Induction of genes expressed in endothelial cells of the corpus callosum in the chronic cerebral hypoperfusion model rat

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Abstract

Background: Cerebrovascular white matter lesions (WMLs) are associated with cognitive impairment in patients with subcortical vascular dementia. We performed a comprehensive gene expression analysis to elucidate genes associated with WML development in a chronic cerebral hypoperfusion rat model.

Methods: Brains of rats with bilateral carotid ligation (2VO, n=10) and sham-operated rats (n=5-10/group) were removed on day 1, 7, or 28 after surgery. Total RNA isolated from the corpus callosum was evaluated by microarray analysis and quantitative reverse transcription-polymerase chain reaction.

Results: On days 7 and 28, WMLs exhibited histologic changes. On day 7, 16 genes were differentially expressed between groups. mRNA levels of Ptprb, Kcnj8, Crispld2, Bcl6b, and Gja5 were differentially expressed in 2VO rats on day 7, but then returned to normal, whereas mRNA levels of Vwf and Trappc6a were upregulated after day 7. Immunohistochemistry showed that GJA5 and vWF were detected in endothelial cells, KCNJ8 in endothelial cells and astrocytes, CRISPLD2 in neurons and astrocytes, and TRAPPC6A in neurons.

Conclusion: Our findings indicate novel genes that may be associated with WMLs development in the chronic cerebral hypoperfusion rat model, and suggest an important role of neurovascular dysfunction in the pathophysiology.

Introduction

Cerebral white matter lesions (WMLs), characterized by hyperintense signals on T2-weighted magnetic resonance images, are observed in healthy elderly people and patients with subcortical vascular dementia [1-5]. These WMLs are thought to be associated with cognitive impairment [2,6]. Neuropathologic changes of WMLs are characterized by rarefaction, demyelination, gliosis, microglial activation, and axonal loss [7-10]. Although the pathogenesis of WMLs remains unclear, some types of WMLs may be due to ischemic brain injury [11]. Experimentally, chronic cerebral hypoperfusion induced by permanent occlusion of the bilateral common carotid arteries in the rat causes WMLs with neuropathologic changes very similar to those of WMLs in patients with chronic cerebrovascular disease such as subcortical vascular dementia [12-17]. This rat model of chronic cerebral hypoperfusion gradually develops impairments of visuospatial learning, fear conditioning, and non-spatial memory during the chronic phase [12,18-20], and is therefore commonly used to represent ischemic WML-associated diseases such as subcortical vascular dementia [12,21]. Although cerebral ischemia is thought to trigger WML development in the chronic cerebral hypoperfusion rat model, the pathogenetic mechanisms remain unclear. Decreased cerebral blood flow (CBF) during ischemia activates or inhibits the expression of various genes that encode proteins that regulate ischemic processes. Identification of genes associated with WML development may contribute not only to elucidate the pathomechanism of WMLs, but also biomarkers and therapeutic targets of ischemic WMLs-associated diseases. In the present study, we performed comprehensive gene expression microarray analyses to investigate genes associated with early-stage development of WMLs induced by chronic cerebral hypoperfusion in the rat.

Materials and Methods

Animals and histologic analysis

All experiments were performed in accordance with the Japanese law for the humane treatment and management of animals, standards relating to the care and management of laboratory animals and relief of pain, and were approved by the Ethics Committee of Animal Research at Oita University (Permission No. K017002). Male Wistar rats (n=48; Kyudo Co, Saga, Japan) weighing 274 - 392 g (9 weeks-old) were used in this study. Animals were deeply anesthetized by intraperitoneal injection of sodium pentobarbital (40 mg/kg). During surgery, a heat lamp was applied to maintain body temperature. Through a midline cervical incision, the bilateral common carotid arteries were carefully separated from the cervical sympathetic and vagal nerves, double-ligated with 5-0 silk sutures, and cut between the ligation (2VO). The sham-operated animals underwent similar procedures, except that the bilateral common carotid arteries were not occluded. After surgery, the rats were kept in cages with food and water available ad libitum. On days 1, 7, 28 after surgery, a subgroup of animals was deeply anesthetized with sodium pentobarbital and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.01 M phosphate buffer (pH 7.4). The brains were removed and fixed in 4% paraformaldehyde in 0.01 M phosphate buffer (pH 7.4) for 12 h at 4°C, and then coronal brain blocks were embedded in paraffin. Coronal brain sections (2 μm thick) were stained with hematoxylin and eosin. The severity of the WML in the corpus callosum was graded as normal (grade 0), mild (grade 1), or marked (grade 2) by two independent investigators blinded to the treatment.

Measurement of cerebral blood flow

CBF was measured using a laser Doppler flow meter (ALF-21, Advance Co, Inc, Tokyo, Japan) as described previously [22]. Briefly, rats were deeply anesthetized by intraperitoneal injection with sodium pentobarbital (40 mg/kg) and allowed to respire spontaneously. After incising the scalp on the right side of the head, a probe (LP-CM, Unique Medical, Tokyo, Japan) was placed between the temporal muscle and the lateral aspect of the skull. CBF recordings of 2VO (n=10) or sham (n=10) rats were obtained just before surgery and on days 1, 7, and 28 after surgery.

RNA extraction

On days 1, 7, or 28 after surgery, rats were anesthetized and decapitated, the brain was removed immediately, and the whole corpus callosum was carefully dissected under a stereomicroscope. Tissues were frozen immediately in liquid nitrogen and stored at -80°C. Total RNA was extracted from frozen tissues using ISOGEN (Nippon Gene, Tokyo, Japan), and then purified using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) in accordance with the manufacturer's instructions.

Gene expression microarray

For the gene-expression microarray, 300 ng of total RNAs extracted from the corpus callosum tissues of 2VO (n=5) or sham (n=5) rats on day 7 after surgery were subjected to microarray analysis as previously described [23]. Briefly, before hybridization, the quantity and quality of the total RNA were evaluated using a spectrophotometer and 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA, USA), respectively. Cy3-labeled cRNA targets were generated using an Agilent Quick Amp Labeling Kit (Agilent Technologies).

A rat 44 K oligoarray was used for hybridization in accordance with the manufacturer's recommendations (Agilent Technologies). A laser confocal scanner (Agilent Technologies) was used to measure signal intensities in the expression microarray analysis. Feature Extraction software (Version 9.5, Agilent Technologies) with the manufacturer's recommended settings was applied for the microarray image analysis.

Gene expression analysis

Analysis of the microarray images was performed with Genespring GX10 Software (Agilent Technologies). For comparison among multiple arrays, probe set data were median-normalized per chip. Data were then filtered based on a signal intensity of 100 or greater and containing no flagged values. The 20,791 probe sets remaining after this filtering were used for further statistical evaluation. For selection of genes differentially expressed between 2VO and sham rats, probe sets were assessed by Mann-Whitney U test ($p < 0.05$). Among these differentially expressed genes, those designated as 'upregulated' were over-expressed more than 1.20-fold compared with the sham controls, and those designated as 'downregulated' were under-expressed by less than 0.83-fold compared with the sham controls. Annotations were provided by Agilent Technologies.

Quantitative reverse transcription-polymerase chain reaction

Reverse transcription was performed using a Transcriptor first-strand cDNA synthesis kit (Roche Diagnostics, Mannheim, Germany) with random hexamer primers, in accordance with the manufacturer's instructions. qRT-PCR was performed using a LightCycler 480 Realtime PCR System with a Universal probe library and a LightCycler 480 probe master (Roche Diagnostics, Penzberg, Germany), in accordance

with the manufacturer's instructions. All the primer sets for genes of interest were designed by Roche Diagnostics (Supplementary Table 1). qRT-PCR was performed using the following cycle parameters: 1 cycle of 5 min at 95°C, followed by cycles of 10 s at 95°C, 10 s at the appropriate annealing temperature, and 1 s at 72°C (Supplementary Table 1). TATA-binding protein was used as a control and relative expression levels were obtained by relative quantification analysis. Dilutions of cDNA synthesized from normal rat corpus callosum were used as standards.

Immunohistochemistry

Proteins were visualized as described previously [24]. Briefly, paraffin-embedded coronal brain sections (2-μm thick) were deparaffinized and rehydrated using standard protocols. Except for anti-CX40 antibody, sections were immersed in 10 mM sodium citrate buffer, pH 6.0 (Iatron, Tokyo, Japan), and autoclaved at 120°C for 10 min for antigen retrieval. The sections were treated with 3% H_2O_2 for 20 min at room temperature (RT) to inactivate the endogenous peroxidase activity. After blocking with 10% goat or rabbit serum (Nichirei, Tokyo, Japan), sections were incubated overnight at 4°C with the following primary antibodies diluted in diluting solution (Dako, Copenhagen, Denmark): rabbit polyclonal anti-potassium inwardly-rectifying channel, subfamily J, member 8 (KCNJ8, Kir6.1) antibody (1:15000, Novus Biologicals, Littleton, CO, USA), rabbit polyclonal anti- Cx40/GJA5 antibody (1:700, Chemicon, Temecula, CA, USA), rabbit polyclonal anti-vWF antibody (1:1000, Dako), rabbit polyclonal TRAPPC6A antibody (1:800, Santa Cruz Biotechnology, Dallas, TX, USA), mouse monoclonal anti-PTPRB antibody (clone 122.2, Abcam, Cambridge, MA, USA), goat polyclonal anti-Bcl-6b antibodies (N-12 and S-13, Santa Cruz Biotechnology), and rabbit immunoglobulin G (IgG; Dako) or mouse IgG (Vector Laboratories Inc.) for the negative control.

The sections were then washed with phosphate-buffered saline (PBS) and incubated with biotinylated anti-rabbit or anti-mouse IgG antibody (Nichirei, Tokyo, Japan) for 1 h at RT. After washing, the sections were incubated with a solution of avidin-conjugated horseradish peroxidase (Vectastain Elite ABC kit; Vector Laboratories Inc) for 15 min at RT, according to the manufacturer's recommendations, and then washed. Peroxidase activity was detected with H_2O_2/diaminobenzidine substrate solution (Vector Laboratories Inc.), and the sections were counterstained with hematoxylin before dehydration and mounting.

For the antigen competition experiments, prior to immunostaining with the anti-Cx40 antibody, anti-TRAPPC6A antibody, or anti-Bcl-6b antibody, the antibodies were preabsorbed with 10 times the amount of the mouse Cx40 control peptide (AG634, Chemicon), human TRAPPC6A GST fusion protein (ag7373, Proteintech Group, Chicago, IL, USA), or Bcl-6b blocking peptides (N-12 or S-13, Santa Cruz Biotechnology), respectively.

The number of immunoreactive vessels in the corpus callosum was counted and the numerical density of immunoreactive vessels was expressed as the number of vessels per 0.6 mm^2.

Double-labeled immunofluorescence

Paraffin-embedded coronal brain sections (2-μm thick) were deparaffinized and rehydrated using standard protocols. For antigen retrieval, sections were immersed in 10 mM sodium citrate buffer, pH 6.0 (Iatron), and boiled at 100°C for 10 min and cooled to RT. After blocking with 10% nonfat dry milk in 1xPBS, sections were incubated overnight at 4°C with a mixture of rabbit polyclonal anti-CRISPLD2 antibody (1:100 dilution, Abgent, San Diego, CA, USA) and mouse monoclonal anti-GFAP antibody (1:200 dilution, Sigma-Aldrich, St Louis, MO, USA). After washing, the sections were incubated with a mixture of Alexa

Fluor 488-conjugated goat anti-rabbit IgG (H+L) antibody (Life Technologies, Carlsbad, CA, USA) and Alexa Fluor 568-conjugated goat anti-mouse IgG (H+L) antibody (Life Technologies) for 1 h at RT. After washing, nuclei were stained with 4'-6-diamidino-2-phenylindole (Life Technologies). The sections were then washed again and mounted. The mounted sections were observed under a LSM710 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany).

For competition experiments, prior to immunostaining, anti-CRISPLD2 antibody was preabsorbed with an excess amount of CRISPLD2 antibody blocking peptide (BP5570a, Abgent).

Western blot analysis

Frozen tissue sections of the corpus callosum excised from the sham rats or 2VO rats on day 7 after the surgery were lysed in sodium dodecyl sulfate (SDS)-modified RIPA buffer (0.1% w/v SDS, 40 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid-NaOH (pH 7.4), 1% w/v Nonident p-40, 0.5% w/v sodium deoxycholate, 150 mM NaCl, 5 mM EDTA acid, 10 mM sodium fluoride, 2 mM sodium orthovanadate, and protease inhibitor cocktail; Sigma-Aldrich, St. Louis, MO, USA). After centrifuging 12,000 rpm for 20 min at 4°C, the supernatants were boiled with Laemmli's sample buffer. Protein was measured using the Bio-Rad protein assay kit (Bio-Rad Labs, Hercules, CA, USA). Fifty micrograms of total protein were separated on a 12% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). After blocking with PBS containing 10% nonfat dry milk for 1 h, the membrane was incubated overnight at 4°C with a rabbit polyclonal anti-CRISPLD2 antibody (diluted 1:500, Abgent) and then incubated for 1 h at RT with peroxidase-conjugated goat anti-rabbit IgG antibody (diluted 1:70,000; Jackson Immunoresearch Labs, West Grove, PA, USA). Signals were detected using enhanced chemiluminescence

reagents (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The same membranes were reprobed with mouse monoclonal anti-α-tubulin antibody (Sigma-Aldrich). For quantification, the signal intensities of CRISPLD2 relative to that of α-tubulin were determined by densitometry using NIH Image J (Version 1.62) software.

Statistical analysis

All CBF, histologic, immunohistochemistry, and qRT-PCR data are presented as mean ± SD. CBF values were analyzed by analysis of variance and Tukey-Kramer post hoc test. Statistical analyses of all other data were performed using the Mann-Whitney U test. A P value less than 0.05 was considered statistically significant.

Results

Characterization of chronic cerebral hypoperfusion model rats

Figure 1A shows temporal profiles of CBF values in the bilateral carotid ligation model (2VO) and sham-operated (sham) groups. Mean CBF values in the 2VO group and sham group were similar before surgery (mean ± SD ml/min/100g, 2VO : sham = 36.3 ± 4.6 : 35.8 ± 3.5). Compared with the sham group, mean CBF values in the 2VO group were significantly decreased on days 1, 7, and 28 after surgery (16.3 ± 3.9 : 34.5 ± 3.9 on day 1; 25.5 ± 3.5 : 36.3 ± 4.6 on day 7; 29.4 ± 2.5 : 38.3 ± 3.4 on day 28). In the 2VO group, mean CBF values on day 7 were significantly increased compared with those on day 1, which were significantly lower than that before surgery. The difference in the mean CBF values between days 7 and 28

was not significant. In the sham group, there was no significant difference in the mean CBF values among time points.

Histologic analysis revealed no pathologic changes in the brains of sham rats on days 1, 7, and 28 after surgery (Figure 1B-G). On day 1 after surgery, focal acute ischemic regions, consisting of rarefaction and ischemic neurons with eosinophilic cytoplasm and pyknosis were observed in the cortex or hippocampus in approximately half of the 2VO rats. The corpus callosum of these rats had mild rarefaction and some reactive astrocytes (data not shown). The other half of the 2VO rats on day 1 after surgery showed no acute ischemic regions in the brain, nor any pathologic changes in the corpus callosum (Figure 1B). No infarct or hemorrhage was observed in the brains of the 2VO rats on days 7 and 28 after surgery. In the corpus callosum of the 2VO rats on days 7 and 28 after surgery, rarefaction, activation of astrocytes, and loss of oligodendrocytes were observed (Figure 1C and 1D; WML grading score, means ± SD, 2VO : sham; day 7, n=6, 1.3 ± 0.52 : 0.17 ± 0.41, p=0.0104 ; day 28, n=5-8, 1.0 ± 0: 0.13 ± 0.35, p=0.0104).

Gene expression microarray analysis and quantitative reverse-transcription polymerase chain reaction

To identify genes associated with WMLs in the early stage of chronic cerebral hypoperfusion, we performed a gene expression microarray analysis using total RNAs extracted from the corpus callosum on day 7 after surgery. We compared the mRNA expression levels between the 2VO and sham groups with analysis of variance ($p<0.05$) and found 16 genes that were significantly differentially expressed more than 1.2-fold in the 2VO group; 10 genes were significantly upregulated and 6 genes were downregulated (Supplementary Table 2).

The expression levels of these 16 differentially expressed genes in the 2VO group were further analyzed by

quantitative reverse-transcription polymerase chain reaction (qRT-PCR) using total RNA extracted from the corpus callosum (Table 1). We compared the expression levels of genes between the 2VO and sham groups on days 1, 7, and 28 after surgery. The expression levels of the potassium inwardly-rectifying channel, subfamily J, member 8 (Kcnj8) gene and protein tyrosine phosphatase, receptor type B (Ptprb) gene were significantly increased on day 1, but were no longer significantly different on days 7 and 28 after surgery in the 2VO group. The expression level of the cysteine-rich secretory protein LCCL domain containing 2 (Crispld2) gene was significantly increased on days 1 and 7, but was no longer significantly different on day 28 after surgery in the 2VO group. The expression level of the gap junction protein alpha5 (Gja5) gene tended to increase on day 1, was significantly increased on day 7, but was not significantly different on day 28 after surgery in the 2VO group. The expression level of the B-cell CLL/lymphoma 6, member B (Bcl6b) gene was significantly increased on day 1, significantly decreased on day 7, and no longer significantly different on day 28 after surgery in the 2VO group. The expression level of the von Willebrand factor homologue (Vwf) gene was not significantly different on at day 1, but was significantly increased on days 7 and 28 after surgery in the 2VO group. The expression level of the trafficking protein particle complex 6a (Trappc6a) gene was not significantly different on days 1 and 7, but was significantly increased on day 28 after surgery in the 2VO group. The expression levels of the eukaryotic translation initiation factor 4E member 3 (Eif4e3) gene and tensin 1 (Tns1) gene tended to be increased on day 7, but were not significantly different on days 1 or 28 after surgery in the 2VO group. The expression levels of the other genes showed no significant differences on days 1, 7, or 28 after surgery.

Immunohistochemistry and immunofluorescence

To investigate cells expressing proteins encoded by genes differentially expressed in the 2VO groups in vivo, we performed immunohistochemical analysis on cerebral tissue sections including the corpus callosum. Cells expressing the KCNJ8, connexin 40 (Cx40)/ gap junction alpha-5 (GJA5), vWF, TRAPPC6A, and CRISPLD2 in the cerebrum of 2VO rats were similar to those in sham rats on days 1, 7, and 28 after surgery. Positive immunoreactivity for KCNJ8 was observed in the vascular endothelial cells (arrowheads in Figures 2A and 2C) and astrocytes (arrows in Figures 2A and 2C). The difference in the density of KCNJ8-positive vessels in the corpus callosum between the 2VO rats and sham rats was not significant (means ± SD, 2VO : sham, 9.7 ± 3.0 : 8.9 ± 5.8 vessels / 0.6 mm^2, p=0.631, n=6). Positive immunoreactivity for Cx40/GJA5 was observed in the vascular endothelial cells (Figures 3A, 3B, 3D, and 3E). The difference in the density of Cx40/GJA5-positive vessels in the corpus callosum between the 2VO rats and sham rats was not significant (means ± SD, 2VO : sham, 1.5 ± 0.53 : 1.2 ± 0.57 vessels / 0.6mm^2, p=0.42, n=6). Positive immunoreactivity for KCNJ8 and Cx40/GJA5 was also detected in the cytoplasm of vascular smooth muscles. Positive immunoreactivity for vWF was detected in the vascular endothelial cells (Figures 4A and 4C). The difference in the density of vWF-positive vessels in the corpus callosum between the 2VO rats and sham rats was not significant (means ± SD, 2VO : sham, 37.0 ± 11.0 : 40.0 ± 12.1 vessels / 0.6 mm^2, p=0.584, n=6). Positive immunoreactivity for TRAPPC6A was not detected in the corpus callosum, but was detected in the cytoplasm of the pyramidal neurons in the cerebral cortex and hippocampus (Figures 5A, 5C, 5E, and 5G). Nonspecific immunoreactivity was unlikely because the positive immunoreactivity was no longer observed in antigen competition experiments (Figures 3C, 3F, 5B, 5D, 5F and 5H) or with negative control antibody (Figures 2B, 2D, 4B, and 4D). Double-labeled immunofluorescence revealed positive immunoreactivity for

CRISPLD2 in the nucleus of glial fibrillary acidic protein (GFAP)-positive glial cells (indicating astrocytes; Figures 6A-F), GFAP-negative glial cells (Figures 6A-F), and neurons (Figures 6J-O). Nonspecific immunoreactivity was unlikely because the positive immunoreactivity for CRISPLD2 was significantly decreased in antigen competition experiments (Figures 6G-I and 6P-R). Western blotting analysis with the anti-CRISPLD2 antibody revealed no significant difference in the CRISPLD2 protein level relative to α-tubulin between the 2VO rats (n=5) and sham rats (n=6; Figures 6S and 6T). Positive immunoreactivity for protein tyrosine phosphatase, receptor type B and Bcl-6b was not observed with the antibodies used in this study.

Discussion

In the present study, we investigated genes associated with early phase WMLs in the 2VO rat, using a comprehensive gene expression microarray analysis. The corpus callosum serves as the most common and easily delineated region of interest for white matter research in the 2VO rat [12]. Temporal changes in CBF values and neuropathologic changes in the corpus callosum of the 2VO rat in the present study were consistent with previous findings [12]. At 7 days after the 2VO surgery, CBF values dynamically decreased and then recovered, and neuropathologic changes in the corpus callosum were minimal. After day 7 following 2VO surgery, CBF gradually stabilized, and neuropathologic changes in the corpus callosum were observed. Based on these findings, we performed a comprehensive gene expression analysis using RNA extracted from the corpus callosum on day 7, when CBF values stabilized and neuropathologic changes in the corpus callosum were observed. Based on the analysis of temporal changes in gene expression by

qRT-PCR, genes differentially expressed in the 2VO group at day 7 were classified into two groups: five genes, Ptprb, Kcnj8, Crispld2, Bcl6b, and Gja5, were upregulated within 7 days after surgery and returned to normal levels after day 7; two genes, Vwf and Trappc6a, were upregulated after day 7. These findings suggest that the expression of these genes in the corpus callosum of the 2VO rats was dysregulated in accordance with the degree of changes in the CBF values and neuropathologic changes in the corpus callosum.

Of the seven genes differentially expressed in the 2VO rats in the present study, the proteins encoded by Kcnj8, Gja5, and Vwf genes are expressed in endothelial cells, as shown in the present study, and the Ptprb gene encodes an endothelial-specific membrane protein, vascular endothelial protein tyrosine phosphatase (VE-PTP) [25,26]. The cerebral endothelium plays a crucial role in regulating cerebral blood flow and in the blood brain barrier (BBB). Disruption of the BBB is observed in chronic cerebral hypoperfusion animals as well as in patients with WMLs [27-31]. Endothelial activation and an increase in biomarkers of endothelial dysfunction are observed in patients with small vessel disease such as lacunar infarction and leukoaraiosis [32-34]. Our findings suggest that endothelial dysfunction has an important role in the development of WMLs in the 2VO rats.

The inwardly rectifying K^+ channel Kir6.1, encoded by the Kcnj8 gene, is expressed in vascular endothelial cells and vascular smooth muscle cells, and forms ATP-sensitive K+ channels by coupling with a sulfonylurea receptor 2B [35-37]. Kir6.1 is also expressed in astrocytes [38]. A study of KCNJ8-null mice indicated that Kir6.1-containing K^+ channels are critical for the regulation of vascular tonus and for vasodilation (Miki et al., 2002). The Cx40/GJA5 protein, encoded by Gja5 gene, is a gap-junction protein [39,40]. Cx40/GJA5 is expressed in endothelial cells, smooth muscle cells of arteries including cerebral

arteries, and the myocardium [41-44]. Cx40/GJA5 expressed in endothelial cells plays a role in vasodilation, like nitric oxide and prostaglandins [45-48]. In addition, Fang and colleagues reported that Cx40/GJA5 is necessary for postischemic tissue survival and reperfusion because Cx40-deficient mice experience a severe reduction in limb perfusion and exhibit profound and rapid failure of ischemic limb survival after femoral-saphenous artery-vein pair resection of a unilateral hindlimb compared to wild-type mice [49]. Recovery of CBF values in 2VO rats between days 1 and 7 after surgery despite permanent occlusion of the bilateral common carotid arteries in the present study suggests the existence of compensatory mechanisms. Compensatory blood flow may be provided through artery dilation, recruitment of nonperfused capillaries, remodeling of microvessels, and arteriogenesis [12]. We speculate that Kir6.1 and Cx40/GJA5 are associated with the regulation of CBF in 2VO rats between days 1 and day 7 after surgery via vasodilation.

vWF is a multimeric glycoprotein that is exclusively synthesized in endothelial cells and megakaryocytes [50]. Along with serving as a protective carrier molecule for clotting factor VIII, its main function is mediating initial platelet adhesion at sites of vascular injury [51]. Our finding that upregulation of Vwf was associated with neuropathologic changes in the corpus callosum after day 7 suggests that vWF is associated with the development of WMLs. Recent findings that the severity of WMLs is associated with increased plasma levels and activity of vWF in elderly people support our hypothesis [33,34]. The role of vWF in the development of WMLs remains unclear, although it has been reported that vWF-deficient mice are protected from brain ischemia/reperfusion injury, indicating that vWF is critically involved in cerebral ischemia [51-53]. BBB permeability in experimental allergic encephalomyelitis is significantly increased in mice with a disrupted Vwf gene compared with wild-type mice, indicating that vWF negatively regulates BBB permeability [54]. We speculate that vWF is associated with regulation of the BBB in the development of

WMLs.

Permanent occlusion of the rodent unilateral middle cerebral artery (MCAO) causes cerebral infarction in the ipsilateral hemisphere due to cessation of blood flow. In 2VO rats, CBF is decreased but maintained and there is no infarction in the brain, as demonstrated in the present study and by Farkas et al [12]. Hori and colleagues investigated gene expression profiles in the brain of the MCAO-mouse by gene expression microarray analysis, and reported upregulation of Kcnj8, Gja5, B-cell chronic lymphocytic leukemia/lymphoma 6 (Bcl6b)/Bcl6-associated zinc finger protein (BAZF), and crispld2/late gestation lung 1 (Lgl1) genes among the differentially expressed genes in the ischemic hemisphere 24 h after MCAO [55]. Both our findings and theirs suggest that Kcnj8, Gja5, Bcl6b/BAZF, and Crispld2/Lgl1 genes are induced in the acute phase of ischemia by both hypoperfusion and nonperfusion. In contrast, they reported downregulation of Ptprb/VE-PTP in the ischemic hemisphere 24 h after MCAO, whereas in the present study Ptprb/VE-PTP was upregulated on day 1 [55]. We speculate that Ptprb/VE-PTP expression is dysregulated differently in hypoperfusion than in nonperfusion. Downregulation of PTPRB/VE-PTP expression enhances endothelial cell permeability, augments leukocyte transmigration, and inhibits VE-cadherin-mediated adhesion, indicating that VE-PTP is required for endothelial cell contact integrity [56]. These findings suggest that differences in the expression of PTPRB/VE-PTP are associated with differences in neuropathologic changes, such as infarct.

CRISPLD2/Lgl1, a glycoprotein, was identified in the fibroblasts of fetal rat lung [57, 58]. Expression of the Crispld2 gene is high in the adult rat lung, heart, and spleen, and in human leukocytes, lung, heart, and intestine, but low in the human brain [57,59]. CRISPLD2/Lgl1 null mice are embryonic lethal by embryonic day 9.5 [60]. CRISPLD2/Lgl1 heterozygous knockout mice appear grossly normal, but lung maturation is

delayed and ureteric bud branching is reduced at the fetal stage [60,61]. Chiquet and colleagues reported an association between the CRISPLD2 gene and non-syndromic cleft lip with or without cleft palate [62]. Yuan and colleagues reported that knockdown of crispld2 results in aberrant patterning of neural crest cells in zebrafish [63]. These findings suggest an essential role of CRISPLD2/Lgl1 in the development of various tissues, including the central nervous system. The function of CRISPLD2/Lgl1 in the central nervous system of adult animals and the role of CRISPLD2/Lgl1 in ischemic conditions, however, remain to be elucidated. Our findings that CRISPLD2/Lgl1 was expressed in astrocytes suggest that induction of CRISPLD2/Lgl1 is associated with the activation of astrocytes in the cerebral white matter in the acute phase of cerebral ischemia.

TRAPPC6A is a subunit of the transport protein particle (TRAPP) I and Π complexes. TRAPP complexes are essential for endoplasmic reticulum-to-Golgi (TRAPP I) and intra-Golgi (TRAPP II) vesicle trafficking [64,65]. Mutations of the Trappc6a gene lead to defects in melanosome formation, implicating the involvement of mammalian TRAPPC6A in vesicle trafficking during melanosome biogenesis [66]. Although the Trappc6a gene is widely expressed, including in the brain [66], the localization and role of TRAPPC6A in the brain remain to be elucidated. The present study revealed that TRAPPC6A localized in the cytoplasm of pyramidal neurons in the cortex and the hippocampus of the adult rat brain. The finding that TRAPPC6A was detected exclusively in neurons suggests that TRAPPC6A is associated with neuronal dysfunction due to chronic hypoperfusion rather than the development of WMLs in the 2VO rat. The recent report that one haplotype from TRAPPC6A is associated with nonverbal reasoning in two cohorts of patients with Alzheimer's disease [67] interests us with regard to the association between TRAPPC6A and cognitive function.

BAZF, encoded by the Bcl6b gene, functions as a transcriptional repressor [68]. BAZF/Bcl6b mRNA in mouse tissues is expressed in the heart, the lung, and activated lymphocytes [68], and is also detected in human umbilical vein endothelial cells [69]. We demonstrated that BAZF/Bcl6b mRNA was expressed in the cerebral white matter of the rat. Our findings that expression of the BAZF/Bcl6b gene was upregulated on day 1, downregulated on day 7, and returned to the normal level on day 28, suggest that dysregulation of BAZF/Bcl6b gene expression in 2VO rats is associated with changes in CBF. Impaired angiogenesis and capillary remodeling, which results in delayed wound healing, are observed in skin-wounded BAZF-deficient mice [69], suggesting that BAZF/BCL6B plays a role in tissue repair and angiogenesis. We speculate that BAZF/ BCL6B has a protective role against acute ischemic damage in 2VO rats.

In the present study, we demonstrated that genes dysregulated by chronic cerebral hypoperfusion were expressed in endothelial cells, glial cells, and neurons, which form the neurovascular unit. The neurovascular unit controls BBB permeability and CBF, and maintains the chemical composition of the neuronal 'milieu', which is required for proper functioning of neuronal circuits [70]. Ueno and colleagues reported that chronic cerebral hypoperfusion induces an increase in BBB permeability to horseradish peroxidase, which appears by day 1, peaks on day 3, and recovers by day 7 in the corpus callosum of 2VO rats [29]. In chronic cerebral hypoperfusion, decreased CBF also recovers by day 7, while WMLs consistently develop, as demonstrated in the present study. In addition, we demonstrated that the expression of genes in the corpus callosum of 2VO rats on day 7 was dysregulated based on CBF values and neuropathologic changes. We also demonstrated that there are few differences in the expression of proteins encoded by these dysregulated genes between 2VO rats and sham rats, suggesting that insufficient posttranscriptional control, translation, or posttranslational modifications of these genes is associated with dysfunction of the neurovascular unit. A

comprehensive understanding of the pathophysiology of the neurovascular unit in chronic cerebral hypoperfusion is crucial toward the discovery of therapeutic targets.

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Author Contribution statement

YA contributed to design and performance experiments, acquisition and analysis of data, writing of the manuscript. KN and NK contributed to design, performance and supervise experiments, analysis of data, writing and review the manuscript. MT contributed to performance experiments. RA contributed to analysis of data. TK, HT, and ME contributed to design and supervision of the study, review of manuscript. MF contributed to review of manuscript.

Disclosure/Conflict of Interest

The authors declare no conflict of interest.

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Titles and legends to figures

Figure 1. Cerebral blood flow (CBF) and white matter pathology of 2VO rats. (A) Temporal profile of CBF. Values are means ± SD, n=10 per group. *$p<0.05$ compared to the sham-operated group. "pre" indicates the CBF before surgery. (B-G) Photomicrographs of hematoxylin and eosin stained sections of the paramedian corpus callosum from 2VO rats (B, C, and D) and sham rats (E, F, and G) on days 1 (B and E), 7 (C and F), and 28 (D and G) after surgery. Bars indicate 50 μm.

Figure 2. Immunohistochemistry for KCNJ8. (A-D) Representative photomicrographs of the paramedian corpus callosum of sham rats (A and B) and 2VO rats (C and D) on day 1 after surgery are shown. Positive immunoreactivity for KCNJ8 was observed in the vascular endothelial cells (arrowheads) and astrocytes (arrows). No immunoreactivity was observed with the negative control antibody (B and D). Cells expressing KCNJ8 in the cerebrum of the 2VO rats were similar to those in the sham rats on days 1, 7, and 28 after surgery. Bars indicates 20 μm.

Figure 3. Immunohistochemistry for Cx40/GJA5. (A-F) Representative photomicrographs of the paramedian corpus callosum of sham rats (A, B, and C) and 2VO rats (D, E, and F) on day 7 after surgery are shown. B and E correspond to the boxed are in A and D, respectively. Positive immunoreactivity for Cx40/GJA5 was observed in the vascular endothelial cells (arrowheads in B and E), and was blocked in the antigen

competition experiment (C and F). Cells expressing Cx40/GJA5 in the cerebrum of the 2VO rats were similar to those of the sham rats on days 1, 7, and 28 after surgery. Bars indicate 20 μm.

Figure 4. Immunohistochemistry for vWF. (A-D) Representative photomicrographs of the paramedian corpus callosum of sham rats (A and B) and 2VO rats (C and D) on day 28 (F and G) after surgery are shown. Positive immunoreactivity for vWF was observed in the vascular endothelial cells (arrowheads in A and C). No immunoreactivity was detected with the negative control antibody (B and D). Cells expressing vWF in the cerebrum of the 2VO rats were similar to those in the sham rats on days 1, 7, and 28 after surgery. Bar indicates 20 μm.

Figure 5. Immunohistochemistry for TRAPPC6A. Representative photomicrographs of the cerebral cortex (A, B, C, and D) and hippocampus (E, F, G, and H) of sham rats (A, B, E, and F) and 2VO rats (C, D, G, and H) on day 28 after surgery are shown. Positive immunoreactivity for TRAPPC6A was detected in the cytoplasm of pyramidal neurons. The positive immunoreactivity for TRAPPC6A was blocked in the antigen competition experiments (B, D, F, and H). Cells expressing TRAPPC6A in the cerebrum of the 2VO rats were similar to those in the sham rats on days 1, 7, and 28 after surgery. Bar indicates 50 μm.

Figure 6. Expression of CRISPLD2 protein. (A-R) Immunofluorescence for CRISPLD2. Coronal sections

from the cerebrum of sham rats (A-C and J-L) and 2VO rats (D-I and M-R) on days 28 after surgery were immunostained with anti-CRISPLD2 antibody (green) together with anti-GFAP antibody (red). Nuclei were stained with 4'-6-diamidino-2-phenylindole (blue). Representative images in the paramedian corpus callosum (A-I) and cerebral cortex (cingulate cortex) (J-R) are shown. Positive immunoreactivity for CRISPLD2 was blocked in the antigen competition experiments (G, I, P, and R). Cells expressing CRISPLD2 in the cerebrum of the 2VO rats were similar to those in the sham rats on days 1, 7, and 28 after surgery. Bars indicate 10 μm. (S, T) Western blotting for CRISPLD2. (S) Representative images of the corpus callosum immunoreactive for CRISPLD2. Total proteins extracted from the corpus callosum of 2VO rats (lanes 1 and 2) or sham rats (lanes 3 and 4) on day 7 after surgery were analyzed by Western blotting with the anti-CRISPLD2 antibody (upper panel). After the detection, the same membrane was reprobed with the anti-α-tubulin antibody (lower panel). (T) For quantification, the CRISPLD2 protein level relative to α-tubulin was determined by densitometry. The difference between the 2VO rats (n=5) and the sham rats (n=6) was not significant (means ± SD, 2VO : sham, 0.61 ± 0.15 : 0.96 ± 0.42 vessels/0.6 mm^2, p=0.17, Mann-Whitney U test). N.S. means not significant.