

Selective Impairment of Working Memory in a Mouse Model of Chronic Cerebral Hypoperfusion

Masunari Shibata, MD; Nobuyuki Yamasaki, MD; Tsuyoshi Miyakawa, PhD;
Rajesh N. Kalaria, PhD, FRCPath; Youshi Fujita, MD; Ryo Ohtani, MD; Masafumi Ihara, MD;
Ryosuke Takahashi, MD; Hidekazu Tomimoto, MD

Background and Purpose—We recently designed a mouse model of chronic cerebral hypoperfusion, in which the cerebral white matter is damaged without significant gray matter lesions. The behavioral characteristics of these mice were studied using a test battery for neurological and cognitive functions.

Methods—Adult C57Bl/6 male mice were subjected to either sham-operation or bilateral common carotid artery stenosis (BCAS) using microcoils with an internal diameter of 0.18 mm. At 30 days after BCAS, 70 animals were divided into 3 groups and subjected to behavioral test batteries. The first group underwent comprehensive behavioral test, including the neurological screen, prepulse inhibition, hot plate, open field, light/dark transition, Porsolt forced swim and contextual and cued fear conditioning (BCAS n=13; sham-operated n=11). The second group was for the working memory task of the 8-arm radial maze test (BCAS n=12; sham-operated n=10), and the third for the reference memory task of the 8-arm radial maze test (BCAS n=13; sham-operated n=11). Another batch of animals were examined for histological changes (BCAS n=11; sham-operated n=12).

Results—The white matter including the corpus callosum was consistently found to be rarefied without clear ischemic lesions in the hippocampus. No apparent differences were observed in the comprehensive test batteries between the control and BCAS mice. However, in the working memory tasks tested with the 8-arm radial maze, the BCAS mice made significantly more errors than the control mice ($P<0.0001$). Again, there were no detectable differences in the reference memory tasks between the groups.

Conclusions—At 30 days after BCAS, working memory deficits as well as white matter changes were apparent in the mice. Working memory deficit was attributable to damage of the frontal-subcortical circuits, suggesting the BCAS model is useful to evaluate the substrates of subcortical vascular dementia. (*Stroke*. 2007;38:2826-2832.)

Key Words: behavioral neurology ■ cerebral blood flow ■ hippocampus ■ leukoaraiosis ■ memory
■ vascular dementia ■ white matter disease

Cerebral blood flow (CBF) is decreased in patients with vascular dementia. Subcortical vascular dementia, the major subtype of vascular dementia, is featured by small vessel disease involving white matter (WM) changes and lacunar infarctions. Arteriosclerosis may induce these WM lesions after longstanding cerebral hypoperfusion.¹ In support for this hypothesis, hypoxia-inducible factor-1 is expressed in cerebrovascular WM lesions.² In addition, WM lesions are observed in rodent models of chronic cerebral hypoperfusion, in which the bilateral carotid arteries are stenosed or ligated,³⁻⁷ and stroke-prone spontaneously hypertensive rats which have small vessel pathology.⁸ Such WM lesions are suggested to contribute to frontal hypometabolism and executive dysfunction.^{9,10}

We recently designed a mouse model of chronic cerebral hypoperfusion¹¹ by placing microcoils bilaterally on the common carotid arteries. These mice invariably exhibited WM changes and have several advantages over other models of chronic cerebral hypoperfusion in rats and gerbils.^{3-5,12-14} First, genetically modulated mice produced to model various diseases can be used.¹⁵ Second, the visual pathway is preserved as compared with the rat model, because blood flow in the common carotid arteries albeit reduced is maintained. Third, the cerebral WM is selectively damaged, yet sparing the gray matter such as the hippocampus if the degree of stenosis is appropriately controlled by adjusting the internal diameter of the microcoils.¹¹

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From the Department of Neurology (M.S., Y.F., R.O., M.I., R.T., H.T.), Graduate School of Medicine, and Horizontal Brain Research Organization (N.Y., T.M.), Kyoto University, Sakyo-ku, Kyoto, Japan; and the Institute for Health and Ageing (R.N.K.), University of Newcastle upon Tyne, Newcastle General Hospital, Newcastle-upon-Tyne, UK.

Correspondence to Hidekazu Tomimoto, MD, Department of Neurology, Graduate School of Medicine, Kyoto University, Sakyo-ku, Kyoto 606-8507, Japan. E-mail tomimoto@kuhp.kyoto-u.ac.jp

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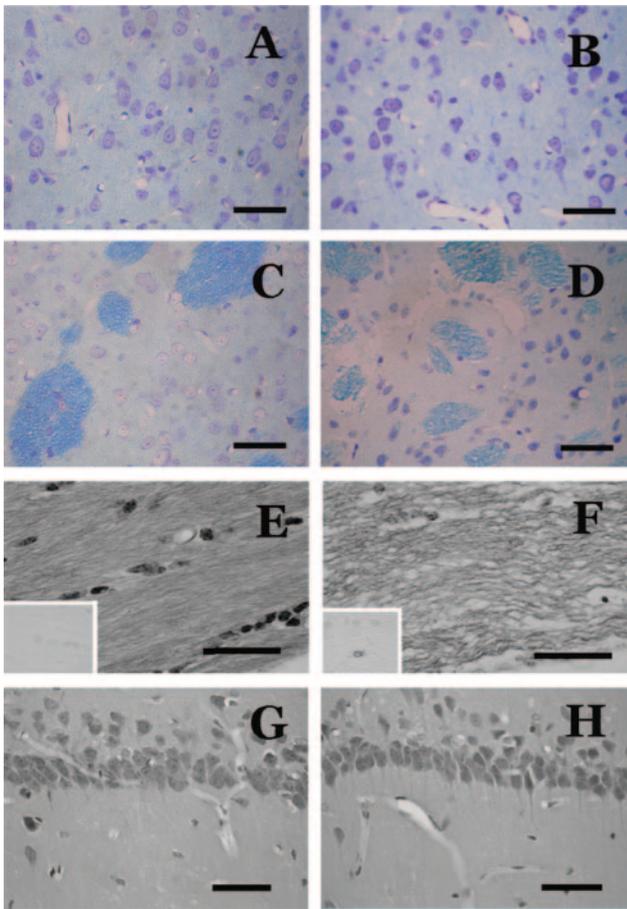


Figure 1. Photomicrographs of Klüver-Barrera staining (A through H) and TUNEL (insets in E and F) in the cerebral cortex (A and B), caudoputamen (C and D), corpus callosum (E and F) and hippocampus (G and H). The left column (A, C, E, and G) indicates the brain from a sham-operated mouse and the right column (B, D, F, and H) indicates a brain after BCAS for 30 days. Note marked vacuoles (F), and intact pyramidal neurons (H) after BCAS. Bars indicate 100 μm (C through F).

In the present study, we used various paradigms to test behavior including working and reference memories in this bilateral carotid artery stenosis (BCAS) mice model of chronic cerebral hypoperfusion. The BCAS model would be useful to explore behavioral substrates of the frontal-subcortical circuit deficits apparent in subcortical vascular dementia.

Materials and Methods

Animals and Experimental Design

Male C57Bl/6 mice (10 to 12 weeks old, 24 to 29 g; Shizuoka laboratory animal center, Hamamatsu) were anesthetized with sodium pentobarbital. Through a midline cervical incision, both common carotid arteries were exposed. A microcoil with a diameter of 0.18 mm was applied to the bilateral common carotid artery, maintaining the rectal temperature between 36.5°C and 37.5°C. Those in the control group were sham-operated, which involved bilateral exposure of the common carotid arteries. The animals were kept in cages for 30 days with food and water ad libitum.

Twenty four mice were examined for histological changes. After BCAS, CBF was measured by laser-Doppler flowmetry at 1, 7 and 30 days, as described previously.¹¹ These mice were deeply anesthetized with sodium pentobarbital and were perfused transcardially with 0.01 mol/L phosphate-buffered saline (PBS) and then with a fixative containing 4% paraformaldehyde and 0.2% picric acid in 0.1

mol/L phosphate buffer (PB, pH 7.4). The brains were postfixed 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 then embedded in paraffin and sliced into 6 μm -thick coronal sections. Sequential sections from every 50 to 100 sections were stained with Klüver-Barrera (KB) and hematoxylin and eosin (H&E) stains. TUNEL staining was done using Apoptag in situ kit obtained from Oncor. The WM changes were evaluated specifically in 4 regions: the optic tract, internal capsule, fiber bundles of the caudoputamen, and corpus callosum. In the coronal plane 0.15 mm anterior to the bregma, the areas of right hemisphere and ventricular space 30 days after the operation were digitized using the NIH image analyzer program, and used as an index of brain atrophy.

Another 70 animals, which were 14 to 16 weeks old were divided into 3 groups and examined 30 days after the operation. The first group of the mice was subjected to a comprehensive behavioral test battery.^{16,17} The battery included the neurological screen, light/dark transition, open field, hot plate, prepulse inhibition and Porsolt forced swim, contextual and cued fear conditioning, which were conducted in this sequence, with each test separated at least by 1 day (BCAS mice, $n=13$; sham-operated, $n=11$). The second group was tested for the working memory task of the 8-arm radial maze (BCAS mice, $n=12$; sham-operated, $n=10$), and the third group for the reference memory task (BCAS mice, $n=13$; sham-operated, $n=11$).

The mice were housed in a room with a 12-hour light/dark cycle (lights on at 7:00 AM) with access to food and water ad libitum. All procedures were performed according to the guidelines of the Animal Use and Care Committee of Kyoto University.

Neurological Screen

A neurological screen was conducted as previously described.¹⁷ The ear twitch, whisker touch and righting reflexes were evaluated.

Startle Response/Prepulse Inhibition Tests

A startle reflex measurement system was used (O'Hara & Co). The test session began by placing a mouse in a plexiglass cylinder for 10 minutes. The duration of white noise as the startle stimulus was 40 ms for all trial types. The startle response was recorded for 140 ms (measuring the response every 1 ms) starting with the onset of the prepulse stimulus. The peak startle amplitude recorded during the 140-ms sampling window was used as the dependent variable. A test session consisted of 6 trial types (ie, 2 types for startle stimulus only trials, and 4 types for prepulse inhibition trials). The intensity of startle stimulus was 110 or 120 dB. The prepulse sound was presented 100 ms before the startle stimulus, and its intensity was 74 or 78 dB. Four combinations of prepulse and startle stimuli were used (74/110, 78/110, 74/120, and 78/120). Six blocks of the 6 trial types were presented in pseudorandom order such that each trial type was presented once within a block. The average intertrial interval was 15 s (range: 10 to 20 s).

Hot Plate Test

The hotplate test for nociception was used to evaluate sensitivity to a thermal stimulus. Mice were placed on a 55.0 (± 0.3)°C hot plate (Columbus Instruments), and latency to the first hind-paw response (a foot shake or a paw lick) was recorded.

Motor Function Tests

Motor coordination and balance were tested with the rotarod test, and neuromuscular strength was tested with wire hang test and grip strength test.¹⁷ In the wire hang test, the mouse was placed on a wire cage lid apparatus (O'hara & Co) to assess balance and grip strength. The mouse was placed on a wire mesh, which was then inverted, and latency to fall was recorded. A grip strength meter (O'hara & Co) was used to assess forelimb grip strength, when mice were pulled back. The rotarod test was performed by placing a mouse on a rotating drum (UGO Basile Accelerating Rotarod), and the time to maintain its balance on the rod was measured. The speed of the rotarod was accelerated from 4 to 40 rpm over a 5-minute period.

Table. General Physical Characteristics and Sensory/Motor Functions of BCAS Mice and Sham-Operated Mice

	BCAS	Sham	<i>P</i> Value
Physical characteristics			
Age, wk	14.1	14.1	
Whiskers, % with	100	100	
Fur, % with normal fur	100	100	
Rectal temperature, °C	36.9 (±0.1)	37.0 (±0.1)	$F_{1,22}=0.495, P=0.4893$
Sensory motor reflex			
Ear twitch, % with quick response	100	100	
Whisker twitch, % with normal response	100	100	
Righting reflex, % with normal response	100	100	
Acoustic startle response, arbitrary unit			
Stimulus intensity=110 dB	1.2 (±0.1)	1.3 (±0.2)	$F_{1,21}=0.042, P=0.8393$
Stimulus intensity=120 dB	1.8 (±0.2)	1.9 (±0.3)	$F_{1,21}=0.094, P=0.762$
Prepulse inhibition (%; stimulus=110 dB)			
Prepulse intensity=74 dB	42.2 (±8.0)	22.3 (±6.7)	$F_{1,21}=3.566, P=0.0729$
Prepulse intensity=78 dB	61.7 (±6.0)	48.9 (±4.0)	$F_{1,21}=3.026, P=0.0966$
Prepulse inhibition (%; stimulus=120 dB)			
Prepulse intensity=74 dB	31.1 (±10.4)	23.6 (±7.0)	$F_{1,21}=0.342, P=0.5651$
Prepulse intensity=78 dB	50.7 (±7.6)	44.2 (±5.6)	$F_{1,21}=0.465, P=0.5027$
Pain test			
Hot plate test (latency; s)	7.7 (±0.6)	7.6 (±0.6)	$F_{1,22}=0.003, P=0.9548$
Motor test			
Wire hang (latency to fall; s)	51.1 (±4.0)	60.0 (±0.0)	$F_{1,22}=4.108, P=0.055$
Grip strength (N)	0.83 (±0.04)	0.81 (±0.04)	$F_{1,22}=0.266, P=0.611$
Rotarod (latency to fall; s; average of 3 trials)			
Day 1	137 (±13)	167 (±14)	$F_{1,22}=2.254, P=0.1475$
Day 2	201 (±17)	226 (±13)	$F_{1,22}=1.274, P=0.2712$

Data represent the mean (±SEM; BCAS, n=13; sham, n=11).

Open Field Test

Locomotor activity was measured using an open field test. Each subject was placed in the center of the open field apparatus (40×40×30 cm; Accuscan Instruments). Total distance traveled (in cm), vertical activity (rearing measured by counting the number of photobeam interruptions), time spent in the center, and the beam-break counts for stereotyped behavior (stereotypic counts) were recorded. Data were collected for 120 minutes.

Light/Dark Transition Test

The apparatus used for the light/dark transition test consisted of a cage (21×42×25 cm) divided into 2 chambers, one of which was brightly illuminated (390 lux), and the other was dark (2 lux). Mice were placed into the dark side and allowed to move freely for 10 minutes. The total number of transitions, time spent in each side, first latency to light side, and distance traveled were recorded by Image LD software (see 'Image Analysis').

Porsolt Forced Swim Test

In the Porsolt forced swim test, the apparatus consisted of 4 plexiglass cylinders (20 cm height × 10 cm diameter). The cylinders were filled with water (23°C), up to a height of 7.5 cm. Mice were placed into the cylinders, and their behavior was recorded over a 10-minute test period (day 1, 2). Data acquisition and analysis were performed automatically, using Image PS software (see 'Image Analysis').

Contextual and Cued Fear Conditioning

Each mouse was placed in a test chamber (26×34×33 cm; O'hara & Co) and allowed to explore freely for 2 minutes. A 55-dB white

noise, which served as the conditioned stimulus, was presented for 30 s, followed by a mild (2 s, 0.3 mA) footshock, which served as the unconditioned stimulus. Two more conditioned stimulus–unconditioned stimulus pairings were presented with 2-minute interstimulus interval. Context testing was conducted 24 hours after conditioning in the same chamber. Cued testing with altered context was conducted 24 hours after conditioning using a triangular box (35×35×41 cm), which was located in a different room.

Data acquisition, control of stimuli, and data analysis were performed automatically, using Image FZ software (see 'Image Analysis'). Images were captured at 1 frame per second. For each pair of successive frames, the amount of area (pixels) by which the mouse moved was measured. When this area was below a certain threshold (ie, 20 pixels), the behavior was judged as 'freezing'. The optimal threshold (amount of pixels) to judge freezing was determined by adjusting it to the amount of freezing measured by human observation.

Eight-Arm Radial Maze Test

The 8-arm radial maze test was conducted as described previously.¹⁷ Each arm (9×40 cm) radiated from an octagonal central starting platform. Identical food wells with pellet sensors were placed at the distal end of each arm. As the initial pretraining, 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. Subsequently, these 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 8 times, using 8 different arms, for each mouse.

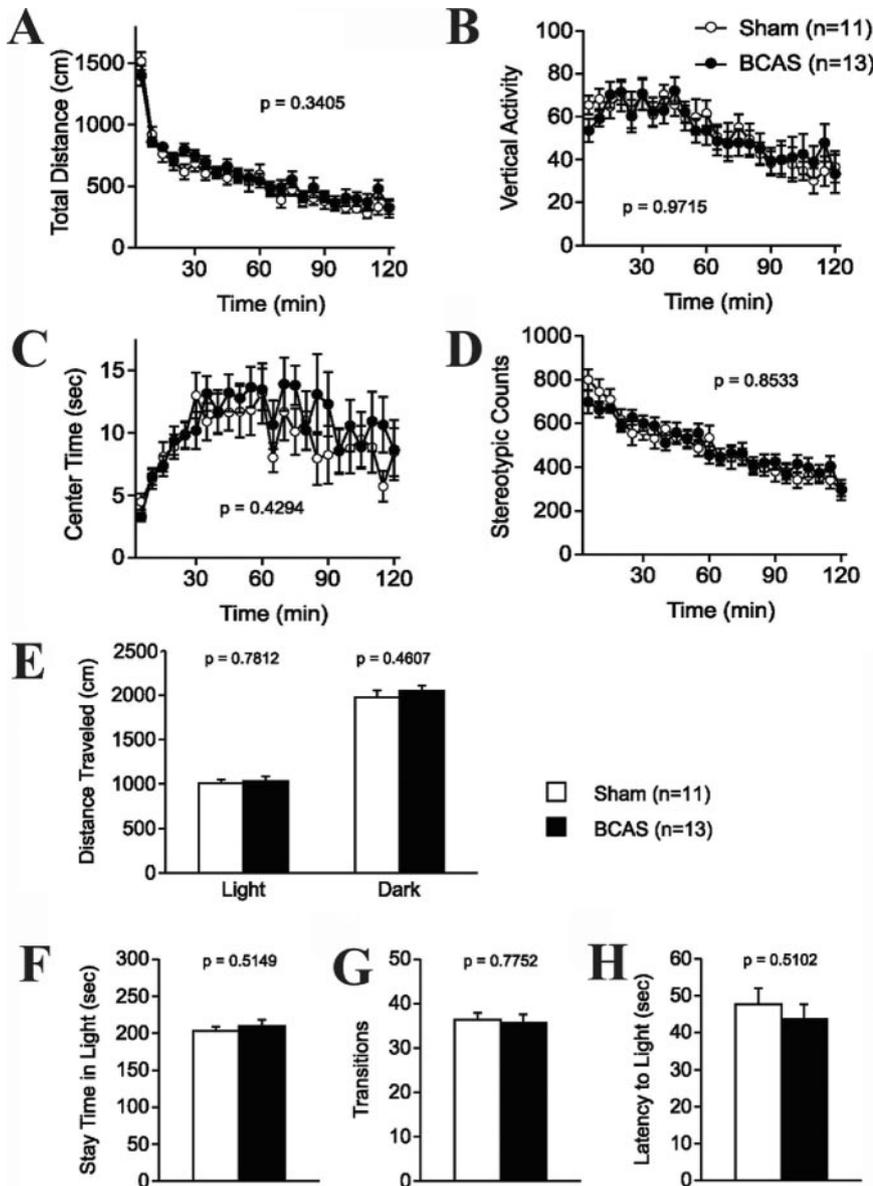


Figure 2. Locomotor activity of the control and BCAS mice in an open field test (A through D). No differences were observed for total distance traveled (group effect, $F_{1,22}=0.949$, $P=0.3405$; A), vertical activity (group effect, $F_{1,22}=0.001$, $P=0.9715$; B), time spent in the center area (group effect, $F_{1,22}=0.648$, $P=0.4294$; C), and the number of stereotypic counts (group effect, $F_{1,22}=0.035$, $P=0.8533$; D). Locomotor activity in the light/dark transition test (E through H). No difference was observed for distance traveled in light (group effect, $F_{1,22}=0.079$, $P=0.7812$) and dark chamber (group effect, $F_{1,22}=0.564$, $P=0.4607$; E), time spent in light (group effect, $F_{1,22}=0.438$, $P=0.5149$; F), number of transitions between the light and dark sides (group effect, $F_{1,22}=0.084$, $P=0.7752$; G), and latency to enter the light side ($F_{1,22}=0.448$, $P=0.5102$; H).

In spatial working memory task of the 8-arm radial maze, all 8 arms were baited with food pellets. Mice were placed on the central platform and allowed to get all 8 pellets within 25 minutes. A trial was terminated immediately after all 8 pellets were consumed or 25 minutes had elapsed. For each trial, choices of arms, latency to get all pellets, distance traveled, number of different arms chosen within the first 8 choices, and the number of revisiting, and omission errors were automatically recorded.

In reference memory task of the 8-arm radial maze, one of the 8 arms was constantly baited by one pellet in a food well and a trial was terminated immediately after one pellet was consumed. Data acquisition, control of guillotine doors, and data analysis were performed by Image RM software (see 'Image Analysis').

Image Analysis and Statistical Analysis

The applications used for the behavioral studies (Image LD, Image OF, Image PS, Image RM, and Image FZ) were based on the public domain National Institutes of Health's Image program.¹⁸ Statistical analysis was conducted using StatView (SAS Institute). Data were analyzed by 2-way ANOVA, or 2-way repeated measures ANOVA, unless noted otherwise. Values in the table and graphs were expressed as mean±SEM.

Results

Histological Findings

All of the mice regained consciousness within a few hours after the operation, but occasionally showed transient ptosis. None of them showed any apparent motor weakness. The CBF values (ratio to the preoperative value) was not changed significantly in the sham-operated mice, but decreased to $72.4 \pm 17.3\%$ at day 1, $77.3 \pm 15.3\%$ at day 7 and $83.4 \pm 13.6\%$ at day 30 after BCAS. The blood pressure measured either at day 1, 7 and 30 days was not different between the BCAS and sham-operated mice. The staining intensity of the myelinated fibers was reduced, and the integrity of the myelin was compromised in the corpus callosum (Figure 1E and 1F), caudoputamen, internal capsule and optic tract, as reported previously.¹¹ The remaining fibers were disorganized and vacuoles were frequently observed in the neuropil. Atrophy was not found in the optic nerve (photo not shown), although being rarefied slightly. Of the brains examined, there were no

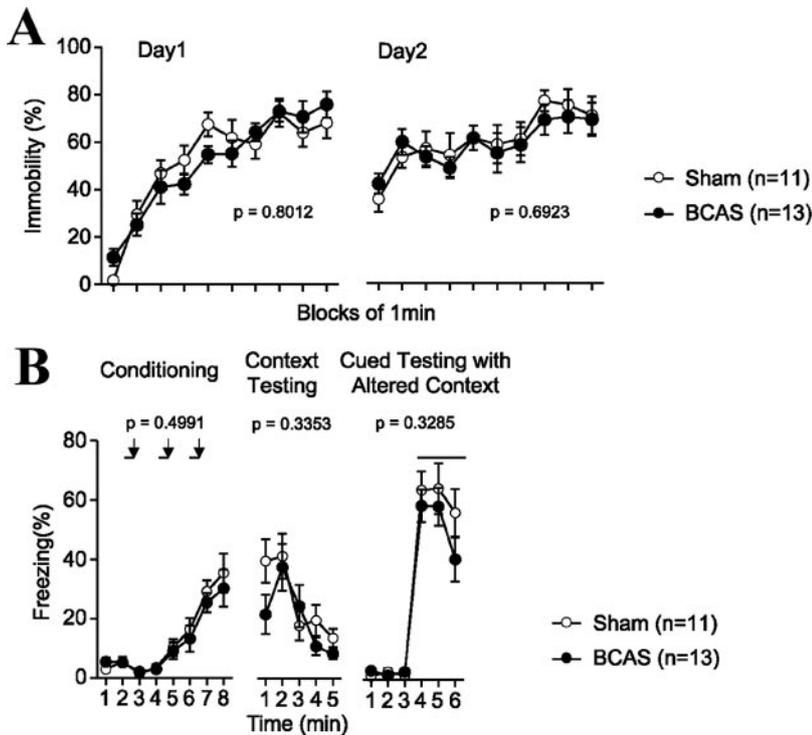


Figure 3. Behavioral despair of mice in the Porsolt forced swim test (A). Mice traveled at day 1 and day 2 (for day1, group effect, $F_{1,22}=0.065$, $P=0.8012$; for day 2, $F_{1,22}=0.161$, $P=0.6923$). Data are given as means (\pm SEM). Percentage of freezing during conditioning, context testing and cued testing with altered context in control and BCAS mice (B). Data are given as means (\pm SEM). In the contextual and cued fear conditioning test, there was no significant difference in freezing during conditioning (group effect, $F_{1,22}=0.472$, $P=0.4991$), context testing (group effect, $F_{1,22}=0.970$, $P=0.3353$), and cued testing with altered context (group effect, $F_{1,22}=0.998$, $P=0.3285$) between the groups.

infarctions or hemorrhage in any gray matter regions including the cerebral cortex, caudoputamen and hippocampus (Figure 1A through 1D, 1G, and 1H). There were only a few TUNEL positive cells in the corpus callosum (Figure 1, insets in 1E and 1F), but not in the hippocampus.

The hemispheric area was 20.4 ± 2.7 mm² (mean \pm SEM) for BCAS mice and 20.9 ± 1.4 mm² for sham-operated mice. The ventricular space area was 0.51 ± 0.2 mm² for BCAS mice and 0.53 ± 0.31 mm² for sham-operated mice. For both areas, there were no significant differences between the groups.

Physical Characteristics, Sensory Motor Reflexes, Nociception, Motor Coordination

As shown in the Table, there were no significant differences between BCAS and sham-operated mice in terms of their physical characteristics. Body weight in BCAS and sham-operated mice were 25.7 ± 0.2 and 25.3 ± 0.2 , respectively, before operation, 23.8 ± 0.2 and 24.1 ± 0.2 after 3 days, and 27.4 ± 0.3 and 27.0 ± 0.4 after 30 days indicating no significant differences between the groups.

There were also no differences in sensory-motor reflexes (percent with quick response of ear twitch, normal response of whisker twitch and righting reflex, acoustic startle response), sensory-motor gating (prepulse inhibition), nociception (hot plate test), and motor coordination (wire hang and rotarod tests).

Locomotor Activity

There were also no differences between BCAS and sham-operated mice for total distance traveled, vertical activity, time spent in the center area, and the number of stereotypic counts in an open field test (Figure 2A through 2D). In the light /dark transition test (Figure 2E through 2H), there was also no differences between the groups for distance traveled

in light and dark chamber, time spent in light, number of transitions between the light and dark sides and latency to enter the light side.

Porsolt Forced Swim Test, and Contextual and Cued Fear Conditioning Test

The ratio of immobility was not different between the groups both at day 1 and day 2 in Porsolt forced swim test (Figure 3A). In the contextual and cued fear conditioning test, the freezing levels during the conditioning period ($P=0.4991$), context testing ($P=0.3353$), and the cued testing with altered context ($P=0.3285$) did not differ between the groups (Figure 3B).

Learning Test

In the working memory task of the 8-arm radial maze, sham-operated mice improved their performance over training, whereas BCAS mice did not and made significantly more errors than the sham-operated control ($P < 0.0001$; 2-way repeated measures ANOVA; Figure 4A). The number of different arm choices in the first 8 entries is another measure of working memory performance. The number ranged from 5.3 for a chance performance to 8 for a perfect performance. However, sham-operated mice improved significantly more than BCAS mice with each consecutive training session ($P=0.0002$, 2-way repeated measures ANOVA; Figure 4B).

On the other hand, there were no significant differences between the groups in the reference memory task of the 8-arm radial maze ($F_{1,20}=2.878$, $P=0.1053$, 2-way repeated measures ANOVA; Figure 4C). After 8-arm radial maze test, the same animals were subjected to the open field test (Figure 4D through 4G). The activity level of BCAS mice was similar to the sham-operated mice (total distance; $F_{1,19}=0.050$,

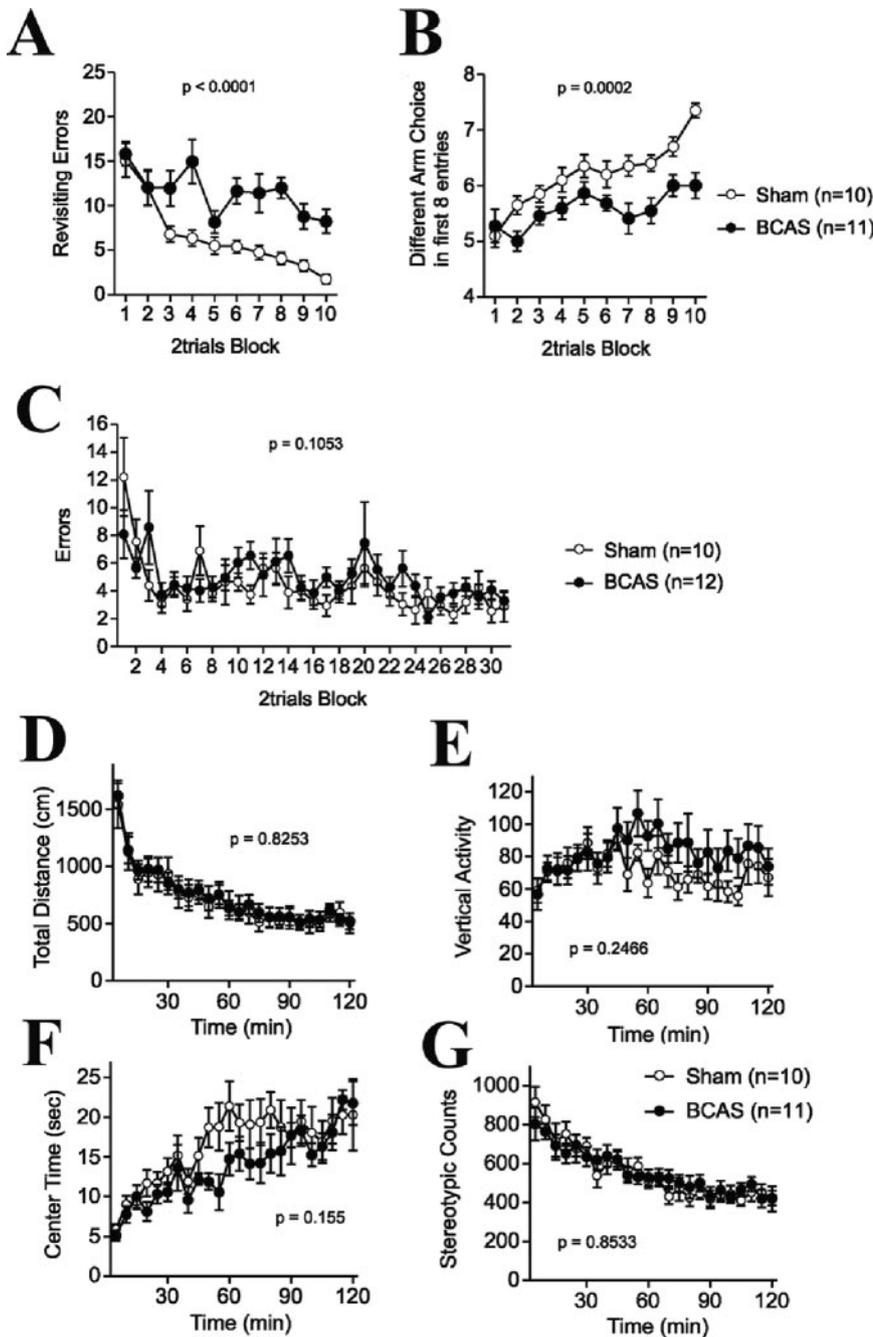


Figure 4. Working memory (A and B) and reference memory (C) of the control and BCAS mice in an 8-arm radial maze test. Data are given as means (\pm SEM) for revisiting errors (A), and different arm choices (B) in working memory task, and errors in reference memory task (C). BCAS mice showed a lesser number of different arm choices (group effect, $F_{1,19}=20.519$, $P=0.0002$; A), and more revisiting errors (group effect, $F_{1,19}=43.267$, $P<0.0001$; B) as compared with the control mice. There was no significant difference in the number of errors in reference memory task between the groups (group effect, $F_{1,20}=2.878$, $P=0.1053$; C). Locomotor activity of the control and BCAS mice in the open field test (D through G). No difference was observed for total distance (group effect, $F_{1,19}=0.050$, $P=0.8253$; D), vertical activity (group effect, $F_{1,19}=0.693$, $P=0.4155$; E), time spent in the center area (group effect, $F_{1,19}=2.194$, $P=0.155$; F), and the number of stereotypic counts (group effect, $F_{1,19}=0.002$, $P=0.9654$; G).

$P=0.8253$, 2-way repeated measures ANOVA). Thus, spatial reference memory required to correctly perform the reference memory task in the 8-arm radial maze does not appear to be affected in the BCAS mice.

Discussion

In the rat model of chronic cerebral hypoperfusion, although we did not find any changes in the hippocampus,⁵ previous studies have reported hippocampal CA1 damages.^{12,13} Hippocampal damages may cause impairment of both reference and working memory, and therefore make it difficult to determine whether the cognitive impairment was a consequence of WM lesions. Variability in hippocampal damage in the rat model may be caused by a relatively severe reduction

of the CBF (30% to 50% of the preoperative values), in which low variability around the threshold may determine the occurrence of hippocampal changes.

In the current mouse model, if the degree of chronic cerebral hypoperfusion is appropriately controlled by changing the internal diameter of the microcoils, the decrease in CBF can be milder to selectively affect the cerebral WM. Using microcoils with a diameter of 0.16 mm, the CBF was decreased to $51.4 \pm 11.5\%$ of the preoperative values with the resultant hippocampal CA1 damage.¹¹ However, using microcoils with a diameter of 0.18 mm or 0.20 mm, the CBF was decreased to $67.3 \pm 18.5\%$ and $77.3 \pm 13.4\%$, respectively, and the histological damages including activation of microglia and astroglia were restricted to the WM.¹¹

The BCAS mice showed no difference to the controls in the comprehensive behavioral tests, including a complete neurological screen, prepulse inhibition, hot plate, open field, light/dark transition, Porsolt forced swim and contextual and cued fear conditioning. Thus, this screen showed that these mice have no deficits in physical characteristics and sensory/motor functions. In addition, BCAS mice showed normal spatial reference memory in the 8-arm radial maze test. Spatial reference memory task was related to cognitive domains thought to rely on the integrity of the hippocampus, and therefore preserved reference memory is in agreement with lack of histological damage in the hippocampus. In contrast, working memory impairment may be attributable to either frontal WM lesions or hippocampal damages which are undetectable by the present methods. In previous studies, working memory deficits have been related either to the hippocampus or frontal-subcortical circuits in the rodent^{14,19,20} and likely primates.^{21,22} Therefore, the disruption of WM tracts especially within the prefrontal cortex may be another mechanism for age-related changes in working memory function.²³

We successfully developed a mouse model of chronic cerebral hypoperfusion, which showed cognitive abnormalities with only a mild damage to the visual system. In the rat model, working memory and gait performances have been shown to be impaired.^{12,13,24} However, the rat model exhibits severe degeneration and atrophy of the optic nerve.^{5,14,25,26} The possibility cannot be ruled out that the visual system impairment may compromise the behavioral test, because visual cues contribute to discrimination even in the rodent.²⁷

In this setting, the rat model is suitable for pharmacological evaluation because of prompt emergence of WM changes and easy applicability of stereotaxic surgery. In contrast, the mouse, which is readily amenable to gene knockout and manipulation and has advantages in cognitive evaluation, can be a model of subcortical vascular dementia suited for pathogenetic analysis and behavioral assessment.

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Disclosures

None.

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Masunari Shibata, Nobuyuki Yamasaki, Tsuyoshi Miyakawa, Rajesh N. Kalaria, Youshi Fujita, Ryo Ohtani, Masafumi Ihara, Ryosuke Takahashi and Hidekazu Tomimoto

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