White Matter Injury After Subarachnoid Hemorrhage
Role of Blood–Brain Barrier Disruption and Matrix Metalloproteinase-9

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Background and Purpose—We recently observed early white matter injury after experimental subarachnoid hemorrhage (SAH), but the underlying mechanisms are uncertain. This study investigated the potential role of matrix metalloproteinase (MMP)-9 in blood–brain barrier (BBB) disruption and consequent white matter injury.

Methods—SAH was induced by endovascular perforation in adult male mice. The following 3 experiments were devised: (1) mice underwent magnetic resonance imaging at 24 h after SAH and were euthanized to determine BBB disruption and MMP-9 activation in white matter; (2) to investigate the role of MMP-9 in BBB disruption, lesion volumes on magnetic resonance imaging were compared between wild-type (WT) and MMP-9 knockout (MMP-9−/−) mice at 24 h after SAH; (3) WT and MMP-9−/− mice underwent magnetic resonance imaging at 1 and 8 days after SAH to detect time-dependent changes in brain injury. Brains were used to investigate myelin integrity in white matter.

Results—In WT mice with SAH, white matter showed BBB disruption (albumin leakage) and T2 hyperintensity on magnetic resonance imaging. MMP-9 activity was elevated at 24 h after SAH. MMP-9−/− mice had less white matter T2 hyperintensity after SAH than WT mice. At 8 days after SAH, WT mice had decreased myelin integrity and MMP-9−/− mice developed less white matter injury.

Conclusions—SAH causes BBB disruption and consequent injury in white matter. MMP-9 plays an important role in those pathologies and could be a therapeutic target for SAH-induced white matter injury. (Stroke. 2015;46:2909-2915. DOI: 10.1161/STROKEAHA.115.010351.)

Key Words: blood-brain barrier ■ brain injuries ■ matrix metalloproteinase-9 ■ subarachnoid hemorrhage ■ white matter

Subarachnoid hemorrhage (SAH) is a devastating cerebrovascular disorder with particularly high mortality and morbidity rates. Many physiological derangements, such as elevated intracranial pressure and decreased cerebral blood flow, are immediately induced by SAH, and these events initiate inflammation and oxidative stress, which result in blood–brain barrier (BBB) disruption, brain edema, as well as neuronal injury. Acute brain edema is recognized as a major consequence of SAH and is identified as an independent risk factor for poor clinical outcome. As yet, the few available SAH therapies focus on preventing aneurysmal rebleeding and prophylaxis for vasospasm, and there are no effective treatments available against early brain injury and consequent edema formation.

Matrix metalloproteinases (MMPs), particularly MMP-9, is well known as a key factor for the development of vasogenic edema after various brain injuries, including SAH. Thus, MMP-9 is upregulated and activated after cerebrovascular disorders in both clinical and experimental settings, and MMP-9 deletion ameliorates brain edema and leads to better neurological recovery in SAH mice. Recently, we observed early white matter injury after experimental SAH. However, the detailed mechanisms involved in such injury remain uncertain. We hypothesized that MMP-9 causes acute BBB disruption in white matter after SAH, resulting in consequent white matter injury. In the present study, we investigated this hypothesis using a mouse model of experimental SAH.

Methods
All animal protocols were approved by the University of Michigan Committee on the Use and Care of Animals. Adult male C57BL/6 mice (Charles River Laboratories, Portage, MI) weighing 22 to 30 g and male MMP-9 knockout (MMP-9−/−) mice with C57BL/6 background (University of Michigan Breeding Core) were housed at a controlled temperature under a 12-hour light-dark cycle. Food and water were available to all animals ad libitum.

Experimental Design
This study included the following 3 experiments. Throughout the current study, we used 42 wild-type (WT) and 27 MMP-9−/− mice. Experiment 1 was conducted in WT mice, and experiments 2 and 3 were conducted in WT and MMP-9−/− mice.

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2909
**Experiment 1**

To determine the extent of BBB disruption and MMP-9 activation/ expression in white matter after SAH, WT mice were divided into sham and SAH groups. Mice underwent magnetic resonance imaging (MRI) at 24 hours after SAH and were then euthanized. Brains were used for immunohistochemistry, transmission electron microscopy, and gelatin zymography.

**Experiment 2**

To investigate the role of MMP-9 in white matter injury, WT and MMP-9−/− mice underwent endovascular perforation. They underwent MRI 24 hours after SAH induction. Severity of SAH, neurological conditions, including mortality, and lesion volumes detected by MRI in each group were compared. We used 28 WT (n=11 for sham and n=17 for SAH) and 13 MMP-9−/− mice in experiments 1 and 2.

**Experiment 3**

To investigate time-dependent changes in white matter, MRI was performed 1 and 8 days after SAH induction in WT and MMP-9−/− mice. Mice without any surgery were set as normal control. Animals were euthanized at day 8, and the brains were used for immunohistochemistry. Mortality, neurological score, and body weight during the observation period were also recorded. In experiment 3, we used 14 WT and 14 MMP-9−/− mice (n=10 for SAH and n=4 for normal control, for each).

**Mouse SAH Model**

SAH was induced by endovascular perforation method as previously described. Details of surgical procedures are presented in the online-only Data Supplement.

**MRI Technique and Measurement of Lesion Volume**

MRI was performed using a 7.0-T Varian MR scanner (Varian Inc, Palo Alto, CA) with acquisition of T2 fast spin-echo and T2* gradient-echo sequences using a field of view of 20×20 mm, matrix of 256×256 mm, and 25 coronal slices (0.5 mm thick). The volume of T2 hyperintensity in the white matter and ventricular volume were measured as previously described.

**Evaluation of SAH Severity and Neurological Scores**

The extent of SAH was assessed using a modified grading system, and the neurological scores were evaluated by a blinded observer at determined time points in each experiment as previously described. Details for the evaluation methods are in the online-only Data Supplement.

**Transmission Electron Microscopy**

Transmission electron microscopy was performed as previously described (see details in online-only Data Supplement).

**Gelatin Zymography for Measurement of MMP Activity**

Activities of MMP-9 and -2 were analyzed by gelatin zymography using commercially available kit (Cosmo Bio, Tokyo, Japan) as previously described. Details are available in the online-only Data Supplement.

**Immunohistochemistry and Histochemistry**

Immunohistochemistry and histochemistry were performed on 10-μm-thick coronal sections (see details in online-only Data Supplement).

**Immunofluorescence**

Double-label immunofluorescence was performed as described previously (see online-only Data Supplement).

**Quantification of Immunostaining**

All analyses were performed by blinded investigator according to previous report (see online-only Data Supplement).

**Statistics**

Data are expressed as mean±SD and analyzed using JMP 7 software (SAS Institute Inc, Cary, NC). Statistical differences among the groups were analyzed using 1-way ANOVA, repeated-measures ANOVA, Spearman rank correlation test, and Log-rank test. A Bonferroni correction was used for multiple comparisons. P<0.05 was considered statistically significant.

**Results**

**SAH Caused BBB Disruption in White Matter**

Mice that underwent endovascular perforation (SAH) but not a sham operation developed white matter T2 hyperintensity...
at 24 hours. SAH also induced albumin leakage (BBB disruption) along the white matter. The area of albumin leakage was significantly larger in SAH- than in sham-operated mice (0.81±0.76 versus 0.04±0.01 mm²; P<0.05; n=4 for each; Figure 1B). There was a good correlation between T2 hyperintensity and albumin leakage (r=0.97; P<0.01; Figure 1C).

An electron microscopic examination showed ultrastructural abnormalities in white matter microvessels after SAH (Figure 1D). Abnormalities included swollen astrocyte endfeet with autophagosomes, tight junction detachment, erythrocytes in capillary lumen (after perfusion), and basement membrane irregularities. Degenerating axons, indicating acute

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**Figure 2.** Subarachnoid hemorrhage (SAH) induces matrix metalloproteinase (MMP)-9 activation and activates astrocytes, oligodendrocyte precursors, and microglia in white matter. **A,** Representative gelatin zymography demonstrates that activity of MMP-9, but not MMP-2, increases in SAH compared with sham mice at 24 h after SAH induction. **B,** Quantification of MMP-9 activity on gelatin zymography. **P**<0.01; values are mean±SD; n=3 for each. **C,** Representative 3,3’-diaminobenzidine (DAB) staining of glial fibrillary acid protein (GFAP), platelet-derived growth factor alpha (PDGFRα), and ionized calcium-binding adaptor molecule 1 (Iba-1) in the corpus callosum in sham and SAH mice at 24 h (Scale bar =100 μm).

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**Figure 3.** Immunofluorescent double labeling for matrix metalloproteinase (MMP)-9 with glial fibrillary acid protein (GFAP; astrocyte marker), platelet-derived growth factor alpha (PDGFRα) (oligodendrocyte precursor marker), ionized calcium-binding adaptor molecule 1 (Iba-1) (microglial marker), CD-31 (endothelial marker), or glutathion S-transferase π (GST-π; mature oligodendrocyte marker). MMP-9-expressing cells were mainly astrocytes and oligodendrocyte precursors and not microglia, endothelial cells, or mature oligodendrocytes. (Scale bar =20 μm).
white matter injury, were also observed after SAH (data not shown). These results strongly suggest that SAH causes BBB disruption in white matter, as well as acute axonal injury.

MMP-9 Activity in White Matter Was Elevated After SAH
Gelatin zymography revealed that MMP-9, but not MMP-2, activity was strongly elevated in the white matter at 24 h after SAH (P<0.01 versus sham; n=3 for each; Figure 2A and 2B). In immunohistochemistry, glial fibrillary acid protein, platelet-derived growth factor alpha, and ionized calcium-binding adaptor molecule 1 expression were prominently observed in the white matter after SAH (Figure 2C). Immunofluorescent double labeling showed that MMP-9–positive cells in white matter were mainly astrocytes (glial fibrillary acid protein positive) and oligodendrocyte precursor cells (platelet-derived growth factor alpha–positive), but rarely microglia (ionized calcium-binding adaptor molecule 1–positive), endothelial cells (CD-31–positive), or mature oligodendrocytes (glutathion S-transferase π–positive; Figure 3).

MMP-9 Deletion Ameliorated Early White Matter Injury
WT and MMP-9−/− mice were examined at 24 hours after SAH induction. Mortality rates were 24% (4/17) and 23% (3/13) in WT and in MMP-9−/− mice, respectively (P=0.98). No sham animal died (n=11). One WT mouse that developed a large hemispheric infarction after SAH was excluded from further investigation. The SAH severity score (degree of bleeding) was equivalent in WT and MMP-9−/− animals (9.0±3.3 and 9.1±4.0, respectively; P=0.95). Neurological scores at 24 hours after SAH were also similar (WT mice, 13.1±3.0; MMP-9−/− mice, 13.8±3.7; P=0.55). However, although all WT mice developed T2 hyperintensity in white matter at 24 hours after SAH (as also shown in our previous study9), this was much less evident in MMP-9−/− mice (Figure 4A). Thus, the volume of white matter T2 hyperintensity in WT mice with SAH was 6.2±3.0 versus 0.06±0.05 mm3 in WT sham mice (P<0.001; n=12 and 11, respectively), whereas it was only 1.0±1.4 mm3 in MMP-9−/− mice with SAH (P<0.01 versus WT with SAH; n=10; MMP-9−/− mice with SAH; Figure 4B). Ventricular volume was significantly greater in WT with SAH mice than in sham mice (15.4±3.3 and 8.9±1.6 mm3, respectively; P<0.001). Ventricular volume after SAH in MMP-9−/− mice (16.2±4.6 mm3) was similar to that in WT mice with SAH (P=0.56; Figure 4C).

Time Course of MRI Findings and Neurological Conditions Following SAH
The time course of neurological deficits, body weight, and MRI changes after SAH was examined in another set of 10 WT and 10 MMP-9−/− mice which underwent endovascular perforation. Mortality rates at 8 days after SAH were 20% (2/10) in each group, and the survivors were used for the following investigation. The initial reduction in neurological score (14.4±2.7 in MMP-9−/− versus 13.6±1.9 in WT) and loss of body weight (10.0%±4.0% in MMP-9−/− versus 11.1%±3.5% in WT) were similar in both genotypes. By 8 days, there were tendencies for MMP-9−/− mice to have a better recovery in neurological deficits (17.1±1.6 versus 15.6±1.6 in WT) and body weight (2.5%±4.5% and 7.1%±3.5% loss in MMP-9−/− and WT mice, respectively). However, these did
MMP-9 Deletion Attenuated White Matter Damage After SAH

White matter injury in WT and MMP-9−/− mice 8 days after SAH was examined using Luxol fast blue staining (Figure 6). Mice without any surgery (n=4 for WT and MMP-9−/− mice) were set as normal control. Estimated Luxol fast blue integrity (% loss of myelin) was equivalent in WT and MMP-9−/− mice without any surgery (data not shown). As expected, decreased myelin integrity was observed in WT mice with SAH compared with controls (24%±4% versus 8%±1% myelin loss, respectively; P<0.01). This loss was ameliorated in MMP9−/− mice (13%±4% myelin loss; P<0.01 versus WT SAH; n=4 for each; Figure 6).

Discussion

The present study contains 3 major findings: (1) experimental SAH induced by endovascular perforation caused acute BBB disruption in white matter; (2) MMP-9 activity in white matter was increased after SAH, and the main sources of MMP-9 were reactive astrocytes and oligodendrocyte precursors; and (3) MMP-9 deletion ameliorated BBB disruption and white matter injury.

Brain edema and BBB disruption are major components of early brain injury after SAH, and brain edema is an independent risk factor of poor clinical prognosis after SAH. Although we recently reported that SAH causes acute white matter injury, the underlying mechanisms are still uncertain. In animal models of other central nervous system injuries, such as trauma and multiple sclerosis, or chronic ischemic stroke, BBB disruption occurs in the early stage of disease and contributes to progression of white matter dysfunction at later time points. It is possible that similar mechanisms are involved in white matter pathology after SAH. However, to our best knowledge, there is no previous study that investigated acute BBB disruption in white matter after SAH. In the present study, T2 hyperintensity was more prominent in white matter than in other cerebral regions 24 hours after SAH, and this was consistent with albumin leakage detected by immunostaining. In addition, transmission electron microscopy showed ultrastructural abnormalities in white matter microvessels, including swollen astrocyte endfeet with autophagosomes, tight junction detachment, erythrocyte trapping within vessel lumens, and basement membrane irregularities. These results clearly demonstrate that BBB disruption occurs acutely in white matter after SAH.

The MMP family, especially MMP-9, is well accepted as key neurovascular proteases that can induce BBB damage and cause edema, hemorrhage, and neuronal death. Increased MMP-9 activity has been reported at 24 hours after SAH. MMP-9 contributes to early brain injury, with MMP-9−/− mice developing less brain edema after SAH. In this study, we confirmed that white matter MMP-9 activity was significantly increased at 24 hours after SAH induction. Activated astrocytes, microglia and, as with our previous report, oligodendrocyte precursors were prominently observed in white matter after SAH. The main MMP-9 sources were activated astrocytes and oligodendrocyte precursors and not activated microglia, endothelial cells, or mature oligodendrocytes.

Oxidative injury caused by excessive hemoglobin and iron, a major hemoglobin degradation product, significantly contributes to brain injury after hemorrhagic stroke. It was reported that astrocytes play an important role in maintaining BBB integrity. Also, MMP-9 can be activated in reactive astrocytes induced by oxidative stress, resulting in BBB disruption after intracerebral hemorrhage or traumatic brain injury. Our current results suggest that similar mechanisms are involved in astrocytic induction of MMP-9 in white matter after SAH, leading to BBB disruption. On the other hand, it is well known that the components of white matter, such as axons and oligodendrocytes, are highly sensitive to oxidative stress, and therefore, white matter is vulnerable to damage in various neurological disorders. As shown in the current study and our previous
results, acute white matter injury, including axonal degeneration and demyelination, occurs at 24 hours after SAH. Oligodendrocyte precursors are considered to maintain homeostasis and mediate long-term repair in white matter after injury. They rapidly proliferate, migrate, and fill in the damaged lesion in response to demyelinating signals to differentiate into mature oligodendrocytes and restore myelin sheaths.33,34 Recently, it was reported that oligodendrocyte precursors can rapidly respond to chronic hypoperfusion-induced white matter injury and secrete MMP-9 that leads to BBB disruption.32 These findings are comparable to our current results. Taken together, oligodendrocyte precursors are considered another player that mediates acute BBB disruption in white matter after SAH. In addition, studies have suggested that other mechanisms, such as inflammation and excitotoxicity, may also contribute to early white matter injury after SAH.35

We previously reported that lipocalin-2 (LCN-2) plays an important role in SAH-induced acute white matter injury.36 LCN-2 has the potential to preserve MMP-9 activity by preventing its degradation,33 and there is a close relationship between LCN-2 expression and MMP-9 activity in a mouse model of breast cancer.34 Similarly, our unpublished data confirmed that the activity of MMP-9 in the white matter after SAH is significantly lower in LCN-2 knockout mice than in WT mice. The effect of LCN-2 on MMP-9–related pathogenesis, such as BBB disruption after SAH shown in the current study, needs further investigation.

Our current results demonstrated that MMP-9 deletion effectively attenuated acute BBB disruption and consequent white matter injury. However, some previous studies indicate that MMP-9 plays a beneficial role in promoting neurovascular remodeling in the delayed stage of stroke recovery.21,35 In adult white matter, it was also reported that MMP-9 promotes white matter remodeling through accelerating angiogenesis in the recovery stage after injury,36 although MMP-9 plays a deleterious role in myelin damage and BBB disruption during acute injury.22 Because of these dual-edged actions of MMPs, the use of a broad-spectrum MMP inhibitor during the first few hours after cerebral ischemia reduced infarction, but delayed use of the same inhibitor worsened outcome.38 In this study, we used MMP-9–/– mice because specific MMP-9 inhibitors are currently unavailable. Future studies should determine the optimal timing of MMP-9 inhibition against SAH-induced white matter injury.

In conclusion, SAH results in rapid activation of astrocytes and oligodendrocyte precursors in white matter. They secrete MMP-9, leading to acute BBB disruption, axonal damage, and demyelination. MMP-9 deletion effectively reduced acute BBB disruption and attenuated consequent white matter injury. Hence, MMP-9 could be a therapeutic target for SAH-induced white matter injury.

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Disclosures
None.

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SUPPLEMENTAL MATERIAL

White matter injury after subarachnoid hemorrhage: role of blood-brain barrier disruption and matrix metalloproteinase-9

Supplemental Methods

Mouse SAH model
Anesthesia was induced by inhalation of 4% isoflurane in a nitrous oxide/oxygen mixture (70/30) and maintained by 2% isoflurane administered through a facemask. A midline skin incision was made in order to expose the left common carotid artery. The external carotid artery (ECA) and its branches were isolated and coagulated; thereafter, an 5-0 nylon monofilament (Ethicon, Somerville, NJ), with heat blunted-tip, was introduced into the left internal carotid artery (ICA) through the ECA stump up to the left ACA near the anterior communicating artery, where resistance was encountered. Then the suture was advanced 3 mm further to perforate the artery and was immediately withdrawn through the ICA into the ECA, allowing reperfusion and producing SAH. Sham control mice underwent the same surgical procedure, without insertion of suture. Body temperature of all animals was maintained at 37.5°C with a feedback-controlled heating pad throughout these procedures. After the surgery, mice were housed in heated cages until recovery.

Evaluation of SAH severity and neurological scores
The basal brain including brainstem was divided into 6 segments to evaluate SAH severity. Each segment was assigned a grade from 0 to 3 depending on the amount of SAH as follows: Grade 0, no SAH; Grade 1, minimal SAH; Grade 2, moderate SAH with recognizable
arteries; and Grade 3, SAH covering the cerebral arteries. The animals assigned a total score ranging from 0 to 18 after summating the scores from all 6 segments.

The evaluation of neurological score consisted of six tests that could be scored 0 to 3 or 1 to 3. These six tests consist; symmetry in the movement of all four limbs; forelimbs outstretching; climbing; side stroking; and response to vibrissae (whisker stimulation). Mice were given a score of 3 to 18 in 1-number step (higher scores indicate greater function).

Transmission electron microscopy
Mice were anesthetized and underwent transcardiac perfusion with 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1mol/L Sorensen’s buffer (pH 7.4). Brains were removed and a 1-mm thick coronal slice was cut with a blade 1mm posterior to bregma. Slices were immersed in the same fixative overnight at 4°C and then post-fixed with 1.0% OsO4 and dehydrated in graded ethyl alcohol. After dehydration, samples were infiltrated with propylene oxide, embedded in Epon, and sectioned. Ultra-thin sections were then stained with uranyl acetate and Raynold’s lead citrate, and evaluated using a Philips CM 100 TEM (FEM Company, Hillsbolo, OR) and digitally imaged using a CCD camera (Hamamatsu ORCA-HR; Hamamatsu Photonics, Hamamatsu, Shizuoka, Japan).

Gelatin zymography for measurement of MMP activity
Brains from sham or SAH mice were removed, and a 1-mm thick coronal slice was cut with a blade 1mm posterior to bregma, and the white matter carefully separated from cortex and basal ganglia. They were then lysed, homogenized and protein concentration measured. Forty micrograms of total proteins were subjected to electrophoresis in polyacrylamide gels containing 0.5 mg/mL gelatin in the presence of SDS under nonreducing conditions, washed twice in 2.5% Triton X-100 for 1 h, rinsed briefly, and incubated at 37°C for 48 hours in 100
mmol/L Tris-HCl (pH7.4) and 10 mmol/L CaCl₂. Then they were stained with Coomassie Brilliant Blue R-250 and destained in a solution of 5% acetic acid and 30% methanol. Zones of enzymatic activity appeared as clear bands against a blue background and their signal intensity were measured using Image-J software (version 1.47; National Institutes of Health, Bethesda, MD).

**Immunohistochemistry and histochemistry**

Mice were anesthetized with pentobarbital (60mg/kg; i.p.) and underwent transcardiac perfusion with 4% paraformaldehyde in 0.1mol/L phosphate-buffered saline (PBS; pH 7.4). Forebrains were removed, fixed in 4% paraformaldehyde for 48 h, immersed in 30% sucrose for 48 hours at 4°C, and then frozen. Fresh frozen forebrains were sliced into 10-µm-thick coronal sections by using a cryostat vibratome (Leica CM 1900; Leica Microsystems, Wetzler, Germany), and sectioned tissues were placed onto individual slides. For immunohistochemistry, anti-albumin (Bethyl Laboratories Inc, Montgomery, TX; 1:1000), anti-glial fibrillary acid protein (GFAP; Millipore, Billerica, MA; 1:400), anti-platelet-derived growth factor receptor alpha (PDGFRα, Abcam; 1:100) and anti-Iba-1 (Wako, Osaka, Japan; 1:400) antibodies were used. Luxol fast blue (LFB) staining was performed to evaluate myelin integrity according to the manufacturer’s instructions (American Mastertech, Rodi, CA).

**Immunofluorescence**

For immunofluorescent labeling, the primary antibodies were anti-MMP-9 (Millipore; 1:100), anti-GFAP (Millipore; 1:400), anti-PDGFRα (Abcam; 1:100), anti-Iba-1 (Wako; 1:400), anti-CD-31 (BD Biosciences, San Jose, CA; 1:200) and anti-GST-π (BD Biosciences; 1:200). The anti-GST-π antibody was used as a marker of mature oligodendrocytes. The appropriate
Alexa-Flour conjugated antibodies (Molecular Probes, Eugene, OR; 1:400) were used as secondary antibodies. The slides were covered with Prolong Gold reagent with DAPI (Molecular Probes), and were observed underneath a fluorescence microscope (Olympus BX51; Olympus, Tokyo, Japan).

Quantification of white matter injury

Ten-µm-thick coronal sections (bregma -1.5 mm to -2.1 mm) were used for each analysis. For quantitative measurements of albumin staining, 4 brain sections were digitized under a 10x magnification using Olympus BX-51 microscope. Albumin positive areas in the white matter tract (corpus callosum, external capsule, and fimbria) were marked and calculated using Image-J. For quantification of LFB staining, 4 brain sections which contained 3 regions of interest (ROI) including the white matter tract were digitized under a 40x magnification. The white matter tract was marked in each ROI, and areas not stained by LFB were determined as the areas of myelin loss. Unmyelinated Schaffer collaterals obtained from same settings for each section served as an internal control.