**Supplementary Information -1**

# Oligodendrocyte precursors induce early blood-brain-barrier opening after white matter injury

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**Supplemental Figures**

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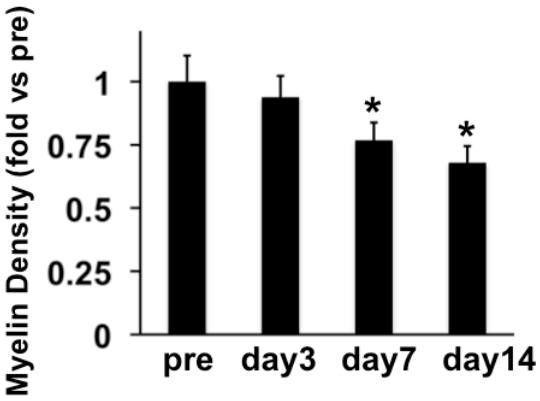


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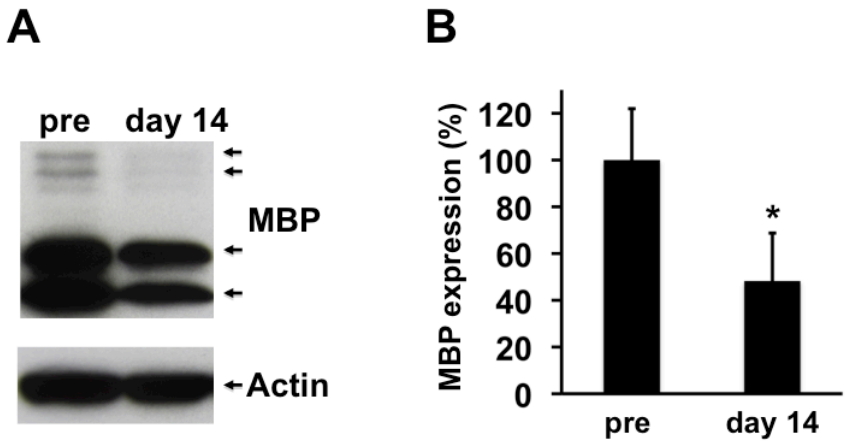

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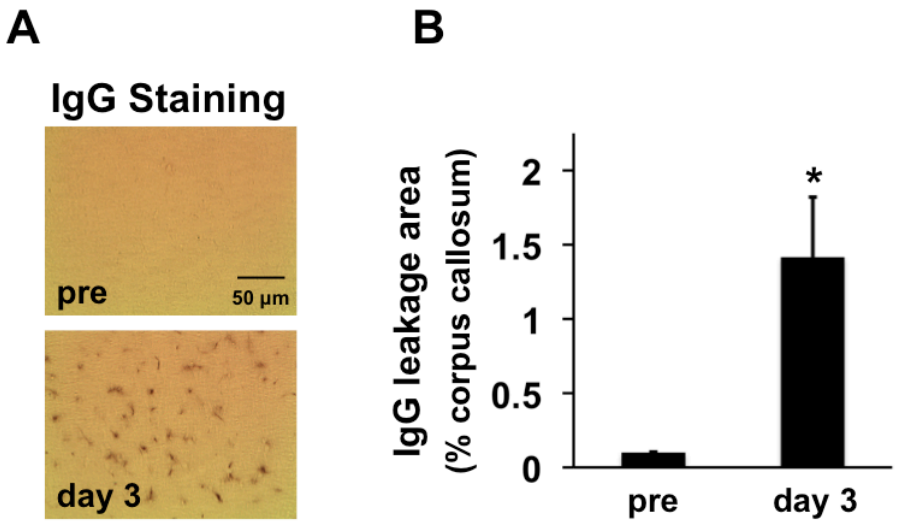


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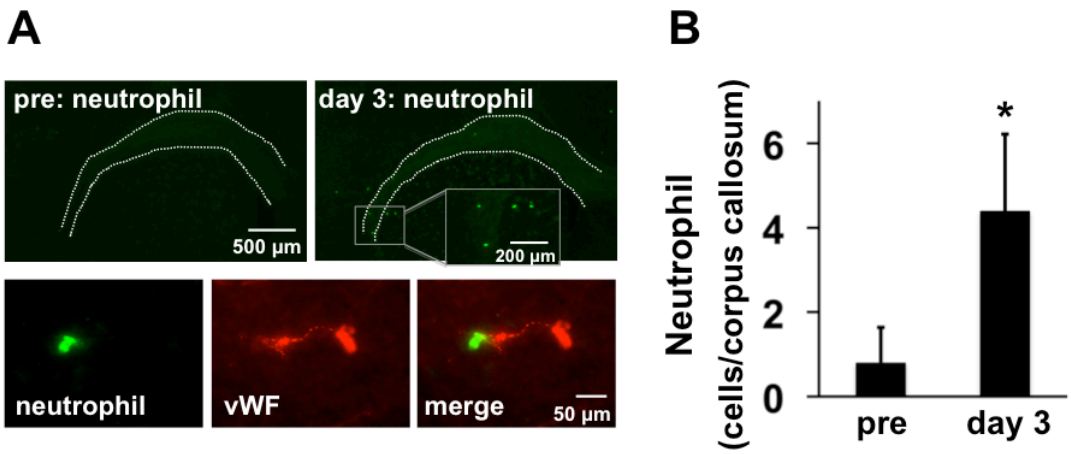

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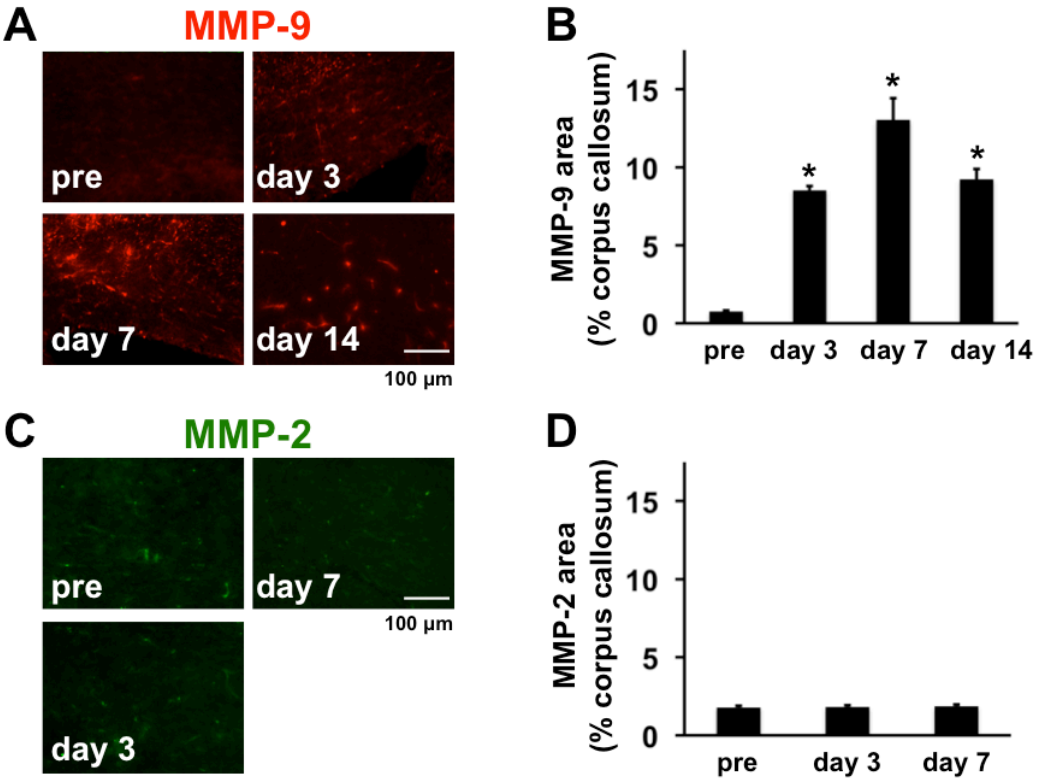


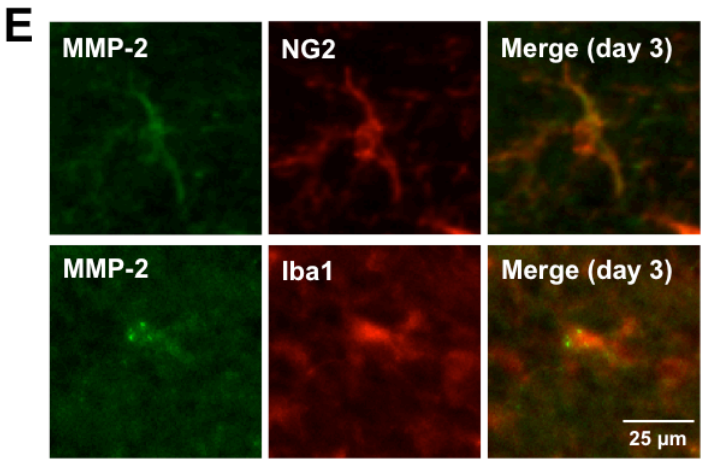


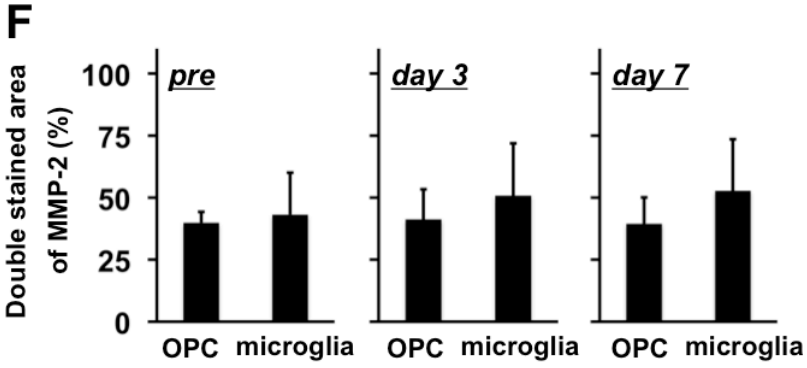

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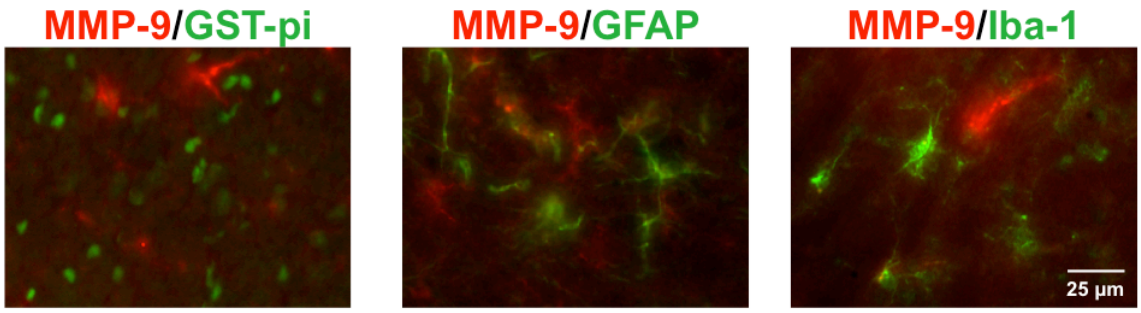


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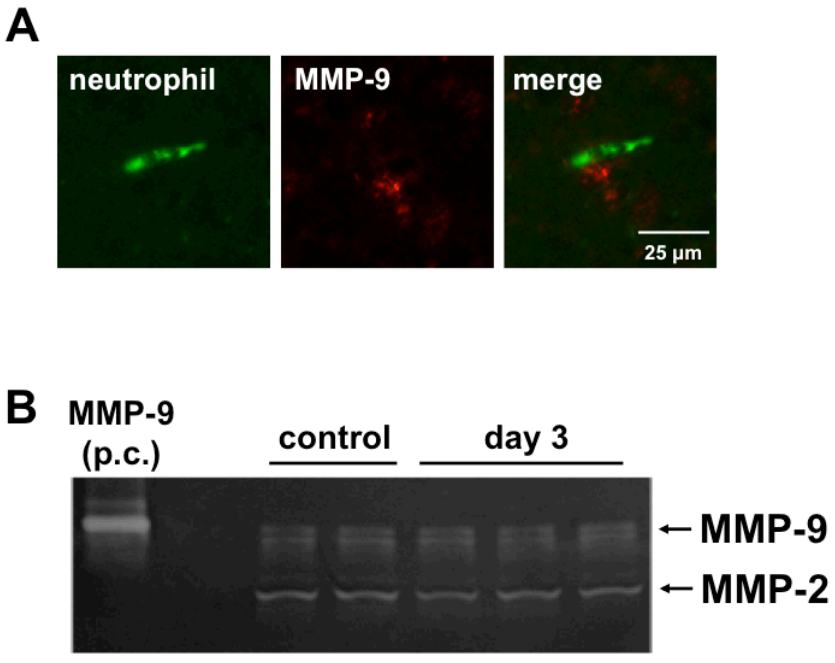

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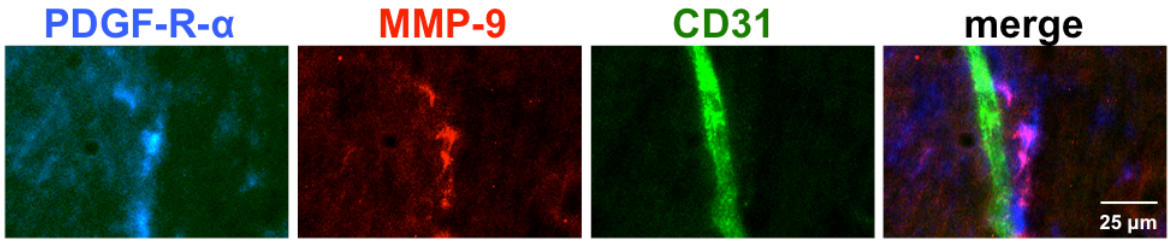


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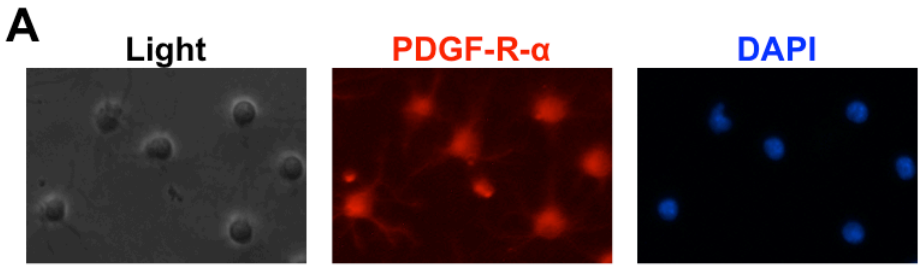


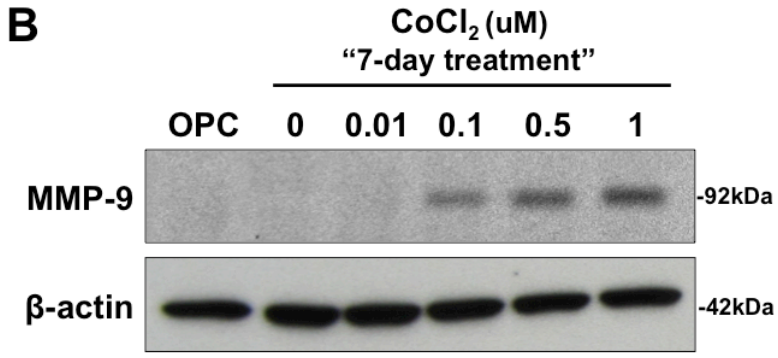

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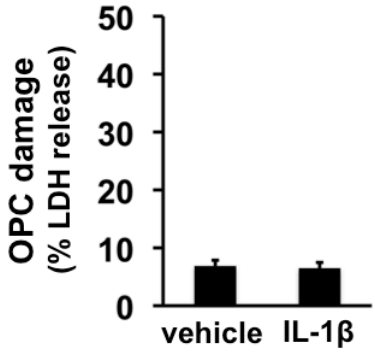


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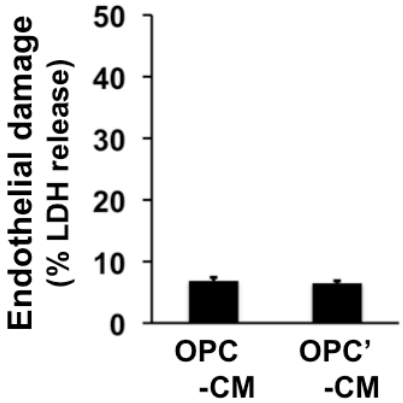

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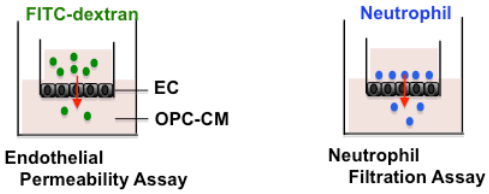


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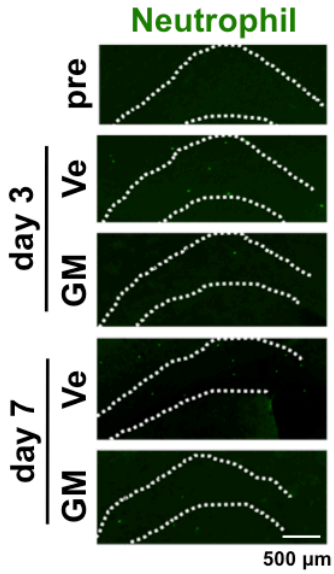

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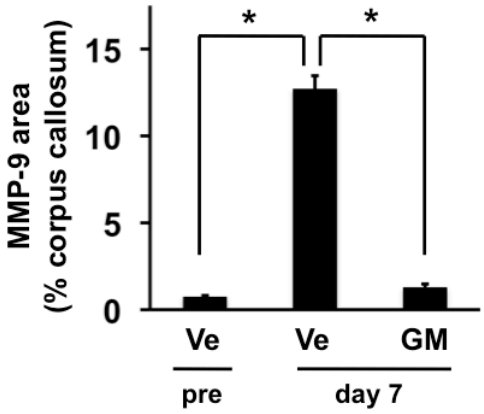


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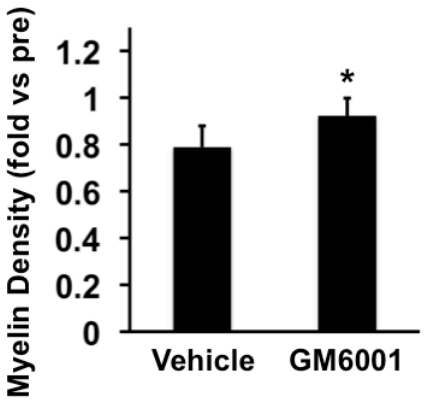

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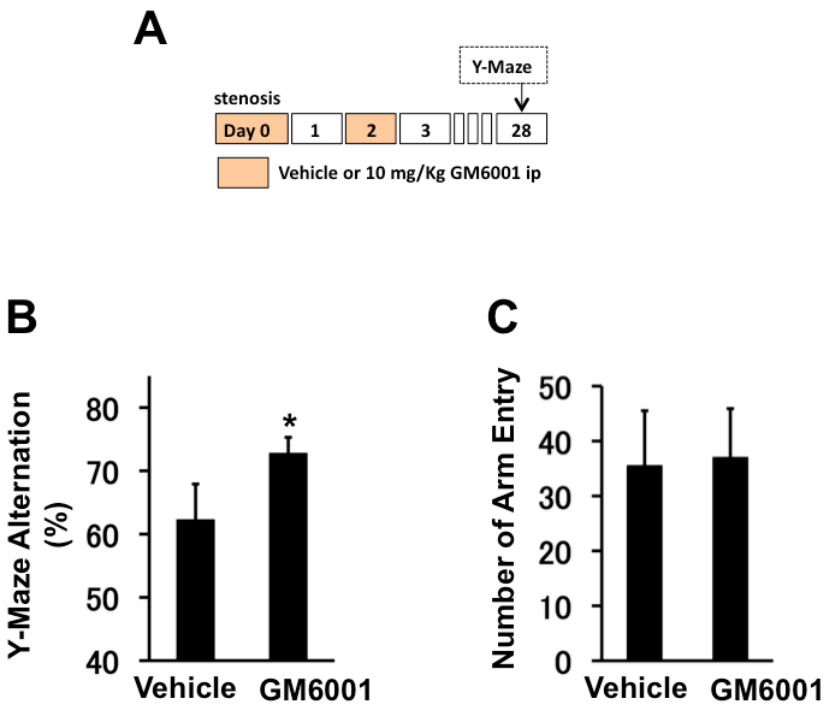

**Supplemental Figure Legends**

**Supplemental Figure S1:** Quantitative data for Figure 1A. Cerebral prolonged hypoperfusion stress induced demyelination in the mouse corpus callosum. N=5. *p<0.05 vs pre

**Supplemental Figure S2:** In addition to the immunostaining approach (Figure 1A and Supplemental Figure S1), we used western blot system to confirm that mice subjected to prolonged cerebral hypoperfusion stress developed white matter demyelination. The expression level of myelin-basic-protein (MBP: one of the major components of myelin) was lower at day 14 compared to pre-treatment group. N=5. *P<0.05 **(A-B)**.

**Supplemental Figure S3:** Before starting the demyelination, BBB leakage occurred at day 3 assessed by IgG staining. N=5. *P<0.05

**Supplemental Figure S4:** In addition to the BBB leakage, neutrophils were infiltrated from blood to brain parenchyma at day 3. vWF is a marker for blood vessels, and neutrophils were observed outside the blood vessels. N=5. *P<0.05

**Supplemental Figure S5:** In addition to the western blot approach (Figure 1B), we used immunostaining method to confirm that levels of MMP-9, but not MMP-2, increased in the mouse corpus callosum after the white matter injury. The number of MMP-9-positive cells was increased after the stress onset, but patterns of MMP-2 signal in corpus callosum were not changed by the chronic hypoxia stress **(A-D)**. MMP-2 positive cells were co-localized with NG2 (OPC marker) or Iba-1 (microglia marker), suggesting that both OPCs and microglia express MMP-2 in the mouse corpus callosum **(E-F)**. N=5. *p<0.05

**Supplemental Figure S6:** As shown in Figure 1C-E, MMP-9 expressing cells were mostly OPCs under acute phase after the injury, but cerebral endothelial cells became to produce MMP-9 at later time points. Here, we confirmed that other cell types such as oligodendrocytes (GST-pi), astrocytes (GFAP), Iba-1 (microglia) did not express MMP-9 in corpus callosum at day 3.

**Supplemental Figure S7:** Our overall hypothesis was that at the acute white matter injury phase, OPCs would produce MMP-9, resulting in the early BBB disruption. Here we confirmed that neutrophil in the blood vessels did not produce MMP-9 at day 3 **(A)**. The brain sections were prepared from mouse brain without PBS-perfusion. Moreover, gelatin zymography showed that plasma MMP-9 levels at day 3 were similar to ones of sham-operated (control) mice **(B)**. Those data suggest that cells/factors in the blood may not play pivotal roles in the acute BBB disruption.

**Supplemental Figure S8:** To confirm that MMP-9-expressing OPCs are closely located to cerebral endothelial cells at day 3, we conducted triple-staining with PDGF-R-α (OPC marker), MMP-9, and CD31 (endothelial marker) antibodies.

**Supplemental Figure S9:** Our primary rat OPC cultures indeed expressed an OPC marker PDGF-R-α **(A)**, and importantly, they responded to chemical hypoxic stress and produced MMP-9 **(B)**. OPCs were subjected to sub-lethal CoCl2 (chemical hypoxic inducer) for 7 days, and then cell lysates were used for western blot analysis. β-actin: internal control

**Supplemental Figure S10:** We treated 100 ng/mL IL-1β to our OPC cultures for 1 hour. After that, cells were switched to standard culture media and maintained for 23 hours. LDH assay showed that the IL-1β treatment did not affect OPC viability. N=3.

**Supplemental Figure S11:** Conditioned media from IL-1β-treated "injured" OPCs (OPC'-CM) degraded ZO-1 in endothelial RBE.4 cell cultures (Figure 2C-E). However, LDH assay showed that the OPC'-CM (24-hour treatment) did not affect endothelial survival. N=3.

**Supplemental Figure S12:** Schematics for endothelial permeability **(A)** and neutrophil filtration **(B)** assays in vitro. EC: endothelial cells (RBE.4 cells), OPC-CM: conditioned media from oligodendrocyte precursor cells

**Supplemental Figure S13:** Representative images of neutrophil staining for Figure 3D. N=5.

**Supplemental Figure S14:** Quantitative data for Figure 3E. GM6001 inhibited MMP-9 production in endothelium at day 7. N=5. *P<0.05

**Supplemental Figure S15:** Quantitative data for Figure 3F. GM6001 inhibited the development of white matter injury at day 7. N=5. *P<0.05

**Supplemental Figure S16:** We examined if the GM6001 treatment reduced the cognitive dysfunction by the prolonged hypoxic stress in mice. GM6001 was treated at day 0 and 2, and we conducted the Y-maze-type spatial learning test at day 28 **(A)**. The GM6001 treatment recovered the spatial learning performance. N=4. *P<0.05 **(B)**. There was no significant difference in the number of arm entry between vehicle and GM6001 groups, indicating that the treatment did not affect motor function. N=4. **(C)**.

## Supplemental Methods

**Prolonged cerebral hypoperfusion stress by bilateral common carotid artery stenosis (BCAS):** All experiments were performed following protocols approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male C57Bl/6 mice (10 to 12 weeks old, 24 to 29 g) were anesthetized with 4.0% isoflurane and maintained on 1.5% isoflurane in 70% $N_2O$ and 30% $O_2$ using a small-animal anesthesia system. Through a midline cervical incision, both common carotid arteries were exposed. A micro-coil with a diameter of 0.18 mm (Sawane Spring Co.) was applied to the bilateral common carotid artery. During the surgery, the rectal temperature was maintained between 36.5°C and 37.5°C using a heating pad. Mice were divided at random into three groups. (i) BCAS group (n=35), (ii) BCAS with GM6001 treatment group (n=10). This group received intraperitoneal injections of 50 mg/kg body weight GM6001 (Millipore) at 0 and 2 days after the operation, (iii) Control sham-operated vehicle-treated groups (n=10).

**Myelin staining:** Mouse coronal sections of 12-μm thicknesses (bregma +0.86 mm to +0.50 mm, to analysis of corpus callosum and bregma +5.80 mm to +6.00 mm, to analysis of cerebellum) were prepared on glass slide using cryostat. Sections were fixed by 4% paraformaldehyde (PFA), and rinsed three times in phosphate-buffered saline (PBS), then permeablize them in PBS-T (PBS + 0.2% Triton X-100) for at least 20 minutes. The sections were incubated with FluoroMyelin green fluorescent myelin stain (1:300, Molecular probes) for 20 minutes at room temperature. After the sections were rinsed with PBS-T, the slides were covered with VECTASHIELD.

**Immunohistochemistry:** Mouse brains were taken out after perfusion with PBS (pH 7.4) and then quickly frozen using liquid nitrogen. Coronal sections of 16-μm thicknesses were cut on cryostat at -20°C and collected on glass slides. Sections were fixed by 4% PFA, and rinsed three times in PBS (pH 7.4). After blocking with 3% BSA, sections were incubated at 4°C overnight in PBS/0.1% Tween/0.3% BSA solution containing primary antibodies – anti-myelin basic protein (MBP) (a marker for mature oligodendrocyte, 1:100, Pierce Biotechnology), anti-GFAP (a marker for astrocyte, 1:100, BD Pharmingen), anti-Iba-1 (a marker for microglia, 1:100, Wako), anti-GST-pi (a marker for mature oligodendrocyte, 1:100, MBL international), anti-NG2 (a marker for OPC, 1:100, Millipore), anti-PDGFR-α [CD140a] (a marker for OPC, 1:100, BD Pharmingen), anti-CD31 (a marker for endothelial cell, 1:100, BD Pharmingen), anti-von Willebrand factor (vWF, a marker for endothelial cell, 1:100, Dako), anti-MMP-9 (1:100, Carbiochem), and anti-neutrophil (1:200, Abcam). Then sections were washed and incubated with secondary antibodies with fluorescence conjugations at

room temperature for 1 hour. Subsequently, the slides were covered with VECTASHIELD mounting medium with DAPI (H-1200 from Vector Laboratories). Immunostaining was analyzed with a fluorescence microscope (Olympus BX51) interfaced with a digital charge-coupled device camera and an image analysis system.

**Western Blotting:** Lysates from the corpus callosum region were prepared in Pro-PREPTM Protein Extraction Solution (Boca Scientific). Samples were heated with equal volumes of SDS sample buffer (Novex) and 10 mM DTT at 95°C for 5 min, then each sample (20 µg per lane) was loaded onto 4–20% Tris–glycine gels. After electrophoresis and transferring to polyvinylidene difluoride membranes (Novex), the membranes were blocked in Brockace (AbD Serotec) for 60 min at room temperature. Membranes were then incubated overnight at 4°C with anti-IL-1β antibody (1:3000, Abcam), anti-MMP-9 antibody (1:3000), MBP antibody (1:1000) or anti-β-actin antibody (1:10000, Sigma Aldrich) followed by incubation with peroxidase-conjugated secondary antibodies and visualization by enhanced chemiluminescence (Amersham).

**Cell Culture:** OPCs were prepared from cerebral cortices of 1-2 day old Sprague-Dawley rats. Dissociated cortex cells were plated in poly-d-lysine-coated flasks, and culture in Dulbecco's Modified Eagle's medium containing 20% fetal bovine serum and 1% penicillin/streptomycin. After the cells were confluent, the flasks were shaken for 1 hour on an orbital shaker (220 rpm) at 37°C to remove microglia. The medium was changed with a new medium and shaken overnight. The medium was then collected and plated on non-coated tissue culture dishes for 1 hour at 37°C to eliminate possible contamination by astrocytes and microglia. The non-adherent cells were collected and cultured in Neurobasal Media containing glutamine, 1% penicillin/streptomycin, 10 ng/mL PDGF, 10 ng/mL FGF, and 2% B27 supplement onto poly-dl-ornithine-coated plates. Rat brain microendothelial cell lines (RBE.4) were cultured in EGM-2MV containing EGM-2MV Single Quots kit onto collagen-coated flasks. Leukocytes were prepared from mouse peritoneal cavity. 24 hours later, non-adherent cells were collected as a leukocyte population, then cultured in RPMI media containing 10% fetal bovine serum, 1% penicillin/streptomycin.

**Cell viability assay:** To ensure that IL-1β has no effect on OPCs viability and OPC conditioned media has no effect on endothelial survival, cytotoxicity was quantified by lactate dehydrogenase (LDH) assay.

**Gelatin zymography:** The collected conditioned medium was concentrated using Microcon (Millipore) with a 10 kDa pore diameter cutoff, then each sample was mixed with equal amounts of SDS sample buffer (Novex) and electrophoresed on 10% SDS-polyacrylamide gels (Novex) containing 1 mg/ml gelatin as the protease substrate. Following electrophoresis, gels were placed in

2.7% Triton X-100 for 1 hour to remove SDS, and then incubated for 16 h at 37°C in developing buffer (50 mM Tris base, 40 mM HCl, 200 mM NaCl, 5 mM $CaCl_2$, and 0.2% Briji 35; Novex) on a rotary shaker. After incubation, gels were stained in 30% methanol, 10% acetic acid, and 0.5% w/v coomassie brilliant blue for 1 hour followed by de-staining. Mixed human MMP-2 and MMP-9 standards (Chemicon) were used as positive controls.

**Y-Maze Test:** The Y-maze test was conducted during the early morning (7:00 to 9:00 AM) and performed 28 days after the BCAS operation. This task is based on spontaneous alternation behavior and is used to measure spatial working memory. The maze consists of 3 arms (40 cm long, 9.5 cm high, and 4 cm wide, labeled A, B, or C) diverging at a 120° from the central point. Each mouse was placed at the center of the start arm and allowed to move freely through the maze during a 10-minute session without reinforcers such as food, water, or electric foot shock. This task was videotaped with a Victor camera (Everio GZ-MG-77-S) and the sequence of arm entries manually recorded. An actual alternation was defined as entities into all the 3 arms on consecutive. The maximum alternation was subsequently calculated by measuring the total number of arm entries minus 2 and the percentage of alternation was calculated as (actual alternation/maximum alternation) x100. The total number of arms entered during the sessions, which reflect locomotor activity, was also recorded.