Myelin Loss Associated With Neuroinflammation in Hypertensive Rats

Fakhreya Y. Jalal, PhD; Yi Yang, PhD, MD; Jeffrey Thompson, BS; Anita C. Lopez, MS; Gary A. Rosenberg, MD

Background and Purpose—Small vessel disease is the major cause of white matter injury in patients with vascular cognitive impairment. Matrix metalloproteinase (MMP)-mediated inflammation may be involved in the white matter damage with oligodendrocyte (Ol) death. Therefore, we used spontaneously hypertensive stroke-prone rats to study the role of neuroinflammation in white matter damage.

Methods—Permanent unilateral carotid artery occlusion was performed at 12 weeks of age in spontaneously hypertensive stroke-prone rats. Following surgery, rats were placed on a Japanese permissive diet and received 1% NaCl in drinking water. MRI, histology, biochemistry, and ELISA characterized white matter lesions, and cognitive impairment was tested by Morris water maze.

Results—White matter damage was observed 4 to 5 weeks following permanent unilateral carotid artery occlusion/Japanese permissive diet. Immunoblotting showed marked reduction in myelin basic protein and upregulation of immature Ols. Mature Ols underwent caspase-3-mediated apoptosis. Morris water maze showed cognitive impairment. Abnormally appearing vessels were observed and surrounded by inflammatory-like cells. IgG extravasation and hemorrhage, indicating blood-brain barrier (BBB) disruption, was closely associated with MMP-9 expression. Lesions in white matter showed reactive astrocytosis and activated microglia that expressed tumor necrosis factor-α. MMP-3 and MMP-9 were significantly increased, and MMP-2 was reduced in both astrocytes and Ol.

Conclusions—We found apoptosis of mature Ols with an increase in immature Ols. Increased MMP-3, MMP-9, and tumor necrosis factor-α were associated with myelin breakdown and BBB disruption. Neuroinflammation is an important factor in white matter damage and Ol death, and studies using this new model can be performed to assess agents to block inflammation. (Stroke. 2012;43:1115-1122.)

Key Words: white matter ▪ vascular cognitive impairment ▪ matrix metalloproteinases

Vascular cognitive impairment is a heterogeneous disease caused by large and small vessel disease.1 White matter injury is commonly seen in small vessel disease, with arteriolosclerosis secondary to hypertension and diabetes.2 As the population increases in age, the incidence of vascular causes of dementia is projected to rise, creating a need for animal models to elucidate the pathophysiology of white matter damage and to test treatments.3 Glutamate-mediated excitotoxicity, inflammatory cytokines, and protease activation are thought to cause oligodendrocyte (Ol) death in acute ischemic injury, but little is known about the mechanisms of white matter injury in chronic vascular disease.4 Damage to small vessels causes an inflammatory response with disruption of blood-brain barrier (BBB) and subsequent accumulation of fluid and macromolecules in white matter.5 In animal models of hypoxic hypoperfusion, damage to the white matter is associated with disruption of the BBB, breakdown of myelin basic protein (MBP), and Ol cell death.6

Matrix metalloproteinases (MMPs) and tumor necrosis factor-α (TNF-α), which are increased in neuroinflammation, are implicated in MBP breakdown and Ol death.7,8 Brain tissues from vascular dementia patients show expression of MMPs in the regions of white matter damage.9 Vascular cognitive impairment patients have disruption of the BBB suggestive of an inflammatory response.10,11 Studies of cerebrospinal fluid show elevated MMPs.12 In acute ischemia, there is early elevation of MMP-2.13 Hypoxia-induced MMP-9 expression leads to vascular leakage.14 Knockout of MMP-9 reduces BBB leakage in acute ischemia.15 TNF-α production by microglia has been demonstrated to participate in Ol death in chronic cerebral hypoperfusion.16

Several animal models are used to study vascular cognitive impairment. Most commonly, bilateral carotid artery occlus-
sion is performed in young normotensive rats, producing hypoxic hypoperfusion. Given that vascular cognitive impairment patients with white matter damage generally are older individuals with vascular disease secondary to hypertension and/or diabetes mellitus, a more appropriate animal model is an older spontaneously hypertensive stroke-prone rat (SHR-SP). These animals develop extensive white matter damage with gliosis, apoptosis of Ols, and BBB damage, but the role of neuroinflammation has not been determined. We hypothesized that white matter injury is a neuroinflammatory process associated with expression of MMPs and cytokines. To study the role of inflammation in vascular cognitive impairment in an animal model, we used a 12-week-old SHR-SP with a unilateral carotid artery occlusion (UCAO) and the Japanese permissive diet (JPD) with added salt in the water. We used MRI to monitor white matter injury and histological/biochemical studies to identify BBB opening, myelin loss, and Ol death and expression of neuroinflammatory proteases and cytokines. A Morris water maze (MWM) was used to test for cognitive impairment.

Methods

A detailed description of methods is found in the online-only Supplemental Methods (http://stroke.ahajournals.org).

Animal Groups and Surgery

All protocols followed the guidelines of the Institutional Animal Care and Use Committee at the University of New Mexico. SHR-SP rats were divided into 2 groups: UCAO/JPD and sham-operated. UCAO, as described in the online-only Supplemental Methods, was performed on 12-week-old male SHR-SP (Charles River Laboratories). Following UCAO, rats were placed on a JPD (18.7% protein, 0.63% potassium, 0.37% sodium; Ziegler Bros, Inc) with 1% sodium chloride added to drinking water for 4 or 5 weeks (end point was determined based on the appearance of neurological symptoms and white matter lesions on T2-weighted MRI). In the sham-operated group, the right carotid artery was isolated, and rats were fed with regular rodent diet with tap water following this procedure.

Physiological Parameters

Body weight and systolic blood pressure were recorded weekly. Plasma pH, PO2, PCO2, glucose, K+, and Na+ levels were also determined.

Histological Analysis

Klüver-Barrera and hematoxylin eosin staining followed standard protocols. For immunohistochemistry, brain sections were stained using anti-MMP-2, anti-MMP-3, anti-MMP-9, anti-glial fibrillary acidic protein, anti-cleaved caspase-3, anti-adenomatous polyposis coli (APC) (CC1), anti-TNF-α, anti-Iba-1, and anti-Cy-3-conjugated affinity pure goat anti-rat IgG antibodies.

Magnetic Resonance Imaging (MRI)

White matter lesions were evaluated using MRI (4.7-T small animal scanner; Bruker BioSpin) weekly following the UCAO/JPD. Detailed descriptions of the method were reported previously.19

Immunoblotting


Evaluation of BBB Permeability by IgG Leakage

We used an ELISA kit (GenWay Biotech, Inc) to measure the levels of brain tissue IgG.

Morris Water Maze

Three weeks following UCAO/JPD, cognitive function was assessed as reported previously.20

Statistical Analysis

Data are expressed as mean±SEM. Statistical significance was set at P<0.05. Data were analyzed by 2-way ANOVA followed by Bonferroni modified t test analysis, and unpaired Student t tests, using Prism 5.0 (GraphPad Software, Inc).

Results

Baseline body weight was similar between UCAO/JPD and sham-operated groups. The UCAO/JPD group increased in body weight during the first week, but had a significant weight loss during weeks 3, 4, and 5 (P<0.01). The sham-operated group gained in body weight throughout the course of the study (online-only Supplemental Figure S1A).

Systolic blood pressure gradually increased in SHR-SP rats from 7 to 12 weeks of age, and continued to increase for 4 weeks post UCAO/JPD; it was significantly different on weeks 3 and 4 compared with the sham group (P<0.001, P<0.01, respectively; online-only Supplemental Figure S1B).

Blood chemistry parameters were not significantly different between groups (online-only Supplemental Table).

Following 4 to 5 weeks of UCAO/JPD, there was a gradual increase in the number of rats developing neurological deficits including lethargy, absence of exploration, gait deficit, hemiparesis, and abnormal circling. T2-weighted images displayed hyperintense areas in the white matter and hippocampus in both hemispheres, and unilaterally in the cortex. Sham-operated rats showed no T2 hyperintensities (Figure 1A).

Myelin loss, using Klüver-Barrera staining, was observed in the external capsule, corpus callosum, and internal capsule of both occluded and nonoccluded sides 4 to 5 weeks following UCAO/JPD (Figure 1B). Most of the myelin loss occurred in a caudal portion of the brain (approximately −2 to −6 mm relative to Bregma). This damage was characterized by increased vacuolation and rarefaction of myelin fibers. No white matter damage was seen in the sham-operated group in either hemisphere. Western blot demonstrated that MBP was significantly decreased in the occluded side of external capsule and corpus callosum, and in the nonoccluded hemisphere in external capsule, corpus callosum, and internal capsule compared with corresponding controls (P<0.05; Figure 1C, D). Immunoblotting with GalC showed immature OL increases in all 3 areas of white matter on the nonoccluded side, and in corpus callosum and internal capsule of the occluded side (Figure 1C, D).

In MWM, rats that received UCAO/JPD demonstrated significantly higher escape latencies during the acquisition trials than did the sham-operated group on days 3 and 4 (P<0.01; Figure 2A).

In the probe trial, in which the platform was removed, rats were required to recall the location of the platform in the northwest quadrant, relying on distal cues. Rats in the sham-operated group had intact memory, evidenced by greater time spent in the northwest quadrant, whereas the UCAO/JPD group spent significantly less time, revealing memory impairments (P<0.01; Figure 2B).
Swimming speeds during 4 days of the acquisition trial were similar (Figure 2C), indicating that deficits in performance by the UCAO/JPD group were not because of poor motor function. In addition, there were no group differences in latencies to find a platform with a visual cue, indicating that visual function was normal after UCAO/JPD (Figure 2D).

White matter IgG content was observed within the area of external capsule and within the corpus callosum of the occluded side. Extravasations of IgG were also demonstrated in the regions of external capsule, corpus callosum, and internal capsule of the nonoccluded side using ELISA (Figure 3A). In addition, MMP-9 was detected in the area of IgG leakage (Figure 3B). MMP-3 was not detected (data not shown). H&E staining showed hemorrhage and perivascular infiltrates of inflammatory-like cells. There was loss of structure in the white matter surrounding the vessels (Figure 3C).

Western blot showed that MMP-2 was present in both latent and active forms and was identified in both sham-
operated and UCAO/JPD groups. MMP-2 was decreased in the external capsule on the nonoccluded side following UCAO/JPD (P<0.05), with no change detected on the occluded side (Figure 4A, B). We detected MMP-3 proform in both groups on both sides (Figure 4A, B). Western blot analysis showed a significant increase in the expression of proMMP-3 in areas of external capsule, corpus callosum, and internal capsule in occluded (P<0.05) and external capsule in nonoccluded brain hemispheres (P<0.01). ProMMP-9 was significantly greater in external capsule, corpus callosum, and internal capsule of the UCAO/JPD group on both sides when compared with the sham-operated group (Figure 4A, B). Strong colocalization of MMP-3 and MMP-9 immunoreactivity with glial fibrillary acidic protein and CC1 was observed (Figure 4C). No colocalization was observed with OX-42 (data not shown), suggesting that astrocytes and Ols are important sources of MMP-3 and MMP-9.

Confocal immunohistochemistry images exhibited many cleaved caspase-3 positive cells in the area of external capsule, colocalized with CC1, suggesting that these Ols were undergoing apoptosis (Figure 5A). Western blot analysis demonstrated increased cleaved caspase-3 in the areas of external capsule, corpus callosum, and internal capsule of both hemispheres (Figure 5B and C). The sham-operated group showed little cleaved caspase-3. Glial fibrillary acidic protein-positive cells were distributed throughout white matter and hippocampus (online-only Supplemental Figure S2A). TNF-α was increased in white matter and was colocalized with Iba-1-positive microglia/macrophages (online-only Supplemental Figure S2B).

**Discussion**

This hypertensive rat model has damage to the white matter similar pathologically to that seen in vascular cognitive impairment patients. Our results indicate that inflammation with MMPs and TNF-α expression is associated with astrogliosis and death of mature Ols. At 12 weeks of age, the SHR-SP were fed the JPD with salt in the drinking water and had a unilateral carotid occlusion. After 4 to 5 weeks, we found death of mature Ols, proliferation of immature Ols,
breakdown of MBP, and opening of the BBB that was associated with an increase in MMPs. Memory impairment was demonstrated by MWM by week 3 following UCAO/JPD. Mature Ols expressed cleaved caspase-3, suggesting apoptosis. We observed an inflammatory response after UCAO/JPD that resulted in the expression of TNF-α in microglia and in increased expression of MMP-3 and MMP-9 in reactive astrocytes and Ols. MMP-9 colocalized with regions where the BBB was disrupted, as shown by IgG extravasation. These results support the hypothesis that inflammation is involved in white matter damage.

Although only 1 carotid was ligated, MRI showed white matter lesions in both hemispheres. A prior study in young, normotensive rats used bilateral occlusion of the carotids to induce white matter damage. When we attempted to occlude both carotids in the 12-week-old SHR-SP, death occurred within 24 hours (data not shown). However, with occlusion of only 1 side, not only did the rats survive, but the injury pattern was apparent in both hemispheres, providing a model pathologically consistent with vascular cognitive impairment. Damage to the nonoccluded hemisphere was greater than that seen in the occluded hemisphere. A previous study demonstrated that wall-to-lumen ratios of arteries from chronically hypertensive rats revert to those of normotensive rats when the vessels are ligated. Additional study will be needed to clarify the mechanism of apparent protection on the occluded side.

Extensive myelin disruption with marked decrease in MBP was seen by 4 weeks after the start of the diet and unilateral carotid occlusion. Ols showed increased expression of cleaved caspase-3 that colocalized with CC1. Mature Ols were targeted for cell death as revealed by the loss of MBP, whereas immature Ols increased in number as shown by GalC. The damage to the myelin could have been caused by a direct attack by the MMPs or loss of Ols leading to breakdown of the myelin sheath. MMP-3 was increased in cells close to the damaged myelin, but not in the vicinity of blood vessels. Our recent study supports the role of MMP-3 and MMP-9 in Ol death following cerebral ischemia. MMP-2, including pro- and active forms, was decreased, suggesting that MMP-2 may have been consumed. Thus, whereas MMP-3 and/or MMP-9 are most likely involved in the damage, MMP-2 may have contributed as well.

Our finding of extensive white matter damage in the SHR-SP with UCAO and JPD resemble the findings in pathological studies in vascular cognitive impairment patients with small
vessel disease. The SHR-SP were studied between age 12 and 16 weeks, making them a more relevant model for the human disease, which is seen mainly in older patients. Studies in human tissues show disruption of the BBB with expression of MMP-2 in reactive astrocytes and MMP-3 in pericytes. MMP-3 and MMP-9 are increased in cerebrospinal fluid of patients with vascular cognitive impairment.

Other injury pathways have been implicated in apoptotic death of Ols, such as oxidative stress, inflammation, or glutamate toxicity. Previous studies demonstrated independent tim-

Figure 4. Effect of UCAO/JPD on MMPs in white matter areas. A, Immunoblots of MMP-2, MMP-3, and MMP-9 from white matter extract of UCAO/JPD and sham-operated (S) groups in regions of external capsule (EC), corpus callosum (CC), and internal capsule (IC) from occluded (right; R) and nonoccluded (left; L) sides. Actin was used as loading control (lower panel). B, Quantification of Western blot analysis for MMP-2, MMP-3, and MMP-9. C, Z-stack showing colocalization of astrocytes (glial fibrillary acidic protein) or Ols (CC1) and MMP-2, MMP-3, or MMP-9. Scale bar=20 μm. *P<0.05, **P<0.01, ***P<0.001 versus corresponding sham-operated group (n=5/group).
In demyelinating transgenic mice, MMP-3 triggered neuroinflammation with microglial induction of TNF-α, and MMP-3 upregulation preceded the onset of disease. Release of MMP-3 from apoptotic neurons activated microglia and induced inflammatory cytokines, suggesting that MMP-3 may be a signaling molecule in neuronal apoptosis. TNF-α and interleukin-1β produced a significant increase in the production of MMP-3 and MMP-9 in cultured astrocytes and microglia. Our data demonstrate that an increase in the expression of MMP-3 and MMP-9 parallel increased microglial reactivity, TNF-α expression, and eventual apoptotic death of Ols.

Hypertensive rats exhibiting white matter damage showed memory deficits in the MWM test by the third week after the UCAO/JPD, which was shown to be unrelated to either visual or motor deficits. In addition, microglial activation that has been observed in the white matter in the present study may be involved in cognitive impairment. Given that the hippocampus was involved in the injury, we cannot exclude a contribution to the memory loss from hippocampal damage, which we plan to explore in future studies.

In conclusion, we have described a model of white matter damage in SHR-SP that has similarities to the hypertensive, small vessel form of vascular cognitive impairment seen in older patients. Our data show an inflammatory response, involving expression of MMPs and TNF-α with BBB opening and demyelination. This animal model combines the major features of small vessel vascular cognitive impairment and it exhibits not only myelin and Ol loss, but also cognitive...
improvement. The expression of MMPs and TNF-α in the regions of inflammatory damage to the BBB and OIs is consistent with earlier studies, but it will have to be confirmed by blocking their action with inhibitors of MMPs. Because of its unique similarities to vascular cognitive impairment, this is a potential model for the study of agents to reduce the inflammatory response and slow progression of white matter damage.

Sources of Funding
This work was supported by grants from the National Institutes of Health (2 R01 NS045847-05A2).

Disclosures
None.

References
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Stroke. 2012;43:1115-1122; originally published online February 23, 2012;
doi: 10.1161/STROKEAHA.111.643080
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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World Wide Web at:
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Supplemental Methods:

1-Unilateral Common Carotid Artery Occlusion in SHR-SP

Rats were deeply anesthetized with 2.0 % isoflurane. A heating pad was used to maintain body temperature at 37 ± 0.05 °C. Under a surgical microscope, a ventral midline incision was made to expose the right carotid artery. After isolation, the right carotid artery was double-ligated permanently with 4-0 silk sutures.

2-Body Weight and Systolic blood pressure (SBP) measurement

The body weight of both experimental and sham operated rats was measured before UCAO/JPD. Weekly measures were made for 4 weeks following UCAO/JPD for both sham and experimental groups.

SBP was measured for UCAO/JPD and its sham-operated group by tail-cuff non-invasive blood pressure measurements using the CODA system (Kent Scientific Inc.). SBP was measured starting week 7 and through to 16 weeks of age in awake rats that were habituated to this procedure. For every data point, seven measurements were performed and mean values were calculated.

3-Blood collection for the measurement of blood parameters

During a brief 2 % isoflurane induced anesthesia, venous blood was withdrawn from the orbital sinus of rats using heparinized capillary tubes one week before UCAO/JPD, and then every week for 4 weeks. Eye lubricant was applied to prevent corneal ulceration. At each time
point the following parameters in blood samples were measured: pH, pCO$_2$, pO$_2$, glucose concentration (Glu), and concentration of electrolytes: K$^+$, and Na$^+$ (iSTAT CG8 + Cartridge and the iSTAT analyzer, Abaxis and Heska Corporation).

4-Tissue preparation for Immunohistochemistry and immunoblotting

Rats were anesthetized with pentobarbital (50 mg/kg, i.p) and transcardially perfused with 0.1 % procaine in cold phosphate buffer followed by 2 % PLP (2 % paraformaldehyde, 0.1 M sodium periodate, 75 mM lysine in 100 mM sodium phosphate buffer, pH 7.4). Brains were removed and post-fixed in 2 % PLP for 24 hours at 4 °C. For cryosectioning, tissues were cryoprotected in 30 % sucrose/2 % PLP at 4 °C, and placed in a Peel-A-Way histology mold (Ted Pella Inc.) containing Tissue-Tek embedding medium (OCT; Sakura Finetek). Tissue was frozen in 2-methylbutane cooled in liquid nitrogen. Brains were kept at – 80 °C until use. Brain tissues were sectioned at 10 µm prior to histological analysis. For immunoblotting, rats were perfused with ice cold saline, brains were removed quickly, frozen in – 80 °C 2-methylbutane, then kept at – 80 °C until use.

5-Immunohistochemistry (IHC)

Brain sections were dried for 20 min followed by incubation with ice cold acetone for 10 min. This was followed by two washes in tap water, and then slides were placed in PBS containing 1 % Tween 20 (PBST) for 15 min. Blocking buffer containing 5 % normal serum (Invitrogen) was used to block nonspecific binding for 30 min. After blocking, slides were incubated with primary antibodies overnight at 4 °C. After 24 hr, slides were washed three times with PBST (6 min each). Secondary antibodies, conjugated to FITC or Cy-3 (Alexafluor 488 or Alexafluor 546; Invitrogen), were applied for 90 min at room temperature. Slides then were washed in PBST and were incubated with 4′-6-diamidino-2-phenylindole (DAPI; Invitrogen) to
label cell nuclei. Primary antibodies and dilutions used for IHC were as follows: rabbit anti-MMP-2 (Chemicon; 1:200), rabbit anti-MMP-3 (Chemicon; 1:175), rabbit anti-MMP-9 (Chemicon; 1:175), mouse anti-glial fibrillary acidic protein (GFAP; 1:400; Sigma), rabbit anti-cleaved caspase-3 (Cell Signaling; 1:200), Cy-3-conjugated Affinity Pure Goat Anti-Rat IgG (Jackson Immuno Research Laboratories, Inc.; 1:50), mouse anti-TNF-α (15 μg/ml; R&D Systems), and mouse anti-APC (CC1;12 μg/ml; Calbiochem). To detect co-localization, double immunofluorescence was performed for MMP-2, MMP-3, MMP-9, with specific cell markers, including glial fibrillary acidic protein (GFAP; marker of reactive astrocytes), and CC1 (a marker of mature oligodendrocytes. In addition, cleaved caspase-3 was also co-labeled with CC1 and a marker of microglia/macrophages (Iba-1) was co-localized with TNF-α. Negative controls were incubated without the primary antibodies or with normal (nonimmune) IgGs from the host species of the primary antibodies and did not exhibit specific immunolabeling (data not shown).

6-Immunoblotting

Frozen coronal sections were cut at 400 um thickness and micropunched biopsies were collected from both brain hemispheres in white matter areas of external capsule, corpus callosum, and internal capsule at – 25°C. Tissues were homogenized in RIPA lysis buffer (50 mM Tris-HCL, pH 7.4, 150 mM NaCl,1 % NP-40, 1 % sodium Deoxycholate, and 0.5 % SDS) with protease inhibitor cocktail (10 ul/ml; Pierce) and centrifuged (12000 rpm, 10 minutes, 4°C). Protein concentration in the homogenates was determined by the Micro BCA protein Assay kit (Pierce). Equal amounts of protein (30 μg) were separated by electrophoresis on 4-20 % SDS-polyacrylamide gels (Bio-Rad Labs). Proteins were transferred to a polyvinylidene fluoride membrane (Millipore). Membranes were then blocked with 5 % nonfat dry milk in Tris-buffered saline containing 1 % Tween 20 (TBST) for 1 hour. Membranes were then incubated in mouse
monoclonal anti-GalC (Chemicon; 1:500), rabbit polyclonal anti MBP (Chemicon; 1:3000), rabbit polyclonal anti-MMP-2 (Chemicon; 1:1000), rabbit monoclonal anti-MMP-3 (Epitomics; 1:1000), rabbit monoclonal anti-MMP-9 (Epitomics; 1:1000), or rabbit polyclonal anti-active caspase-3 (Abcam; 1:1000) primary antibodies in blocking buffer overnight at 4 °C. Membranes were then washed four times for 5 min each in TBST. Membranes were incubated with horseradish peroxidase-linked secondary antibodies in blocking buffer for 1 hour, after which they were washed 3 times 15 min each in TBST, all at room temperature. Bands were visualized by using Super Signal West Pico Chemiluminescent substrate (Pierce) which was used for MBP, MMP-2, MMP-3, MMP-9, and cleaved caspase-3. For GalC, 10 % SuperSignal West Femto Substrate was added to West Pico detection reagent for a better visualization of this antibody. Equal protein loading was determined using rabbit anti-actin IgG (Sigma-Aldrich; 1:7500). Protein bands were visualized on X-ray film. Optical density of bands was quantified using ImageJ software (National Institutes of Health).

7-Evaluation of BBB Permeability by IgG Leakage

White matter samples from external capsule, corpus callosum, and internal capsule and standards were incubated in 96-micro well plate which was coated with affinity purified anti-Rat IgG antibodies. IgG present in samples or standard reacts with the anti-IgG antibodies which have been adsorbed to the surface of the well bottoms. After removal of unbound proteins by washing, anti-IgG antibodies conjugated with horseradish peroxidase (HRP), were added. Anti-IgG-HRP antibodies formed complexes with previously bound IgG. Following detection using the chromogenic substrate, 3,3′,5,5′-tetramethylbenzidine (TMB), the concentration of IgG in samples were measured by spectrophotometer at a wavelength of 450 nm and quantified relative to standards.
8-Morris Water Maze

Spatial learning and memory were tested on experiment week 3. The Morris water maze consists of a circular pool (180 cm in diameter and 60 cm high), which was virtually separated into four quadrants (NW, SW, NE, and SE) and was filled with opaque water at a temperature of 22.0 ± 1.0 °C. A plexiglas escape platform was submerged 1.5 cm below the water surface at a fixed location (North West quadrant; NWQ) in the pool. The pool was located in a room containing several extramaze location cues. In the acquisition phase, rats were given 4 trials each day for 4 consecutive days. In each trial rats were placed in the tank facing the edge at one of three random starting positions (SW, SE, or NE), with no two successive trials from the same starting position. Rats were allowed to swim until they found the platform or for 60 sec if they failed to find it. Once the rat located the platform, it was allowed to remain on it for 30 sec. During this phase rats learned to navigate to the hidden platform during each 60 sec trial session. If a rat failed to reach the platform it was guided to platform and was allowed to remain on it for 30 sec. The time each rat took to locate the platform, and the average swim speed were recorded and analyzed with a video camera and ANY-maze (Version 4.70) computerized software from San Diego Instruments. Twenty four hours after the final trial a probe trial was performed, in which the platform was removed and the rat was allowed to swim for 60 sec in the pool. The time the rat spent in the NWQ, former location of the platform, was recorded. Following the probe trial a cue test was performed to determine whether the animals had any vision impairments. In this test the platform was raised, a colored ball was placed on it, and the time the rat took to reach the platform was recorded for 60 sec.
Images for Klüver-Barrera, H & E, microglia/TNF-α, astrocytes, IgG/MMP-9 were acquired using Olympus BX-51 bright field and fluorescence microscope (Olympus America Inc.) equipped with an Optronics digital camera and Picture Frame image capture software (Optronics). Confocal images were obtained using a laser scanning confocal microscope (Zeiss LSM 510, Carl Zeiss Microimaging) to confirm co-labeling.
**Supplementary Table 1.** Physiological parameters in both sham-operated and UCAO/JPD groups.

<table>
<thead>
<tr>
<th>Animal Age</th>
<th>Group</th>
<th>Glu (mg/dL)</th>
<th>Na⁺ (mmol/L)</th>
<th>K⁺ (mmol/L)</th>
<th>pH</th>
<th>pCO₂ (mmHg)</th>
<th>pO₂ (mmHg)</th>
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<tr>
<td>12 weeks</td>
<td>Baseline n=12</td>
<td>159.7 ± 6.96</td>
<td>141.2 ± 0.49</td>
<td>3.97 ± 0.14</td>
<td>7.44 ± 0.01</td>
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<td>13 weeks</td>
<td>Experimental n=6</td>
<td>152.8 ± 9.72</td>
<td>141.3 ± 0.56</td>
<td>3.65 ± 0.17</td>
<td>7.41 ± 0.01</td>
<td>40.1 ± 2.92</td>
<td>50.3 ± 2.28</td>
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<td>Sham n=6</td>
<td>163.2 ± 9.83</td>
<td>136.5 ± 2.95</td>
<td>4.52 ± 0.44</td>
<td>7.42 ± 0.02</td>
<td>33.6 ± 2.01</td>
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<td>14 weeks</td>
<td>Experimental n=6</td>
<td>163.7 ± 7.72</td>
<td>140.50 ± 0.67</td>
<td>3.78 ± 0.21</td>
<td>7.39 ± 0.01</td>
<td>44.0 ± 1.86</td>
<td>48.8 ± 5.17</td>
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<td>Sham n=6</td>
<td>158 ± 7.73</td>
<td>141 ± 1.41</td>
<td>4.13 ± 0.09</td>
<td>7.43 ± 0.02</td>
<td>41.2 ± 3.47</td>
<td>50 ± 1.89</td>
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<td>15 weeks</td>
<td>Experimental n=6</td>
<td>171.2 ± 16.4</td>
<td>139.0 ± 1.15</td>
<td>3.90 ± 0.27</td>
<td>7.43 ± 0.01</td>
<td>39.1 ± 2.79</td>
<td>50.3 ± 3.43</td>
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<td>Sham n=6</td>
<td>165 ± 25.0</td>
<td>141 ± 0.68</td>
<td>4.08 ± 0.38</td>
<td>7.46 ± 0.02</td>
<td>30.6 ± 2.10</td>
<td>51.3 ± 4.53</td>
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<td>16 weeks</td>
<td>Experimental n=6</td>
<td>175.3 ± 7.80</td>
<td>138.5 ± 0.85</td>
<td>4.47 ± 0.21</td>
<td>7.44 ± 0.02</td>
<td>38.9 ± 2.30</td>
<td>53.8 ± 5.70</td>
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<tr>
<td></td>
<td>Sham n=6</td>
<td>164 ± 10.2</td>
<td>143 ± 0.49</td>
<td>4.52 ± 0.12</td>
<td>7.44 ± 0.01</td>
<td>34.0 ± 2.44</td>
<td>56.0 ± 2.45</td>
</tr>
<tr>
<td>17 weeks</td>
<td>Experimental n=3</td>
<td>146.7 ± 13.4</td>
<td>137.3 ± 0.33</td>
<td>4.53 ± 0.62</td>
<td>7.43 ± 0.00</td>
<td>39.1 ± 2.98</td>
<td>49.3 ± 1.20</td>
</tr>
<tr>
<td></td>
<td>Sham n=6</td>
<td>168 ± 11.8</td>
<td>143.0 ± 0.48</td>
<td>4.68 ± 0.36</td>
<td>7.45 ± 0.01</td>
<td>35.4 ± 1.06</td>
<td>54.0 ± 3.94</td>
</tr>
</tbody>
</table>
Supplementary Figure 1. A comparison between UCAO/JPD and sham-operated group in body weight and SBP. A) Effect of UCAO/JPD on body weight (UCAO/JPD group, n=25; sham-operated group, n=10). B) Effect of UCAO/JPD on SBP (UCAO/JPD group n=10; sham-operated group n=6). UCAO/JPD occurred at time denoted by the arrow. Data are expressed as mean ± SEM. ** P < 0.01, *** P < 0.001 compared to the sham-operated group.
Supplementary Figure 2. Increased microglia and astrocytes reactivity following UCAO/JPD. A) Distribution of astrocytes in UCAO/JPD and sham-operated groups. B) Double-staining of Iba-1 (green) and TNF-α (red), showing the expression of TNF-α in microglia/macrophages (Iba-1 positive cells) in white matter area of UCAO/JPD group. Insert shows co-localization of Iba-1 and TNF-α. External capsule (EC), corpus callosum (CC), internal capsule (IC), hippocampus (HIP). Scale bar=100 μm.