat model of chronic cerebral hypoperfusion. Dysfunction in olfactory threshold, identification, or discrimination is an early sign of cognitive impairment in patients with various neurodegenerative diseases.11 The poor performance of rats with BCCAo in the odor discrimination task might be comparable with the olfactory dysfunction in patients...
with diverse neurodegenerative diseases. To our knowledge, impaired odor discrimination in a rat model of chronic cerebral hypoperfusion has not been reported before. In addition, we confirmed our previous results about the protective effect of cilostazol against cognitive impairments in a rat model of chronic cerebral hypoperfusion.8

Loss of Oligodendrocytes and Attenuated Myelin Density Induced by Chronic Cerebral Hypoperfusion

Major components of the white matter are neuronal axons, the surrounding myelin sheath, and myelin-producing oligodendrocytes.1 In previous studies, neuroinflammation has been suggested...
as a key pathophysiology of white matter injury in animal models of chronic cerebral hypoperfusion. The loss of oligodendrocytes or compromised oligodendrogenesis has been reported in a mouse model of cerebral hypoperfusion. In our study, the loss of oligodendrocytes, attenuation of myelin density, and concomitant neuroinflammation were prominent. Neuroinflammation and the loss of myelin were attenuated by cilostazol treatment, but the loss of oligodendrocytes could not be prevented. However, previous studies using a similar rodent model of chronic cerebral hypoperfusion showed that cilostazol could protect oligodendrocytes. A longer time interval before histological study and different cilostazol treatment protocol in our study might have led to this discrepancy. Myelinated axons survived longer than oligodendrocytes in a mouse model of primary oligodendropathy,
suggesting that the myelin sheath can remain supportive for axons independently from oligodendrocytes.14 The protective effect of cilostazol against white matter injury might be selective to myelin, but not to oligodendrocytes. However, the selective protective effect of cilostazol needs to be confirmed in other experimental design using different animal models.

Figure 3. A, Selected regions of interest for imaging study (red line). B, Serial change of diffusion tensor imaging parameters. *P<0.05; n=4 to 5 in each group. C, Representative tractography. D, Quantitative measurement of tractography fiber variables. *P<0.05; n=3 to 4 in each group. AD indicates axial diffusivity; BCCAo, permanent occlusion of the bilateral common carotid arteries; FA, fractional anisotropy; MD, mean diffusivity; ns, not significant; RD, radial diffusivity; and T2WI, T2 weighted image.

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Structural Derangement at the Nodes of Ranvier After Chronic Cerebral Hypoperfusion

Ankyrin-G is a key cytoskeletal molecule, which is present at the nodal area and binds various nodal or paranodal proteins.1,15 Proteolysis of ankyrin-G disrupts integrity of the nodes of Ranvier and detaches myelin from the axonal surface.15 Caspr
Disintegration of the White Matter Based on Diffusion Tensor Imaging

An increased radial diffusivity has been reported in animal models of dysmyelination or demyelination. Therefore, reduced fractional anisotropy and increased radial diffusivity in the optic chiasm of rats with BCCAo might suggest white matter disintegration. In our study, decreased axial diffusivity in the optic chiasm of rats with BCCAo might suggest white matter disintegration. Protective effect of cilostazol against white matter tracts. Further increase of mean diffusivity in the corpus callosum 5 weeks after BCCAo surgery might reflect ongoing encephalomalacic changes and disintegration of the white matter. Neuroinflammation-associated water accumulation in the affected tissue might be attributed to the increases of radial diffusivity, axial diffusivity, and mean diffusivity. Even 5 weeks after BCCAo surgery, we found no such increase in the cilostazol-treated group.

In conclusion, white matter injury after chronic cerebral hypoperfusion can be characterized by disintegration of the white matter, which is demonstrated by the loss of oligodendrocytes, attenuation of myelin density, structural derangement at the nodes of Ranvier, and disintegration of white matter tracts. Protective effect of cilostazol against white matter disintegration might suggest a therapeutic strategy for patients with vascular dementia.

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Characterization of white matter injury in a rat model of chronic cerebral hypoperfusion
Supplemental Methods

Experimental groups
Male Wistar rats (aged 3 months; Charles River, Gapyeong, Republic of Korea) were used in the experiments. Rats were housed in an environmentally controlled vivarium at Konkuk University (22 ± 1°C, 50 ± 10% humidity, and lights on from 7 a.m. to 7 p.m.). Food and water were available ad libitum. Rats were allocated randomly to sham-operated or BCCAo groups with a ratio of 1:3 to 4. Rats in the BCCAo group were subjected randomly to the cilostazol or vehicle treatment (Fig IIA). Four rats in the BCCAo group died within 3 days after the surgery, leading to a mortality rate 9.3% (4/43). The blindness rate was 15.4% (6/39). Blinded rats were excluded from behavioral tests. A separate cohort was used for imaging. Rats were coded with numbers, and investigators were blinded to the treatment groups.

Surgical procedure
Rats were anesthetized using a mixture of 5% isoflurane and 95% oxygen, and a mixture of 3% isoflurane and 97% oxygen was administered during the surgery. Rectal temperature was maintained at 37 ± 0.5°C with a heating pad during the surgery. In the BCCAo group, the bilateral common carotid arteries were carefully exposed through a midline incision and were permanently double-ligated with silk sutures. The same surgical procedure was performed in the sham-operated group except for the occlusion of the bilateral common carotid arteries. Rats in the BCCAo group were subjected randomly to the cilostazol or vehicle treatment.

Cilostazol treatment
Cilostazol was supplied by Otsuka Pharmaceutical (Tokushima, Japan). Rats were administered 60 mg/kg cilostazol or vehicle for a period of 4 weeks after surgery once a day using oral gavage. Cilostazol was dissolved in 2 mL 10% Tween-80 solution. Rats in the vehicle treated group received an equal volume of 10% Tween-80 solution.

Experimental timeline
The timeline of the experiment is shown in Fig IIB. Behavioral tests were performed in the following order: water maze task, odor discrimination task, and finally, the novel object recognition and location tests were performed consecutively 4 weeks after surgery. Serial brain MR imaging was performed before the surgery, and 1 and 5 weeks after surgery. For histological evaluation, rats were sacrificed after all behavioral experiments were performed. A separate cohort was used for imaging study. Rats were coded with numbers, and investigators were blinded to the treatment groups until the end of the data analysis.

Water maze task
Rats were evaluated in the Morris water maze. The test was performed according to a validated protocol described previously. Normal visual function is mandatory for the recognition of visual cues on the surrounding curtains; however, BCCAo surgery may damage visual functions. Therefore, a blind test was performed, as described previously, prior to the water maze task to exclude blind rats. The maze was a round tank, 1.83 m in diameter and 0.58 m deep, filled up to a depth of 35.5 cm with tepid water (26 ± 1°C), which
was made opaque with white paint. A movable circular platform, 12 cm in diameter, was located 2 cm below the water surface. The maze was surrounded by white curtains, on which black visual stimuli with various shapes and sizes were placed. A camera was located above the center of the maze, which relayed images to a videocassette recorder and the HVS Image Analysis Computer System (Hampton, United Kingdom). Four consecutive sessions, each consisting of five trials for two days (alternating two or three trials per day), were conducted on eight consecutive days. The hidden platform was always located in the southeastern quadrant of the pool. Rats were submerged gently into the water, facing towards the inside wall of the tank. Every trial started at different points, alternating the four quadrants. Rats were handled gently for 10 min daily for 7 days before the test. In the maze, rats were allowed to swim for a maximum time of 90 s. Rats were allowed to remain on the platform for 30 s at the end of each trial. Performance was evaluated for time latency, path length, and search error data of all trials. Measurement of the search error was based on the average distance between the rat and the platform during the trial. The distance was sampled 10 times/s during each trial, and these distances were averaged in 1-s bins. The cumulative search error is the sum of these 1-s averages of the proximity measure corrected for the specific platform location and start location by subtracting the proximity score that would be produced by a perfect performance in the trial. A probe trial was conducted 1 min after every 10th training trial. The entire training procedure included two probe trials for each rat, while the rats swam with the platform retracted to the bottom of the pool for 30 s. After recording the swimming path, the platform was raised to its normal position for the completion of the trial. Swimming time spent in the target quadrant with a retracted platform, and the number of crossings above the retracted platform site was used as parameters for the retention of spatial memory. Six rats per group were assessed.

**Odor discrimination task**

Odor discrimination task, which is not dependent on the visual function, was performed according to a validated protocol described previously.² Tests were performed in a 48 cm × 25.5 cm × 20 cm (height) sized transparent acrylic box divided into two chambers by a sliding opaque black acrylic board. Rats were allowed to explore the testing area for 10 min per day for 3 days. Then, rats were tested with the forced-choice discrimination task as follows. At the beginning of each trial, the rat was placed in the start chamber with the divider closed. Two ceramic pots, 8 cm in diameter and 4.5 cm in height, were placed in the test chamber. They were filled evenly with 50 cm³ of bedding and 50 µl of the diluted odorant applied on the top center of the bedding. Another 50 cm³ of bedding was added to bury the odorant. Food was buried at the bottom of rewarded pot marked with S-limonene (lemon) scent, while the unrewarded pot without food was scented with R-limonene (citrus). The two pots were placed randomly in the test chamber to minimize visual cues based on the spatial location of pots. When the divider was removed, the rat entered the test chamber, and was allowed to dig in the pots until it retrieved the reward. The odor discrimination task consisted of 15 consecutive trials. A trial was considered correct when the rat dug in the rewarded pot first. Rats were allowed to self-correct after digging in the unrewarded pot. If the rat failed to dig in either pot after 120 s, the trial was terminated, and a reward was placed on the top of the rewarded pot to enhance feedback learning. Every 5th trial was performed as a probe trial, in which the rewarded pot was presented with the rewarded scent only to confirm that the rat was not guided by the odor of the reward itself. Once the rat continued to dig in the rewarded
pot during the probe trial, a reward was dropped onto the bedding to maintain odorant-reward association. A video camera was positioned in front of the apparatus, and all behaviors were videotaped for analysis. Time latency to reward retrieval and percentage of correct trials were evaluated for each rat in 15 trials. Four to six rats per group were assessed.

**Novel object recognition and location tests**

Novel object test was performed according to a validated protocol described previously.1 Rats were placed in a 40 cm × 40 cm × 40 cm (height) sized box, and were allowed to explore the testing area 10 min per day for 3 days. After 24 h, two objects were placed in the testing area, and the rat was allowed to explore the two objects in the testing area for 5 min before being returned to the cage. After a 3 h-interval, one of the objects was either relocated or replaced with a new object, and the rat was allowed to explore the testing area once again for 2 min. Novel object exploration was registered if the center of the rat’s head was oriented within 45° of the object and within 4 cm of it. Climbing over or sitting on an object was not included. A video camera was positioned over the arena, and exploratory behaviors were videotaped for later analysis. Exploratory time spent at the novel object was recorded, and percentage exploratory preference was computed as [time at novel object/(time at novel object + time at old object)] in a blinded manner. Six rats per group were assessed.

**Immunohistochemistry**

One week after all behavioral experiments, rats were perfused transcardially with 0.01 M phosphate-buffered saline (PBS), and subsequently with 4% paraformaldehyde (PFA) under deep anesthesia. The brain was removed and post-fixed in 4% PFA for 2 days, cryoprotected in 0.01 M PBS containing 30% sucrose for 3 days, frozen on powdered dry ice, embedded in Tissue-Tek (Sakura), and sectioned on a microtome at 40 µm thickness in the coronal plane. Free-floating sections were stored in a cryoprotective solution (30% ethylene glycol, 25% glycerol, 25% 0.1 M phosphate buffer, 20% distilled water) at 4°C. Sections were washed with 0.3% Triton X-100 in 0.01 M PBS (PBST), and then incubated in blocking serum (10% fetal horse serum in PBST) for 2 h. Subsequently, sections were incubated in the primary antibody solution for 20 h at room temperature.

To evaluate microglial activation and white matter damage, mouse anti-CD11b (Serotec, 1:1000), rabbit anti-Iba-1 (Wako, 1:1000), mouse anti-oligodendrocyte (Millipore, 1:5000), and rabbit anti-myelin basic protein (MBP; Abcam, 1:1000) antibodies were used. Five to six rats per group were assessed.

To visualize structural changes in the nodes of Ranvier, mouse anti-contactin-associated protein (Casp, Millipore, 1:1000) and rabbit anti-ankyrin-G (Santa Cruz Biotechnology, 1:1000) antibodies were used. Sections were washed in PBST and incubated in Alexa® 488 conjugated donkey anti-mouse and Alexa® 568 conjugated donkey anti-rabbit secondary antibody solutions (Invitrogen, 1:200) for 3 h at room temperature. After washing with 0.01 M PBS, stained sections were mounted on resin-coated slides, covered with ProLong® Gold anti-fade reagent (Invitrogen), and sealed with nail polish. Sections were imaged with a confocal microscope (Olympus, FV1000), and fluorescence signal intensity was measured using the FV10-ASW 2.0 analysis program (Olympus). Colocalization of nodal and paranodal proteins at the nodes of Ranvier was assessed using ankyrin-G and Caspr double immunostaining. The Caspr-immunopositive paranodal area was selected as region of interest (ROI) to measure colocalization with the
ankyrin-G signal. Colocalization coefficient was calculated with the FV10-ASW 2.0 analysis program (Olympus). One 0.5mm² ROI was selected per section in the corpus callosum (bregma -2.04 to -3.60 mm; 6 sections per rat) for 6 rats per group.

To confirm white matter damage, diaminobenzidine staining for MBP was performed as follows: sections were quenched endogenous peroxidase with 3% hydroxide and 10% methyl alcohol and incubated in the blocking serum (10% normal horse serum in 0.3% triton X-100 with PBS). Subsequently, sections were incubated in a rabbit anti-MBP primary antibody solution (1:1000, Abcam) for 1 h at room temperature and overnight at 4°C. After incubation, sections were washed in PBS with 0.3% Triton X-100 and incubated in a secondary antibody solution (biotinylated horse anti-rabbit antibody, Vector, 1:200) for 1 h at room temperature. Sections were then incubated in extravidin® peroxidase solution (Sigma Aldrich, 1:1000) for 1 h and visualized with diaminobenzidine solution (DAB kit, Vector). Stained sections were mounted on resin-coated slides and dried for one week. Slides were then defatted in xylene and cover-slipped with permount® (Fischer scientific) reagent. One to three semi-quantitative scale for myelin density was used for quantitative evaluation. Normal myelination was scored 3. Six rats per group were assessed.

To evaluate neuronal damage in the hippocampus, thionin staining was performed as follows: sections were mounted onto resin-coated slides and dried for 10 days. Sections were hydrated using subsequently diluted concentration of ethanol and distilled water. Sections were stained in the thionin solution for a few minutes, washed in distilled water and ethanol, dehydrated using subsequently increased concentration of ethanol, defatted in xylene and cover-slipped with permount® (Fischer scientific) reagent. Hippocampal neuronal damage was evaluated using a semi-quantitative neuronal survival score: 3, no damage; 2, scattered ischemic neurons in CA1; 1, moderate ischemic damage in CA1; 0, severe damage to pyramidal cells in CA1. Six rats per group were assessed.

Diffusion tensor imaging (DTI) protocol

Rats were anesthetized using a mixture of 5% isoflurane and 95% oxygen, and were maintained on a mixture of 3% isoflurane and 97% oxygen during imaging. Rats were placed in a prone position in a non-magnetic home-made holder with head fixation during MR imaging. MR images were obtained with a 3.0 T MR scanner (Signa HDx; GE Healthcare, Milwaukee, WI, USA) with a T/R Wrist coil. Data acquisition was performed with the following parameters: repetition time (TR) = 3 s; echo time (TE) = 87 ms; slice thickness = 2 mm; field-of-view (FOV) = 7 cm; matrix of 64 × 64 using zero-fill interpolation = 256 × 256. Total imaging time was 20 min. Acquisition of diffusion-weighted images was set by two b values = 0 and 600 s/mm² along with 12 different gradient directions. DTI scan time was 5 min 15 s. Coronal T2-weighted fast spin echo sequence was performed with the following parameters: TR = 2 s; TE = 77 ms; slice thickness = 2 mm; FOV = 7 cm; matrix of 320 × 320 using zero-fill interpolation = 512 × 512; scan time = 1 min 56 s. Raw data of DTI were transferred into DICOM format, and processed using the DTI Studio Software v2.3 (Johns Hopkins University, Baltimore, MD, USA). After generating diffusion tensor matrices from sets of twelve diffusion-weighted images and b = 0 images, the three eigenvalues and eigenvectors were calculated by matrix diagonalization. The DTI parameter maps of fractional anisotropy (FA), axial diffusivity (AD), radial diffusivity (RD), and mean diffusivity (MD) were calculated.
Image analysis

The optic chiasm (OC), corpus callosum (CC), and both external capsules (EC), where white matter tracts are the most abundant in the rat brain, were selected as ROIs. These ROIs were drawn manually based on coronal T2-weighted MR images at the appropriate section (OC at bregma -0.22 mm; CC and EC at bregma -0.58 mm), and transferred to identical sites on other parameter maps for further calculations (Fig 3A). Four to five rats per group were assessed.

White matter tractography was also produced to visually compare whole hemispheric fiber trajectories. Each fiber tract was seeded from the CC and EC at the same ROIs. Each fiber trajectory was summated from five coronal sections to compare the whole hemispheric white matter. Length and density of fiber tracts were analyzed for quantification. Three to four rats per group were assessed.

Cerebral blood flow and metabolism measurement

In our previous study, cerebral blood flow and metabolism were measured by $^{18}$F-fluorodeoxyglucose positron emission tomography scanner using same rat model. First 5-min and between 60- and 80-min scans of $^{18}$F-fluorodeoxyglucose uptake were used as indicators of cerebral blood flow and metabolism, respectively. As time went on after the surgery, cerebral blood flow and metabolism decreased chronically (Fig 1).

Statistical analyses

All results are expressed as mean ± standard error of mean (SEM). One-way analysis of variance (ANOVA) followed by the post hoc Fisher’s protected least significant difference test was conducted when appropriate. Serial data of the water maze task or odor discrimination task were analyzed with one-way repeated measures ANOVA followed by the post hoc Fisher’s protected least significant difference test. Parameters of DTI, which was performed twice (1 and 5 weeks after surgery) in the same cohort of rats, were analyzed using the paired samples t-test. A value of $p < 0.05$ was considered statistically significant. Data analyses were performed with SPSS software version 12.0.

References

Supplemental Figures

Figure I

Decreased cerebral blood flow and metabolism in a rat model of chronic cerebral hypoperfusion. Data were presented from our previous study with personal permission. Representative positron emission tomography coronal images superimposed on CT images and temporal profiles of the in the cortex, striatum, and hippocampus (n =3).

A. Mean normalized $^{18}$F-fluorodeoxyglucose uptake during the first 5 min represents chronically reduced cerebral blood flow.

B. Mean normalized $^{18}$F-fluorodeoxyglucose uptake between 60 and 80 min represents chronically reduced cerebral metabolism.
Figure II

A. Allocation of rats into groups.
B. Time line of the experiment.
Figure III

A. Thionin staining of the hippocampus. Scale bar = 100 µm.
B. Semi-quantitative analyses. ns = not significant; n = 6 in each group.
Figure IV

A. Diaminobenzidine staining for myelin basic protein. Scale bar = 100 µm.

B. Semi-quantitative analyses. *p < 0.05; ns = not significant; n = 6 in each group.