SUPPREMENTAL MATERIAL

Oxidative stress interferes with white matter renewal after prolonged cerebral hypoperfusion in mice

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Supplemental Methods:

Cell culture – Primary cortical OPCs were prepared and maintained according to our previous work [1]. To differentiate OPCs to myelin basic protein-positive oligodendrocytes, the culture medium was switched to Dulbecco's Modified Eagle's Medium (DMEM) containing 1% penicillin/streptomycin, 10 ng/ml ciliary neurotrophic factor (CNTF), 15 nM triiodo-L-thyronine (T3), and 2% B27 supplement (DMEM medium). To mimic chronic mild-hypoxic condition, OPCs were incubated with non-lethal cobalt chloride ($CoCl_2$ from Sigma). Edaravone (Mitsubishi Tanabe Pharma) were dissolved in dimethysulphoxide. The final concentration of dimethysulphoxide in the culture medium was less than 0.1%, which had no effects on OPC survival and function. In vitro experiments were performed in duplicate, repeated 3-6 times independently.

In vitro chemical hypoxic stress – OPC cultures were treated with sub-lethal (1 uM for 7 days) CoCl2 to induce a prolonged chemical hypoxic conditions as described before [2, 3]. Hypoxic conditions were confirmed by increase of HIF-1alpha expression.

Cell proliferation/survival assay - Cell proliferation/survival was assessed by water-soluble-tetrazolium (WST) assay (Dojindo) according to the manufacturer's instruction.

ROS assay - Intracellular levels of ROS were evaluated using dichlorofluorescein diacetate (DCF-DA) as a fluorescence probe. To detect ROS, the cells were pre-incubated at 37°C for 1 h with culture medium containing 10 mM DCF-DA dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 0.1% (vol/vol). The culture medium was then replaced with DMEM medium containing $CoCl_2$ with/without Edarabone. After 24h, the fluorescence intensity for oxidized DCF-DA (excitation 488 nm and emission 525nm) in each well was measured by fluorescence microplate spectrophotometer.

Immunocytochemistry - After cells reached 70–80% confluence, they were washed with ice-cold PBS (pH 7.4), followed by 4% PFA for 15 min. After being further washed three times in PBS containing 0.1% Triton X-100, they were incubated with 1% bovine serum albumin in PBS for 1 h. Then cells were incubated with primary antibodies against MBP (1:100, abcam), GST-pi (1:100), and PDGFR-α (1:200) at 4°C overnight. After washing with PBS, they were incubated with secondary antibodies for 1 hour at room temperature. Finally, nuclei were counterstained with DAPI.

Cerebral prolonged hypoperfusion model - All experiments were performed following an institutionally approved protocol in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. For inducing cerebral chronic hypoperfusion stress, a microcoil (0.18 mm diameter, Sawane Spring Co.) was applied to bilateral common carotid arteries. Male C57-black-6 (C57BL/6) mice (sham: 22 mice, vehicle-group: 37 mice, edaravone-group: 38 mice, 10weeks old, Charles River Institute) were anesthetized with 4.0% isoflurane and then maintained on 1.5% isoflurane in 70% N_2O and 30% O_2,

maintaining the rectal temperature between 36.5°C and 37.5°C. The operation time was approximately 20 min per mouse, and the interval between the two microcoils was 5 min. The cerebral blood flow was measured before/after the micro-coil placement as described previously [21]. The radical scavenger edaravone (3 mg/kg ip) or vehicle was treated twice per week starting at day 0 until the mice were sacrificed. Animals were sacrificed on day 0, 7, 14, and 28. All in vivo experiments and measurements were performed in blinded and randomized manner. Animal numbers for each experiment are described in figure legends.

OPC differentiation assay in vivo - To detect cell differentiation, mice were intraperitoneally injected (50 mg/kg, bromodeoxyuridine (BrdU), Sigma-Aldrich) three times a day with 4-hour interval at day 14. The mice were sacrificed 14 days after the BrdU injection, and mouse brains were used for BrdU staining.

Cognitive test - Spontaneous alternative Y-maze cognitive test [4] was conducted between 7:00 AM to 9:00 AM at day 28. Mouse was placed at the center of the start arm and allowed to move freely through the maze in a 8-minute session. This task was videotaped and the sequence of arm entries manually recorded in a blinded manner. 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 (as locomotor activity) minus 2 and the percentage of alternation was calculated as [actual alternation/maximum alternation] x100%. Y-maze animals were not used for histological, biochemical, MRI experiments.

Magnetic resonance imaging (MRI) – MRI was conducted once at day 28 after the surgery. Imaging employed a 4-channel phased array receiver coil inside a volume radio frequency (RA) transmitter on a 9.4 Tesla magnet (Bruker BioSpin Corporation, Billerica, MA). Multi-slice two-dimensional image acquisition covered whole brain from olfactory bulb to cerebellum using coronal slices with a thickness of 400-microns and an isotropic in-plane resolution of 150 microns. Diffusion tensor imaging (DTI) provided a white matter index in the form of fractional anisotropy (FA). Segmented echo planar imaging provided efficient temporal acquisition of DTI using six directions at a b-value of 1000 sec/mm^2, plus an additional acquisition with no diffusion weighting (averaged twelve times). Analysis resampled all MRI onto the Allen Mouse Brain Atlas at 250-micron coronal levels and a resolution of 125 microns in coronal planes. Maps of FA were computed for each animal, and results were averaged across animals within each cohort for comparison with histology. MRI animals were not used for histological, biochemical, and cognitive experiments.

Immunohistochemistry - Mouse brain was removed at days 0, 7, 14, and 28, and postfixed for 24 h in 4% paraformaldehyde (4% PFA) in phosphate-buffered saline (PBS) at 4°C before cryoprotection by bathing in 30% sucrose. Sixteen-μm-thick coronal sections were incubated overnight with anti-BrdU (1:50; Oxford Biotechnology, denature with 37°C for 30 min in 1N HCl). Double immunofluorescence staining was performed by simultaneously incubating the sections overnight at 4°C with anti-GST-pi (1:200, MBL; oligodendrocyte marker), anti-PDGFRα (1:100; SantaCruz, or anti-CD140a, 1:100, BD phamamigen; OPC maker), anti-Ki67 (1:100, abcam; proliferative cell marker). After washing with PBS, they were incubated

with secondary antibodies (1:200; Jackson Immunoresearch Laboratories) for 1 hour at room temperature, and the slides covered with VECTASHIELD with DAPI (Vector Laboratories). Immunostaining was analyzed with a fluorescence microscope (Olympus BX51).

Fluoromyelin staining – Twelve-μm-thick coronal sections (bregma +0.86 mm to +0.50 mm) were incubated with FluoroMyelin Green fluorescent myelin stain (1:300, Molecular probes) for 20 minutes at room temperature. Semi-quantification of the intensity of fluoromyelin staining was conducted on 10x magnification images and viewed using a Nikon upright microscope. Intensity of fluoromyelin staining were analyzed by quantifying mean intensity of the entire field of view for 3 brain sections of each animal, using ImageJ analysis software with no thresholds set.

Western blotting - Tissue samples of corpus callosum and cell culture were dissected in Pro-PREPTM Protein Extraction Solution (Boca scientific). Samples were heated with equal volumes of SDS sample buffer (Novex) and 10 mM dithiothreitol (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), then incubated overnight at 4 ◦C with primary antibodies against phospho-cAMP response element-binding protein (pCREB) (1:3000, upstate), MBP (1:1000, Thermo scientific; a marker for myline sheath), HIF1α (1:3000, abcam; a marker for hypoxic conditions), PDGFRα (1:3000), GST-pi (1:5000), or β-actin (1:10000, Sigma Aldrich) followed by incubation with peroxidase-conjugated secondary antibodies and visualization by enhanced chemiluminescence (Amersham). The Oxyblot protein oxidation detection kit (Chemicon) was used following the manufacturer's instructions.

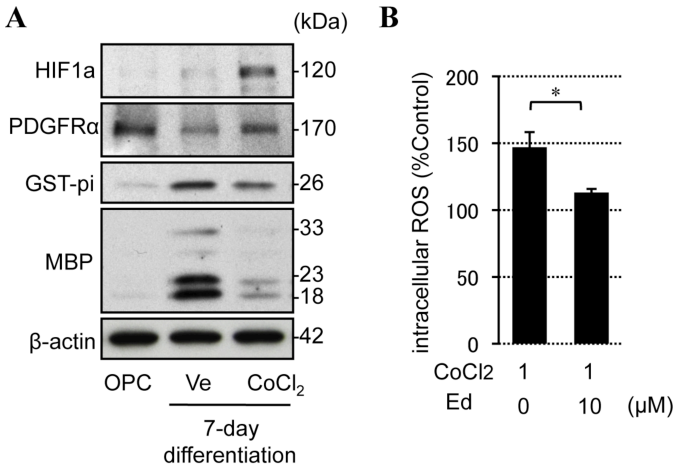
TUNEL - We detected in situ DNA fragmentation by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining using an 'DeadEnd™ Fluorometric TUNEL System' (Promega) on the 16-μm-thick free-floating coronal sections were used following the manufacturer's instructions.

Cell counting for brain sections - An investigator blinded to the experimental groups counted the number of stained cells in lateral side of corpus callosum (0.25 mm^2) of GST-pi- and PDGFRα- stained section and in the corpus callosum of three predefined BrdU, and TUNEL sections (bregma +1.18 mm, +0.98 mm, and +0.74 mm).

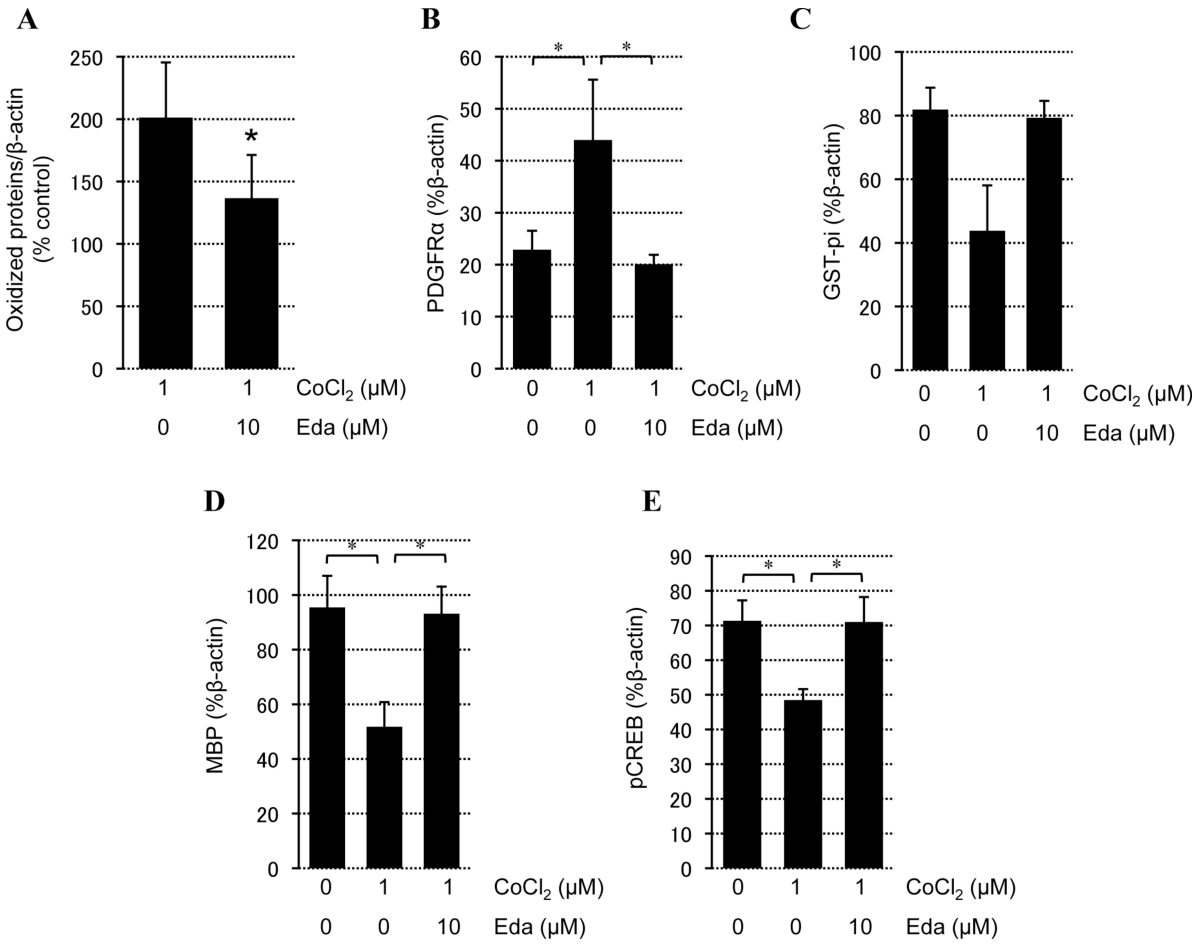
Statistical analysis – Power estimates were calculated based on α=0.05 and β=0.8 to obtain group sizes appropriate for detecting effect sizes in the range of 30-50% for in vivo models and 40-50% for cell cultures models. Statistical significance was evaluated using the unpaired t-test to compare differences between the two groups and a one-way ANOVA followed by Tukey's honestly significant difference test for multiple comparisons. Data are expressed as mean ± S.D. A p value of <0.05 was considered statistically significant.

Supplemental Figures and Figure Legends:

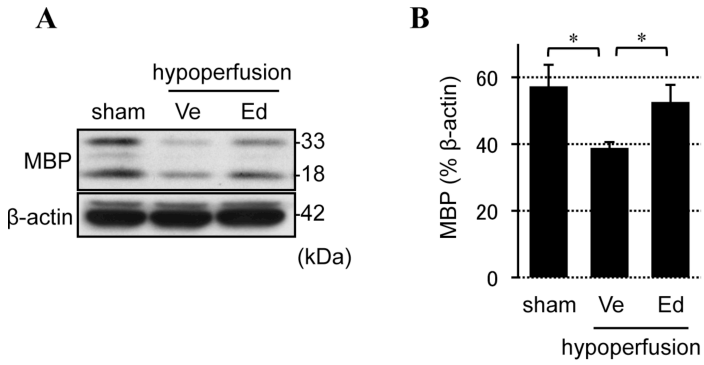
Supplemental Figure I: A. Representative western blot images for cultured rat OPCs with $CoCl_2$ treatment (1 μM for 7 days). HIF1α is a marker for hypoxic conditions and β-actin is an internal control. PDGF-R-α: OPC marker, GST-pi and MBP: oligodendrocyte marker **B.** DCF assay for 7-day CoCl2-treated OPCs with or without edaravone. Values are mean ± SD of N=6. **P*<0.05.



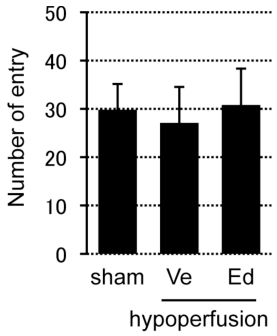
Supplemental Figure II: Quantitative data of western blot experiments for Figure 1. Values are mean ± SD of N=3 (Supplemental Figure IIA, IIB, and IIE) or N=4 (Supplemental Figure IIC and IID). *P<0.05.



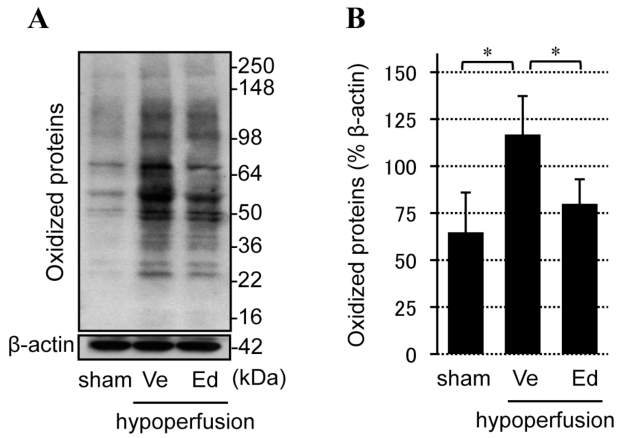
Supplemental Figure III: In addition to myelin staining (Figure 2A-B) and MRI analysis (Figure 2C), MBP western blot approach confirmed that 28-day cerebral hypoperfusion induced white matter damage. Ve; vehicle, Ed; edaravone treatment group. Values are mean ± SD of N=5. **P*<0.05.



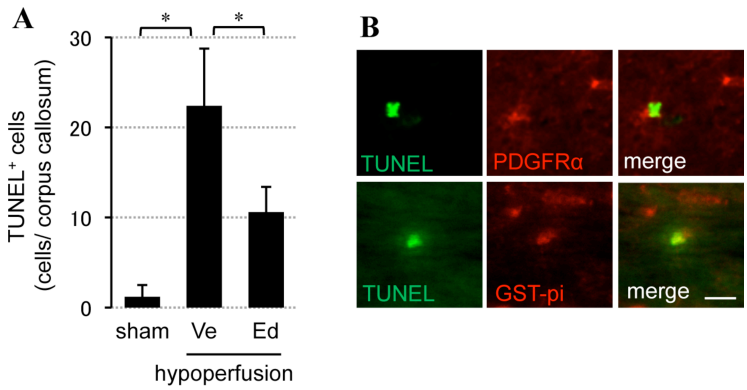
Supplemental Figure IV: Numbers of entry (index of locomotor activity) at 28 day in the Y-maze test. Ve; vehicle, Ed; edaravone treatment group. Values are mean ± SD. N= 10.



Supplemental Figure V: The oxiblot assay confirmed that 28-day cerebral hypoperfusion induced excessive oxidative stress in the mouse white matter. Importantly, a radical scavenger edaravone ameliorated the ROS accumulation. β-actin is an internal control. Ve; vehicle, Ed; edaravone treatment group. Values are mean ± SD of N=3. *P<0.05.



Supplemental Figure VI: A. Numbers of TUNEL–positive (TUNEL^{+}) cells in corpus callosum. TUNEL staining was conducted 28 days after the stress onset. **B.** Double staining of TUNEL with PDGFRα (OPC) or GST-pi (mature oligodendrocyte) in vehicle groups at day 28. Bar = 10 μm. Values are mean ± SD of N=5. **P*<0.05.



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