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Research Paper

Expression and distribution of generated neurons and endogenous precursors in rat cerebral cortical venous ischemia

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ABSTRACT

Neurogenesis in the subventricular zone (SVZ), subgranular zone (SGZ), and cerebral cortex is now a familiar event to confirm by cerebral arterial ischemia in rat models. However, it remains unclear whether cerebral venous ischemia (CVI) alone causes neurogenesis, and where that neurogenesis occurs. After creating CVI rat models via a two-vein occlusion (2-VO) method, neurogenesis was immunohistochemically evaluated by double-labeling 5-bromo-2′-deoxyuridine (BrdU)-positive cells with neuronal nuclei (NeuN) or doublecortin (DCX) antibody. Fifty Wistar rats were divided into two major groups (BrdU-NeuN and BrdU-DCX) and then separated into two subgroups (2-VO or sham). The total number of double-positive cells expressed inside a predefined region of interest (ROI) covering the ischemic area was compared between the two subgroups. Then, we divided the ROI into six sections to evaluate and compare the distribution of double-positive cells generated in each section between the two subgroups. The 2-VO subgroup presented more double-positive cells than the sham group in both BrdU-NeuN and BrdU-DCX groups, while the BrdU-DCX+2-VO group showed a characteristic distribution of double-positive cells in ROI 2 and ROI 3, suggesting areas of the ischemic core and penumbra, with a significant difference compared to the BrdU-DCX+sham group. This study demonstrates that CVI has the potential to induce endogenous neurogenesis, with significant numbers of both newly generated neurons and precursors observed in the ischemic area. The distribution of these cells suggests that the cortex could be the main origin of neurogenesis after cortical CVI.

1. Introduction

Endogenous neurogenesis in the adult brain is no longer a novel phenomenon, as neuronal stem cells can be driven from specific locations under peculiar conditions. Neurogenesis in at least two lesions, the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the hippocampus, is now a familiar event that can be confirmed after focal brain ischemia, especially in cerebral arterial infarction (CAI) rat models (Arvidsson et al., 2002; Wang et al., 2011; Zhang et al., 2001). In addition, there are increasing reports suggesting that neural stem cells also exist in the cerebral cortex, caudate nucleus, and striatum (Bernier et al., 2002; Gould et al., 1999; Gu et al., 2000). However, unlike the SVZ and SGZ, there is a relatively low level of neurogenesis under physiological conditions in the latter regions (Tamaki et al., 2017).

Cerebral venous ischemia (CVI) is a rare cause of cerebral stroke that differs substantially in its mechanism and outcome. However, CVI is potentially a significant complication of neurosurgical procedures in daily clinical settings, as we often face poor outcomes by injuring cortical and bridging veins during surgery (Sindou et al., 2005). There has also been considerable interest in CVI due to the development of skull base neurosurgery (Adachi et al., 2017; Mizutani et al., 2018).

According to previous basic studies, the local pathogenesis of CVI and CAI is quite different. Unlike CAI, which presents severe ischemia in a brief period and causes necrotic cell death, CVI is characterized by prolonged slow ischemia with a small ischemic core and extensive penumbra (Kimura et al., 2005; Takeshima et al., 2015). It is already known that the morphology of cell death in CVI is different from that in CAI (Nishioka et al., 2006), which suggests that neurogenesis in CVI may also be different.

Most of the previous reports on the induction of neurogenesis via cerebral ischemia are based on CAI alone or by making a

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photothrombotic stroke on the cortex, indiscriminately blocking blood flow of both cerebral arteries and veins (Arvidsson et al., 2002; Gu et al., 2000). Recently, Tamaki et al. reported that neurogenesis occurs in the rat brain cortex by modulating transient cortical spreading depression (CSD) with additional CVI (Tamaki et al., 2017). However, it remains unclear whether CVI alone causes neurogenesis, and where neurogenesis occurs in the cortex of a rat brain.

This study aimed to investigate whether endogenous neurogenesis was induced by CVI alone, obtain a novel finding of neurogenetic distribution in CVI rat models, and explore the origin of endogenous neural stem cells.

2. Material and methods

2.1. Animals

Fifty Wistar rats (7–8 weeks old, CLEA Japan, Inc., Osaka, Japan), weighing 250–300 g, were used in this study. The rats were housed and maintained on a 12-hour light-dark cycle in individual cages with free access to food and water.

All experimental protocols were approved by the Animal Welfare Committee of Nara Medical University (approval numbers: 12246, 12471, 12535, 12554, 12721, 12729).

2.2. Surgical preparation

The rats were anesthetized with chloral hydrate (36 mg/100 g body weight) via intraperitoneal injection after premedication with 0.5 mg atropine sulfate. Mechanical ventilation (model 683; Harvard Apparatus, Holliston, Massachusetts, USA) was maintained during the procedure after intubating with a silicon tube (outer diameter, 2.5 mm). Additional shots of chloral hydrate (12 mg/100 g body weight) were injected through a peritoneal catheter every hour to maintain anesthesia. The rectal temperature was maintained at 37 °C using a feedback-controlled heating pad (CMA 150; Carnegie Medicine AB, Stockholm, Sweden). An arterial line inserted into the tail artery was used for blood gas sampling and continuous monitoring of mean arterial blood pressure during the vein occlusion procedure. PaO_2, $PaCO_2$, and arterial pH were measured using a blood gas analyzer (ABL330; Radiometer, Copenhagen, Denmark), while arterial blood pressure was continuously monitored through an intra-arterial catheter connected to a pressure transducer (Polygraph System RM-600; Nihon Koden, Tokyo, Japan). A polyethylene catheter (Biomark Center, Inc., Osaka, Japan) was inserted into the right femoral vein for drug administration. After fixing the rats to a stereotactic frame (SR-6; Narishige Inc., Tokyo, Japan), a left parietal cranial window just caudal from the coronal suture was carefully created to expose the cortical veins. The dura mater was intact.

2.3. Cerebral venous infarction

We chose the previously described rat two-vein occlusion (2-VO) method (Kimura et al., 2005; Takeshima et al., 2011), which can create CVI within an interaural line distance of 6.5–9.0 mm. Two adjacent superficial bridging veins near superior sagittal sinus were occluded using Rose Bengal dye (50 mg/kg) in combination with fiber optic illumination (100-W mercury lamp [6500–7500 lx, 540 nm]). Rose Bengal dye was slowly injected intravenously through the right femoral catheter. The target veins were pinpoint illuminated for 10 min each, which formed intravascular clots to block the venous stream. The sham group had the same protocol as above, except for the illumination procedure, in which none of the cortical veins were occluded. After skin suturing, animals were returned to their cages.

2.4. BrdU injection and immunofluorescence staining

Neurogenesis was immunohistochemically evaluated by double-labeling 5-bromo-2′-deoxyuridine (BrdU)-positive cells with neuronal nuclei (NeuN) or doublecortin (DCX) antibody. NeuN is a biomarker specifically expressed in neurons, while DCX is a biomarker of neural precursor cells. Fifty male Wistar rats were randomly divided into two major groups: BrdU-NeuN (n = 25) and BrdU-DCX (n = 25). The two groups were then separated into two subgroups according to the procedure they underwent: the 2-VO group (n = 15) or the sham group (n = 10). BrdU, a thymidine analog that labels dividing cells, was intraperitoneally injected into all groups at 50 mg/kg, twice per day after the operation. The BrdU-NeuN group received shots during days 4–6, while the BrdU-DCX group received shots during days 1–14. Euthanasia was performed on day 35 in the BrdU-NeuN group and on day 15 in the BrdU-DCX group, followed by transcardiac infusion of saline. The dosage and period of BrdU injection were based on a previous study as there are no optimal data for BrdU injection in venous infarction (Arvidsson et al., 2002). The brains were excised, cut into coronal blocks containing the parietal ischemic area (2 mm thick, 5 mm caudal from the coronal suture), and embedded in optimal cutting temperature compound. Brain Section (3 μm thick) were prepared using a cryostat.

The sections were then incubated with primary and secondary antibodies. BrdU detection was performed using sheep polyclonal primary antibody (1.25:100; Anti-BrdU antibody–Proliferation Marker ab 1893, Abcam plc, Cambridge, UK) and donkey anti-sheep secondary antibody (1:200; Alexa Fluor™ 488 donkey anti-sheep IgG, Invitrogen, Thermo Fisher Scientific, Oregon). Mouse monoclonal primary antibody (1:100; Anti-NeuN–clone A60, Millipore Corp, CA, USA) and goat anti-mouse secondary antibody (1:200; Alexa Fluor™ 546 goat anti-mouse IgG, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) were used to stain NeuN. The antibodies for DCX were mouse primary antibody (1.2:200; mouse monoclonal antibody to DCX [3E1]: IgG–M-1648–100, Biosensis, Thebarton, SA) and goat anti-mouse secondary antibody (1:200; Alexa Fluor™ 546 goat anti-mouse IgG, Invitrogen, Thermo Fisher Scientific, Oregon).

2.5. Histopathological analysis and cell count

After prefixing a large region of interest (ROI) covering the entire ischemic region on the surgical side, BrdU-NeuN or BrdU-DCX double-positive cells expressed in the ROI were manually counted cell-by-cell with a fluorescence microscope (BZ-X710; KEYENCE, Osaka, Japan) using a × 20 objective. The ROI was divided into six sections (ROI 1–6, 1.0 × 0.75 mm^2 each) for distributional evaluation (Fig. 1). In detail, by fixing ROI 2 in the center of the ischemic region, 3 × 2 ROI sections covered the entire insulted surface and cerebral cortex below, and the ROI was identical in all rats. ROI sections on the contralateral side were set as mirror images of the surgical side.

To investigate endogenous neurogenesis induced by CVI, the total number of BrdU-NeuN double-positive cells in the whole ROI was independently compared between the ipsilateral and contralateral subgroups. To assess the expression of endogenous precursors, the total number of BrdU-DCX double-positive cells was independently compared ipsilaterally and contralaterally as described above. For the analysis of neurogenetic distribution, we evaluated the homogeneity of double-positive cell expression per subdivided ROIs on the surgical side and compared the number of BrdU-NeuN or BrdU-DCX double-positive cells located in each ROI between the two subgroups.

2.6. Statistical analysis

All statistical analyses were performed using IBM SPSS 26 (IBM Corp, Armonk, New York, USA). Physiological variables were compared using paired t-tests. An unpaired t-test was used to compare the number of double-positive cells generated between the 2-VO group and sham group

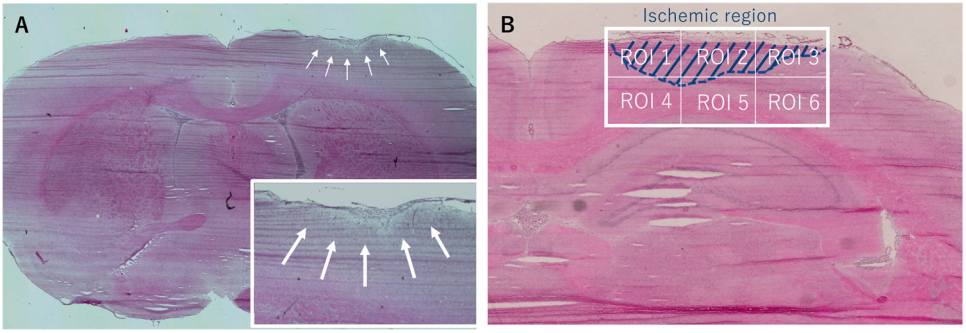

Fig. 1. Microscopic photograms after two-vein occlusion (2-VO) and region of interest (ROI) setting. A: Ischemic damage due to 2-VO is shown on the surgical side of the brain (white arrows). The magnification of the whole brain is × 4 objective while the inset is × 20 objective. B: ROI setting on the ischemic cortex is shown. Six Section (1.0 × 0.75 mm^2 each) are prepared to cover the whole insulted area and nearby cortex. The whole ROI is fixed by placing ROI 2 at the center of the ischemic region, including the surface of the cortex. ROI 4–6 are located below ROI 1–3. There is no difference of ROIs' size and location among individuals. The magnification is × 20 objective.

on each side. The Mann–Whitney U-test was used to compare the number of double-positive cells generated between the surgical side and contralateral side in the 2-VO group or the sham group. The Kruskal–Wallis H-test was also used for the evaluation of homogeneity of double-positive cell expression per subdivided ROIs. Data are presented as mean ± SEM. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Physiological data

Thirty specimens were available for the evaluation from the 50 male Wistar rats that entered the protocol. Ten rats that were intolerant to anesthesia were dropped out during the early phase. Ten brain sections that were too damaged because of either surgical or histopathological management were excluded. Consequently, 30 specimens were available for immunohistochemical evaluation (Fig. 2), and venous ischemia was confirmed in all 2-VO specimens within an interaural line distance of 6.7–8.6 mm. The average of body weight was 278.5 ± 2.8 g with minimal weight difference and only a little gain was observed before and after BrdU injections (BrdU-NeuN group: 309.4 ± 3.9 g, BrdU-DCX group: 308.7 ± 4.5 g). The mean arterial blood pressure and blood gas levels (PaO_2, $PaCO_2$, and pH) also showed no statistical differences before and after the 2-VO procedure (data not shown).

3.2. Immunohistochemical evaluation for neurogenesis and endogenous precursors

Immunofluorescence staining demonstrated BrdU-NeuN double-positive cells and BrdU-DCX double-positive cells in the ischemic area of each major group (Fig. 3).

In the assessment of the total number of BrdU-NeuN double-positive cells on the surgical side, the BrdU-NeuN+ 2-VO group showed approximately 10-fold higher double-positive cells than the BrdU-NeuN+sham group, with significant differences (37.7 ± 13.4 vs. 3.9 ± 1.4 cells, $p = 0.02$). On the contralateral side, there was also a similar trend of BrdU-NeuN double-positive cell generation between the two subgroups, with significant differences (8.4 ± 2.5 vs. 1.9 ± 1.1 cells, $p = 0.04$). In an additional assessment between the surgical and contralateral side, the total number of double-positive cells in the BrdU-NeuN+ 2-VO group showed a significant difference between the sides ($p = 0.03$); however, the contralateral side expressed only 22.3% of the cells on the surgical side (Fig. 4A).

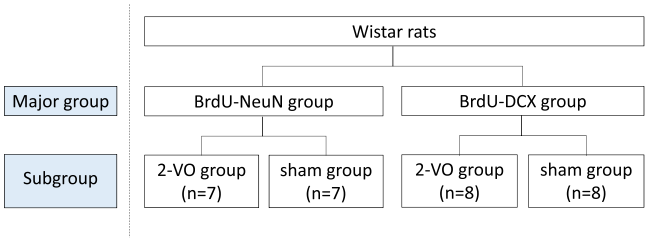

Fig. 2. Flow diagram of the group assignment in this study. Fifty male Wistar rats are prepared and randomly divided into two major groups: BrdU-NeuN (n = 25) and BrdU-DCX (n = 25). The two groups are then divided into two subgroups according to the procedure they underwent: the 2-VO group and the sham group. Individuals who are intolerant to anesthesia and those with extremely damaged brain sections are excluded from the protocol. Thirty specimens are available for immunohistochemical evaluation. 2-VO, two-vein occlusion.

In the assessment of the total number of BrdU-DCX double-positive cells, statistical differences were observed only on the surgical side of the BrdU-DCX+ 2-VO group between the two subgroups (61.6 ± 18.1 vs. 7.4 ± 3.0 cells, $p = 0.01$). The contralateral side barely showed a statistical difference between the two subgroups (26.6 ± 11.1 vs. 5.5 ± 2.4 cells, $p = 0.05$). In the assessment between the surgical and contralateral side, the total number of double-positive cells in the BrdU-DCX+ 2-VO group did not show any differences between the two sides ($p = 0.06$) (Fig. 4B).

3.3. Distribution of double-positive cells

BrdU-NeuN or BrdU-DCX double-positive cells were detectable in all six ROI sections on the surgical side (Table 1). However, in the evaluation of the homogeneity of double-positive cell expression per ROI, the BrdU-DCX+ 2-VO group showed statistical differences ($p = 0.006$), while the BrdU-NeuN+ 2-VO group did not ($p = 0.20$).

In the comparison of the number of double-positive cells located in each ROI, the BrdU-NeuN+ 2VO group generated more double-positive cells than the sham group in ROI 2 and 3 (ROI 2, 8.3 ± 3.9 vs. 0.9 ± 0.5 cells, $p = 0.06$; ROI 3, 11.3 ± 5.4 vs. 0.6 ± 0.3 cells, $p = 0.05$), but there were no significant differences between the two subgroups in any of the six ROI sections. However, the BrdU-DCX+ 2-VO group showed almost 67% of the whole number of double-positive cells located in ROI 2 and 3, with significant differences compared to that of the sham group (ROI 2, 19.5 ± 4.5 vs. 1.4 ± 0.9 cells, $p = 0.002$; ROI 3, 22.0 ± 7.0 vs. 1.1 ± 0.8 cells, $p = 0.01$). Some double-positive cells in the BrdU-DCX+ 2-VO group were found in ROI 4 and 5 near the SVZ or SGZ, but there was no significant difference compared to the sham group (ROI 4; 9.0 ± 8.4 vs. 0.1 ± 0.1 cells, $p = 0.16$, ROI 5; 1.5 ± 0.7 vs. 0.6 ± 0.3 cells, $p = 0.14$). The distributions of these double-positive cells were similar in that they both tended to be located in the cerebral cortex just below and lateral to the insulted surface of the ischemic area (Fig. 5).

4. Discussion

This study was designed to confirm whether CVI alone can induce neurogenesis. Our results demonstrate the potential of CVI to generate new neurons and endogenous precursors in the ischemic core and its

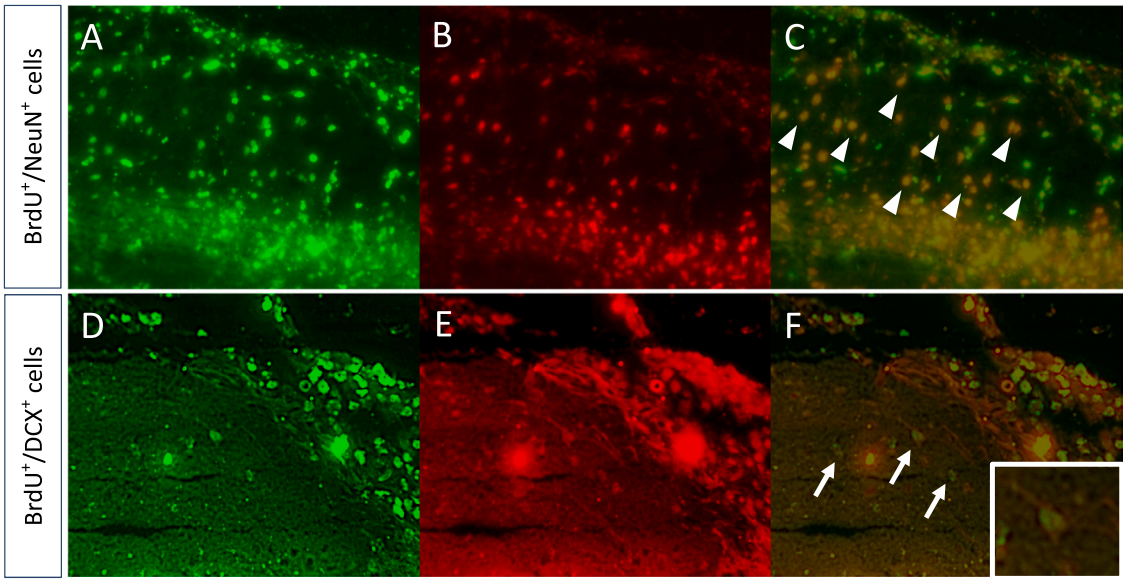

Fig. 3. Immunofluorescence micrographs of generated neurons and endogenous precursors in the insulted cortex. A: Newly generated cells on day 35 after CVI via the 2-VO procedure are shown as green BrdU-positive cells. B: Neurons are indicated by red NeuN-positive cells. C: Co-localization of BrdU-positive cells and NeuN-positive cells by merging micrographs to identify the generated neurons (arrowheads). D: BrdU-positive cells on day 15 after 2-VO. E: Red DCX-positive cells indicate the neural precursors. F: BrdU-DCX double-positive cells (arrows) are newly formed neural precursors awaiting cell replacement. A typical double-positive cell is presented in the inset. magnification used is × 20 objective. CVI, cerebral venous infarction; 2-VO, two-vein occlusion; BrdU, 5-bromo-2′-deoxyuridine; NeuN, neuronal nuclei; DCX, doublecortin.

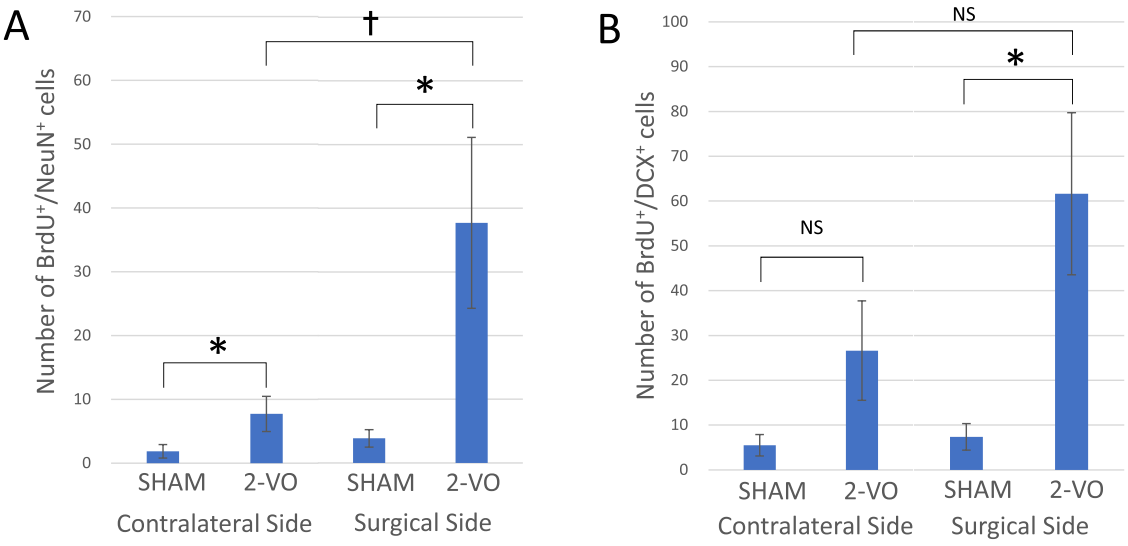

Fig. 4. Number of generated neurons (A) and precursors (B) in the large region of interest (ROI) of the two-vein occlusion (2-VO) group and sham group. A: Comparing the 2-VO group to the sham group, about ten-fold of BrdU-NeuN double-positive cells are detected with significant differences on the surgical side (37.7 ± 13.4 vs. 3.9 ± 1.4 cells, $p = 0.02$) and about three-fold of the double-positive cells with a significant difference on the contralateral side (8.4 ± 2.5 vs. 1.9 ± 1.1 cells, $p = 0.04$). However, there are only 22.3% more double-positive cells on the contralateral side than on the surgical side in the 2-VO group, which is statistically significant ($p = 0.03$). B: The total number of BrdU-DCX double-positive cells in the large ROI show a significant difference only on the surgical side (61.6 ± 18.1 vs. 7.4 ± 3.0 cells, $p = 0.01$), but not on the contralateral side (26.6 ± 11.1 vs. 5.5 ± 2.4 cells, $p = 0.05$). Data are shown as the mean ± SEM, *2-VO vs. sham, †surgical side vs. contralateral side, $p < 0.05$. BrdU, 5-bromo-2′-deoxyuridine; NeuN, neuronal nuclei.

lateral side. In addition, precursors induced by CVI were observed near the SVZ and SGZ. Thus, this study is the first to demonstrate both the expression and distribution of neurogenesis in a rat cortical CVI model.

4.1. Potential for neurogenesis after CVI

This study is unique in two aspects: it is a pathophysiological study of CVI, a relatively rare condition than CAI, and it is focused on neurogenesis in cerebral ischemia localized to the cerebral cortex. The animal model used in this basic research was cerebral cortical venous occlusion, the pathology of which is relevant to venous sinus thrombosis or intraoperative cortical venous injury in the clinical setting. Occlusion of deep cerebral veins is known to be a similar condition, but it may be different from cerebral cortical venous occlusion in terms of neurogenesis. Since ischemia in this 2-VO model occurs in the cerebral cortex and white matter just below it, and not in the deep brain or basal ganglia, the association with the SVZ or SGZ in the deep brain becomes a very important factor to consider when considering ischemia-induced

Table 1
Comparisons of BrdU-NeuN or BrdU-DCX double-positive cells in each region of interest on the surgical side.

Type of cells and groups		Number of cells in each ROI, n					
		ROI 1	ROI 2	ROI 3	ROI 4	ROI 5	ROI 6
$BrdU^+$/ $NeuN^+$ cells at Day 35	2-VO	9.3 ± 4.4	8.3 ± 3.9	11.3 ± 5.4	0.9 ± 0.5	1.9 ± 1.2	6.1 ± 4.1
	sham	0.7 ± 0.4	0.9 ± 0.5	0.6 ± 0.3	0.0 ± 0.0	0.6 ± 0.4	0.9 ± 0.4
	p	0.05	0.06	0.05	0.06	0.18	0.12
$BrdU^+$/ DCX^+ cells at Day 15	2-VO	5.5 ± 2.5	19.4 ± 4.5	22.0 ± 7.0	9.0 ± 8.4	1.5 ± 0.7	4.1 ± 2.7
	sham	3.5 ± 1.7	1.4 ± 0.9	1.1 ± 0.8	0.1 ± 0.1	0.6 ± 0.3	0.6 ± 0.6
	p	0.26	0.002 *	0.01 *	0.16	0.14	0.12

Data are shown as means ± SEM, 2-VO vs. sham; unpaired t-test, * $p < 0.05$.
BrdU, 5-bromo-2′-deoxyuridine; NeuN, neuronal nuclei; ROI, region of interest; 2-VO, two-vein occlusion.

neurogenesis. Therefore, the results of this study are clinically significant.

Previous reports using the middle cerebral artery occlusion (MCAO) model, in which ischemia reaches the basal ganglia, have shown that ischemia-induced neuroblasts migrate from the SVZ, resulting in delayed expression of generated neurons (Arvidsson et al., 2002). This fact indicates that the pathogenesis of MCAO has the potential to induce neurogenesis from the SVZ or SGZ, which is supported by many other reports (Arvidsson et al., 2002; Wang et al., 2011; Zhang et al., 2001). In contrast, it remains unclear whether neurogenesis occurs in CVI, and whether it occurs through ischemia localized in the cerebral cortex far from the SVZ and SGZ. Although Tamaki et al. confirmed the expression of neurogenesis by adding CVI as a means of strongly modifying the effects of CSD (Tamaki et al., 2017), our study shows that CVI alone has the potential to induce neurogenesis in a model where ischemia is localized in the cerebral cortex and does not extend deep into the brain. Moreover, comparison of the distribution of neurogenesis obtained in this study with that of ischemia reaching the deep brain may provide further insight into its pathogenesis.

4.2. Characteristics of CVI and neurogenesis

CVI is often considered a mild ischemic condition. Unlike MCAO, which induces intense cerebral ischemia over a short period of time, previous studies of cerebral hemodynamics employing the 2-VO model showed that cerebral blood flow was reduced to about 50% for a long period of time (Takeshima et al., 2015) and it has a small ischemic core and larger “ischemic penumbra area” (Kimura et al., 2005). However, the “ischemic penumbra area” is not a salvageable environment from a critical situation. Pathophysiological studies of CVI have identified long-lasting apoptotic cell death in these areas from 4 to 168 h after 2-VO (Nishioka et al., 2006). With the characteristic of CVI progressing apoptosis slowly, but certainly, we expected that CVI would also have the potential for neurogenesis by its continuous stimulation, which might differ from that of CAI.

The concept of neurogenetic potential has been reported previously. It is illustrated as a pool of immature neurons generated in surplus but selectively eliminated by apoptosis when they are no longer recruited to the functional neural circuits in the cerebral cortex (Biebl et al., 2000; Blaschke et al., 1996; Kempermann et al., 2003; Miyata et al., 2010). Appropriate functional stimulations recruit more new neurons. Liang et al. demonstrated that SVZ-derived neurogenesis after CAI is activity-dependent, region-specific, and sensitive to modulation, and the synaptic connections formed by these newborn cells are functionally critical for poststroke recovery (Liang et al., 2019). The results of these studies suggest that a milder degree of local ischemia may be preferable for precursor cells. In the present study, we investigated the expression and distribution of neurogenesis in a CVI rat model with 2-VO and observed the development of new progenitor cells and neurons, especially in the ischemic core and penumbra area. This indicates that even the milder and local environment created by cerebral cortical vein occlusion has the potential to induce neurogenesis and to generate and integrate newly formed neurons into the local tissue. Moreover, apoptotic cell death in the ischemic core and penumbra area, as previously reported in 2-VO pathophysiological studies (Nishioka et al., 2006; Takeshima et al., 2011), may have demonstrated the dropout of overexpressed precursors generated by CVI. We previously reported that continuous intraventricular administration of brain-derived neurotrophic factor in a 2-VO rat model reduced cerebral infarct size and suppressed local apoptosis (Takeshima et al., 2011). However, the appropriate modification technique and the proportion of endogenous precursors generated or dropped out via CVI are not known. Further studies are required to investigate these aspects in detail.

4.3. Role of neurogenesis in CVI

In this study, the ROI was widely fixed around the insulted area to ensure detection of cell expression because of the following concerns: there could be little amount of cell expression, and we lack evidence to

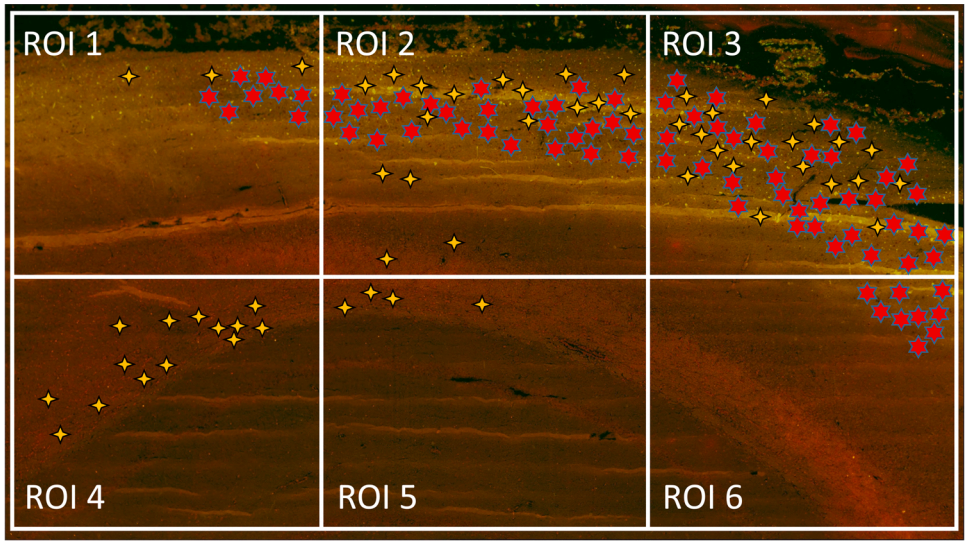

Fig. 5. Schematic merged diagram of the distribution of neural precursors on day 15 and generated neurons on day 35 after two-vein occlusion (2-VO) in a representative specimen. Most of the generated neurons are distributed in ROI 1–3 & 6 (red stars), especially in the cortex where regular neurons are usually established. Neural precursors (yellow stars) are already located in ROI 2 and 3, even with half the duration after the operation. Despite the similar distribution of the two double-positive cells, some BrdU-DCX double-positive cells are detected in ROI 4 and 5, which are located near the subgranular and subventricular zones. The magnification is × 20 objective.

predict where the cells will be generated. However, double-positive cells were successfully detected in the prefixed ROI, showing a specific distribution by dividing it into six sections. Most BrdU-DCX double-positive cells were distributed in ROI 2 and 3 in this study. This differs from the distribution of precursors observed in MCAO models, which is medially and dorsally to the ischemic area (Arvidsson et al., 2002).

The specific results of this study on their expression may reveal their role in ischemia-induced neurogenesis. We speculate that endogenous precursors are promoted to migrate in the vicinity of the ischemic area to directly repair the damaged area and accomplish neuronal replacement. Bacigaluppi et al. have reported the important functions of endogenous neural precursor cells through generation of new cells and through secretion of factors to preserve brain tissue homeostasis and eventually repair pathological conditions, including ischemic strokes (Bacigaluppi et al., 2020). Vascular endothelial growth factor (VEGF), for example, is a well-known factor secreted by neural precursors, which induces angiogenesis in the ischemic area and plays a beneficial role in reducing infarct size (Harms et al., 2010; Jin et al., 2002; Kimura et al., 2005; Miyake et al., 2013; Sun et al., 2003).

Previous studies have shown that the penumbra develops lateral to the ischemic core after 2-VO and that apoptosis progresses slowly inside it (Kimura et al., 2005; Nishioka et al., 2006). The similar greater distribution of BrdU-NeuN and BrdU-DCX double-positive cells generated in ROI 2 and 3 than in ROI 1 leads to a hypothesis that more neurons and neural precursors are generated inside the penumbra than in the medial side of the ischemic core, suggesting that these cells are responsible for not only neural replacement but also neurogenetic support to inhibit apoptotic cell death (Ploughman et al., 2009; Schäbitz et al., 2007; Takeshima et al., 2011).

In contrast, in the setting of a fixed-size ROI in each rat, we should consider the issue of individual differences in infarct size. We have previously reported several studies using the same method in Wister rats to create 2-VOs within an interaural line distance of 6.5–9.0 mm and have shown low variability in infarct area. In a 2-VO experimental setup with rats weighing 230–300 g, the infarct area at 24 h after 2-VO was 0.95 ± 0.26 mm^2 (Kimura et al., 2005). In another experimental setup of rats weighing 240–280 g, the infarct size at 7 days after 2-VO was 0.69 ± 0.27 mm^2 (Takeshima et al., 2011). In addition to the above facts, in this study, the small difference in body weight before 2-VO in both groups suggested that the individual differences in infarct area were minimal.

4.4. Origin of neurogenesis in cortical CVI

The results of this study provide interesting insights into the neurogenetic origins of CVI, in which ischemia is localized in the cerebral cortex. The characteristic distribution of BrdU-DCX double-positive cells that were significantly expressed in ROI 2 and 3 suggests that neurogenesis after cortical CVI may have occurred in the cortex near the ischemic core. In contrast, we found BrdU-DCX double-positive cells in ROI 4 and 5, which were located near and rostral to the SVZ and SGZ, distantly from the distribution in ROI 2 and 3. Although there was no statistically significant difference to prove this, it was an interesting result that neurogenesis after cortical CVI also originates from major neurogenetic regions. These results suggest that neurogenesis after cortical CVI occurs in both the cortex and SVZ or SGZ, but may be mainly in the former regions, where the precursors are more likely to reach the ischemic area because of the shorter distance.

Previous studies have supported these hypotheses. Nakagomi et al. demonstrated that pial ischemia-induced neural stem/progenitor cells after cortical CAI are generated in the cortex from the microvascular pericytes/perivascular cells (Nakagomi et al., 2011). Ohira et al. reported neural progenitor cells in the neocortical layer 1 of adult rats and observed that their proliferation was highly activated by global forebrain ischemia (Ohira et al., 2010). Shimada et al. reported that mild cortical strokes by distal MCAO not penetrating the striatum activate local cortical stem/progenitor cells but do not induce neuroblast migration from the SVZ niche using DCX or PSANCAM and DiI labeling (Shimada et al., 2010). Different locations of ischemia with different ischemic models show the possibility of variation in the expression of neurogenesis. Therefore, the kinetics of neurogenesis in cortical CVI may also be specific, and the results of our study are very interesting.

Although some of the precursors may be generated from the SVZ or SGZ, a chronological examination of endogenous precursor expression may reveal neurogenetic origins via CVI and a unique course toward the ischemic area. To obtain significant evidence on this interesting issue, more detailed studies of the temporal and spatial changes in the expression of endogenous precursors after CVI are needed.

4.5. Limitations

There are some limitations to our research. First, our research revealed neurogenesis after cortical venous infarction, but did not clarify the correlation between neurogenesis and the degree of cortical damage. Second, although 2-VO is a specific and reproducible method to create cortical venous infarction, histological damage was difficult to avoid because of the 3-μm fresh frozen sections. Fresh frozen and 3 μm were the appropriate fixation and thickness, respectively, to confirm and show the best expression of double-positive cells. Thus, we were careful about counting double-positive cells by not counting gathered and suspected cells at the edge or foldings to avoid the edge effects. In addition, as mentioned in Discussion, there is the issue of individual differences in rats, which has not been completely resolved.

The period of BrdU injection and observation after 2-VO was based on a previous study of neurogenesis after MCAO. We thought that five weeks of observation would be enough to confirm new neurons, as venous infarction stimulation is smaller and continuous than MCAO. Two weeks of serial injections was also thought to provide the maximum expression of neural precursor cells before division into neurons. However, we consequently made a chronological limitation as those periods became too long for detecting ongoing neural replacement. We could not track the precursors' migration as they were already in the ischemic area within 15 days in our study. Further research with a brief evaluation interval might provide an answer to the origin or tract of endogenous neural stem cells migrating in the cerebral cortex.

5. Conclusions

This study demonstrates that CVI has the potential to induce endogenous neurogenesis. Significant numbers of both generated neurons and precursors were observed in the ischemic area, suggesting subsequent neural replacement and neurogenetic support for self-repair. The distribution of generated neurons and precursors in our study led us to hypothesize that both the cortex and the SVZ or SGZ could be the origin of neurogenesis after CVI; however, further research is needed to obtain definitive evidence.

Ethical statement

All methods have been reported in accordance with the Animal Research Reporting In Vivo Experiments (ARRIVE) guidelines. All experimental procedures were conducted in accordance with the Institutional Guidelines for Animal Experimentation and the Guidelines for Proper Conduct of Animal Experiments by the Science Council of Japan. All experimental protocols were approved by the Animal Welfare Committee of Nara Medical University (approval numbers: 12246, 12471, 12535, 12554, 12721, 12729).

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CRediT authorship contribution statement

Tae-Kyun Kim: Data curation, Investigation, Software, Formal analysis, Visualization, Writing – original draft. **Yasuhiro Takeshima:** Conceptualization, Funding acquisition, Methodology, Validation, Writing – review & editing. **Yukiteru Ouji:** Investigation, Software, Visualization, Writing – review & editing. **Fumihiko Nishimura:** Writing – review & editing. **Ichiro Nakagawa:** Writing – review & editing. **Young-Soo Park:** Writing – review & editing. **Masahide Yoshikawa:** Supervision. **Hiroyuki Nakase:** Project administration.

Consent for publication

All authors have read and accepted responsibility for the content of the manuscript.

Conflict of interest

The authors have no conflict of interest to declare.

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