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Systemic Factors Trigger Vasculature Cells to Drive Notch Signaling and Neurogenesis in Neural Stem Cells in the Adult Brain

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Received May 4, 2018; accepted for publication October 25, 2018; first published online in STEM CELLS *EXPRESS* October 31, 2018.

http://dx.doi.org/10.1002/stem.2947

Key Words. Blood-brain barrier • Neural stem cells • Neurogenesis • Vascular endothelial growth factor

ABSTRACT

It is well documented that adult neural stem cells (NSCs) residing in the subventricular zone (SVZ) and the subgranular zone (SGZ) are induced to proliferate and differentiate into new neurons after injury such as stroke and hypoxia. However, the role of injury-related cues in driving this process and the means by which they communicate with NSCs remains largely unknown. Recently, the coupling of neurogenesis and angiogenesis and the extensive close contact between vascular cells and other niche cells, known as the neurovascular unit (NVU), has attracted interest. Further facilitating communication between blood and NSCs is a permeable blood-brain-barrier (BBB) present in most niches, making vascular cells a potential conduit between systemic signals, such as vascular endothelial growth factor (VEGF), and NSCs in the niche, which could play an important role in regulating neurogenesis. We show that the leaky BBB in stem cell niches of the intact and stroke brain can respond to circulating $VEGF_{165}$ to drive induction of the Notch ligand DLL4 (one of the most important cues in angiogenesis) in endothelial cells (ECs), pericytes, and further induce significant proliferation and neurogenesis of stem cells. STEM CELLS *2019;37:395–406*

SIGNIFICANCE STATEMENT

The leaky blood-brain barrier in niches of the intact and stroke brain can respond to circulating $VEGF_{165}$ to drive neural stem cells (NSCs) activation and neurogenesis. Vascular endothelial growth factor ($VEGF_{165}$) induces expression of the Notch ligand DLL4 in endothelial cells, pericytes, after stroke or oxygen-glucose deprivation. The enhanced DLL4-Notch signaling and crosstalk between vasculature cells and NSCs regulate the activities of NSCs when triggered by systemic stroke-induced factors.

INTRODUCTION

Understanding the mechanisms that drive the restorative process in the brain is critical to the discovery of ways to therapeutically enhance it after injury or disease. Although the role of endogenous stem cells in this process has not been fully elucidated, it is now well documented that adult neural stem cells (NSCs) residing in the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the hippocampus are induced to proliferate and differentiate into new neurons after injury such as stroke [1–10]. In the last decade, a growing literature indicates that brain niches are far more extensive than once thought. Thus, in addition to the SVZ and SGZ, midline ventricular structures known as circumventricular organs (CVOs) and sites along the third ventricle (3 V) wall and fourth (4 V) ventricle recesses also have been found to contain pockets of NSCs. Importantly, in all these niche sites, stem cell proliferation is dramatically upregulated and NSC differentiation is shifted toward a neuronal fate after experimental stroke from middle cerebral artery occlusion (MCAO) [11–13].

However, the role of injury-related cues in driving this process and the means by which they communicate with NSCs remains largely unknown. As signals must travel long distances from the infarct to reach far-off niches, a systemic route seems both plausible and likely. One of the unique structural traits of stem cell niches is the extensive and close contact between vascular cells and niche cells [14, 15]. This arrangement makes vascular cells a potential conduit between systemic signals and NSCs and may play

important role in regulating neurogenesis. And indeed, the cross-talk between these two cell types, endothelial cells (ECs) and NSCs, via Jagged-dependent Notch signaling is now beginning to emerge as an important avenue for maintaining NSC quiescence [16]. Also, as shown in other important work [17], vascular cells (i.e., ECs and pericytes) from non-neurogenic cortex as well as from the SVZ were able to promote NSC proliferation and neurogenesis in vitro, indicating the crucial regulation of NSC activity by vascular cells even in non-neurogenic regions under certain circumstances. Thus, the regulation of NSC activity in the adult brain is highly complex and well-regulated in order to maintain NSC quiescence, proliferation, and differentiation, especially after injury.

Furthermore, facilitating communication between blood and NSCs is a permeable blood-brain barrier (BBB) found in most (i.e., SVZ and CVOs) but not all (exception = SGZ) niches [13]. Because of this unique penetrability not found elsewhere in the brain, most niches are well positioned to respond to the ever-changing composition of blood in order to sustain homeostatic functions. Moreover, after stroke, the BBB becomes further disrupted. In our earlier studies, this enhanced leakiness has been positively correlated with increased stem cell proliferation and neurogenesis in the SVZ and CVOs after stroke [13, 18]. More recently, we showed that even the SGZ whose BBB is not porous in the normal brain becomes leaky after MCAO [19]. The heightened permeability in brain niches may increase niche access to systemic factors especially after stroke and/or facilitate greater contact between NSCs and cells of the BBB (i.e., ECs, pericytes, and astrocytes), all of which could be potentially important for signaling stem cell activities.

Although local hypoxic and tissue-secreted factors in the niche have been widely studied as triggers for stem cell proliferation in normal and injured brains [4, 20–35], only a few reports have shown a role for circulating factors regulating downstream stem cell responses and those do not offer an underlying pathway/mechanism [36–38]. It is well established that after stroke, the levels of circulating growth factors and cytokines rise dramatically in blood [23–26, 39–59]. One particularly important factor is vascular endothelial growth factor (VEGF), which is associated with enhanced angiogenesis and neurogenesis in the ischemic brain [23, 24, 39, 60–63].

Systemic factors such as VEGF-A, VEGF-C ,and other factors, working directly through their receptors, are known to affect either ECs to increase angiogenesis or NSCs to increase neurogenesis [16, 32, 39, 64–84]. However, no one has heretofore postulated and shown that circulating factors, facilitated by the leaky BBB in the niche, can induce the cross communication between these systems (from EC to NSC or from NSC to EC). Supporting this notion is the tight coupling of angiogenesis and neurogenesis [85] and the discovery that direct cell-cell contact between EC and NSC is essential for maintenance of stem identity in NSCs [16]. However, the underlying mechanism has not been delineated. In both EC:EC or NSC:NSC communication, Notch signaling has been implicated as the downstream mediator. Moreover, the established role of VEGF-DLL4 signaling in angiogenesis could likewise be pivotal in NSC activity regulation as well. Therefore, in this study, we examined whether the leaky BBB in niches of the intact and stroke brain can respond to circulating $VEGF_{165}$ to drive induction of the Notch ligand DLL4 in ECs and pericytes and whether these changes lead to enhanced Notch binding/signaling in stem cells causing a rise in proliferation and neurogenesis.

Materials and Methods

Animals, Antibodies and Reagents

Adult male Sprague-Dawley rats, adult male CD-1 mice, and adult male C57/BL mice were used in our experiments. All procedures in this study were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the International Animal Care and Use Committee of the Thomas Jefferson University. For antibodies and reagents, please see Supporting Information Material and Methods.

Cell Culture

Cultures of bEnd.3 cell line (ATCC, Virginia, USA) were used for $VEGF_{165}$ treatment assay and oxygen-glucose deprivation (OGD) assay. The culture was maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

In Vitro $VEGF_{165}$ Treatment

During $VEGF_{165}$ treatment, bEnd.3 ECs were grown with 100 ng/ml $VEGF_{165}$ in low growth factor (0.1% fetal bovine serum [FBS]) or serum containing media (10% FBS) compared to the respective control groups. For details, please see Supporting Information Material and Methods.

OGD and Hypoxia

Cell cultures were then washed twice with Hanks' balanced saline solution and OGD media (glucose and phenol red-free DMEM was deoxygenated by gassing with 95% nitrogen and 5% CO_2 for 15 minutes) as described before [86] and in Supporting Information Material and Methods. The time points used in the experiments were selected based on our unpublished empirical data and the literature [87, 88] and MCAO procedure.

In Vivo $VEGF_{165}$ Infusion and Bromodeoxyuridine Administration

In adult male rats or mice, recombinant $VEGF_{165}$ or $VEGF_{165}$-biotin were infused intravenously via femoral vein. Briefly, the femoral vein was catheterized and connected to a micro-osmotic pump (for dosage and timing, please see details below in groups 6–7).

Focal Ischemic Stroke Model: MCAO

Adult male Sprague-Dawley rats weighing 275–300 g and adult male C57/BL mice weighing 25 g were used, and MCAO was performed as described in Supporting Information Material and Methods.

Behavioral Tests

To evaluate neurological function, all rats were subjected to a battery of tests at postoperative 24 hours, 3 days, 7 days, and 14 days as described in Supporting Information Material and Methods.

Animal Treatment Protocol and Bromodeoxyuridine Administration

Animals were divided into seven different groups according to the experiments. For more details, please see Supporting Information Material and Methods.

Immunostaining

Animals were perfused with cold (4°C) paraformaldehyde (4%). The brains were then processed as described in Supporting Information Material and Methods.

Transmission Electron Microscopy

Rats received MCAO and their controls were prepared for electron microscopy (EM) as described in Supporting Information Material and Methods.

RNA Isolation and cDNA Synthesis

Rat brain tissue was harvested for Reverse transcription polymerase chain reaction (PCR) as described in Supporting Information Material and Methods.

Real-Time PCR Analysis

Real-time PCR was carried out as described in Supporting Information Material and Methods.

Quantitative Analysis

Quantitative analysis was carried out as described in Supporting Information Material and Methods.

Statistical Analysis

All data are presented as the mean ± SEM. Statistical analysis of cell counts was performed using Student's *t* test or the one-way analysis of variance followed by post hoc Bonferroni test. A p value <.05 was considered significant.

Results

Leaky BBB in SVZ and Median Eminence in Normal Brain

Earlier studies indicated that the SVZ and CVO niches in the normal brain are highly vascularized regions containing leaky capillaries associated with a permeable BBB not seen elsewhere in the brain [13, 14]. In the current study, we used electron microscopy to further examine the BBB in blood vessels of the SVZ and a CVO niche, the median eminence (ME). We found that tight junctions, part of the essential components of an intact BBB, were oftentimes lacking between ECs in vessels of the SVZ (Fig. 1C, 1D) as compared to the non-niche brain region of the striatum (Fig. 1A, 1B). In the ME, we observed capillaries with many small fenestrations that connected by a thin diaphragmatic layer to separate blood from brain (Fig. 1E, 1F). These observations are consistent with a leaky BBB that is unique to brain niches.

Stroke Increases BBB Leakiness in Brain Stem Cell Niches

In order to assess the integrity of the BBB in the niche after stroke, we examined both BBB permeability and ultrastructure in the SVZ. Although the SVZ in the normal brain is permeable to small molecules such as peripherally infused sodium fluorescein (376D) [13, 14], in these experiments on the MCAO brain, we tested BBB leakage of larger molecules such as 40 kDa FITC-dextran. To do so, 40 kDa FITC-dextran was infused into the femoral vein 1 day after MCAO and allowed to circulate for 10 minutes. Under fluorescent microscopy, we observed FITC-dextran extravasated from blood vessels near the SVZ and distributed into the surrounding parenchyma (Fig. 2A, 2B). To further investigate the leakiness of the BBB around the niches to VEGF, 0.5 mg/kg biotinylated $VEGF_{165}$ was infused via the femoral vein after MCAO. We visualized greater leakage of $VEGF_{165}$-biotin into the parenchyma from vessels in the infarct penumbra and around the SVZ after MCAO than in control brains (Fig. 2C–2E). Negative results from the control group are not shown here.

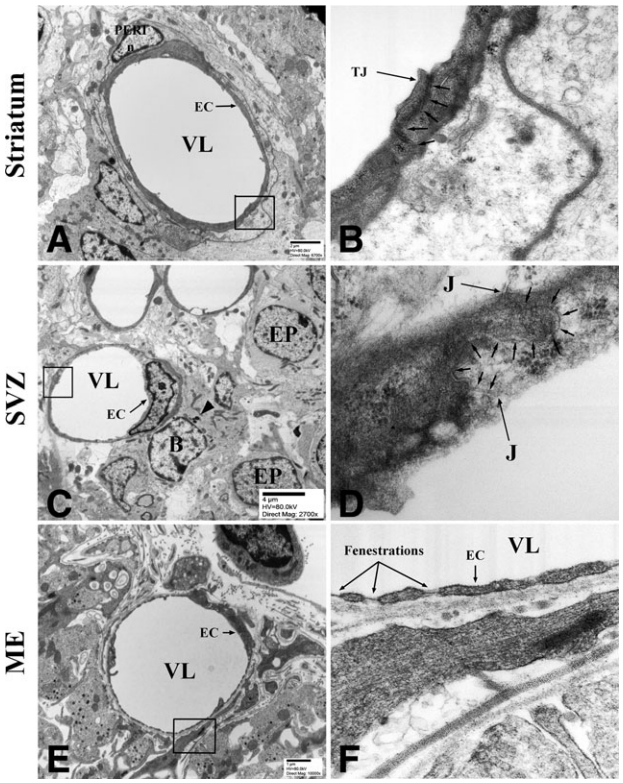

Figure 1. Electron micrograph of blood-brain barrier (BBB) in normal brain. **(A, B):** Note evidence of an intact BBB in blood vessels (VL), including TJ between ECs (rectangle in **A** at higher power in **B**) and apposing PERI n. **(C, D):** Note lack of TJs between ECs in the SVZ niche (see arrow J, rectangle in **C** at higher power in **D**) and their proximity to other niche cells, including the slow cycling stem cells or B cells (recognized by dense bodies in the cytoplasm: arrowhead in **C**, and EPs. **(E, F):** Circumventricular organ niche ME with fenestrated capillaries. Scale bars: 2 μm in **A**, 4 μm in **C**, and 1 μm in **E**. Abbreviations: EC, endothelial cell; EP, ependymal cell; ME, median eminence; PERI n, pericyte nucleus; SVZ, subventricular zone; TJ, tight junction; VL, vessel lumen.

Similarly, transmission electron microscopy analysis showed increased numbers of pinocytotic vesicles (AKA vesiculovesicles or vesiculo-vacuolar organelles [VVOs]) in ECs of SVZ capillaries 72–96 hours after MCAO (Fig. 2H–2J) compared to ECs of SVZ capillaries in the intact brain (Fig. 2F, 2G). As VVOs are highly associated with a state of hyperpermeability, these results combined with those of $VEGF_{165}$-biotin permeability indicate that stroke increases BBB leakiness in the SVZ niche.

Elevated Systemic VEGF Mimics Stroke to Induce Neurogenesis

After stroke, the levels of circulating growth factors and cytokines rise dramatically in blood [23–26, 39–59]. One critically important factor is $VEGF_{165}$ which significantly rises in the blood and brain after stroke. As we found that the BBB in niches is highly

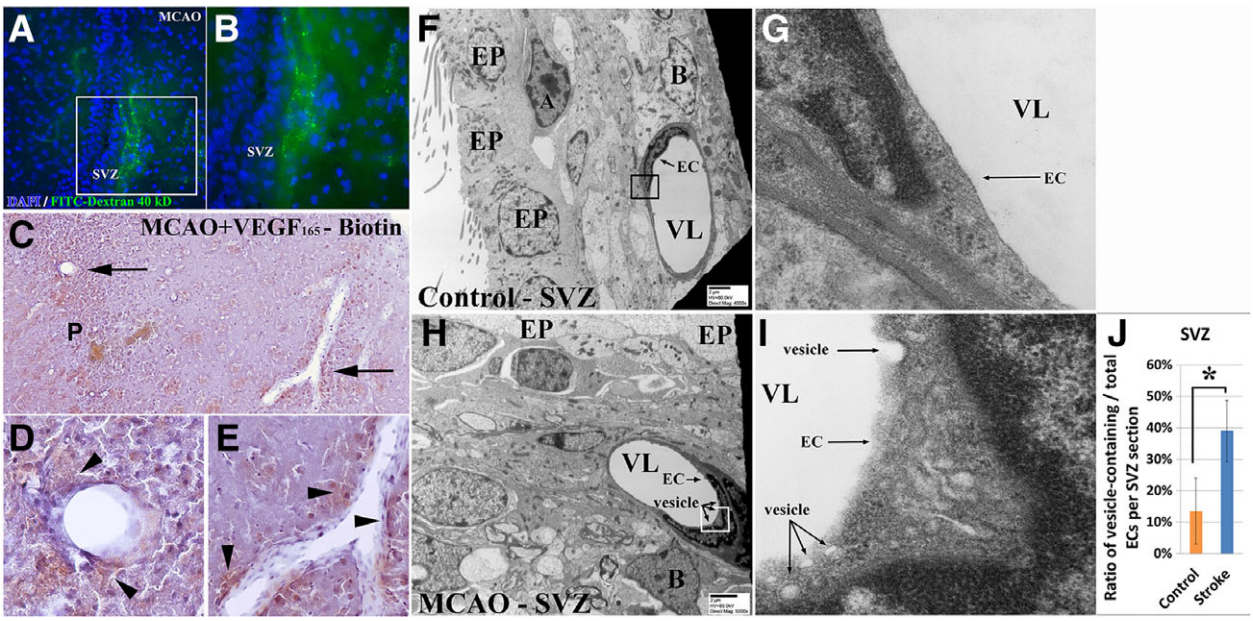

Figure 2. Niches are hyperpermeable after MCAO. **(A, B):** Forty kilodaltons FITC-dextran extravasation from blood vessels adjacent to SVZ 1 day after MCAO. Low **(A)** and high **(B)** power views of SVZ. **(C–E):** Biotinylated rVEGF leakage into the parenchyma (**P**, arrows in **C** and arrowheads at high power in **D and E**) from vessels near SVZ after MCAO. **(F–I):** Electron micrograph of niche vasculature in control **(F, G)** and after MCAO **(H, I)** brains. Note intact blood-brain barrier in blood vessels (VL) without evidence of vesiculo-vacuolar organelles (VVOs). Note greater numbers of VVOs after MCAO in low **(H)** and high **(I)** power. Pinocytotic vesicle containing ECs of SVZ capillaries were also quantified in **(J)**. Rectangles shown in left column at low power and right column in higher power. Data are expressed as mean ± SEM; *$p < .05$. Scale bars: 2 μm. Abbreviations: A, type A cells or neuroblasts; B, type B cells; EC, endothelial cell; EP, ependymal cell; MCAO, middle cerebral artery occlusion; SVZ, subventricular zone; VEGF, vascular endothelial growth factor; VL, vessel lumen.

permeable to FITC-dextran40, the size of $VEGF_{165}$ (dimer: 39 kDa) after stroke, these substances may have relatively unrestricted access to niche cells, including those at a great distance from the infarct. Indeed, we found a transient rise in VEGF levels in the infarcted side of the brain (Supporting Information Fig. S1). In an effort to understand how circulating VEGF mediates its effects, we next determined whether ECs in niche blood vessels express vascular endothelial growth factor receptor 2 (VEGFR2), the main receptor mediating VEGF-A effects. We found that RECA-1+ ECs in the SVZ and ME (Fig. 3A, 3B) labeled for the VEGFR2 receptor are consistent with their ability to bind and respond to circulating VEGF in normal stem cell niches. Although in normal brain, SVZ NSCs do not express VEGFR2 in vivo [89, 90], this phenotype may change after injury. We did not find that VEGFR2 was expressed by Nestin+ NSCs after we stained post-MCAO brain sections (Supporting Information Fig. S2).

In order to directly test whether amplification of circulating VEGF can mimic stroke and induce NSC proliferation and neurogenesis in the niche, we continually infused 1 mg/kg $VEGF_{165}$, the predominant VEGF isoform, or normal saline into 9-week-old CD-1 mice via the femoral vein connected to an ALZET micro-osmotic pump for 3 days. All mice were administered bromodeoxyuridine (BrdU) as described in Materials and Methods section to assess cell proliferation in the SVZ and SGZ niches. We found a significant increase in the number of BrdU-labeled cells in the SVZ as compared to the saline group ($p < .05$; Fig. 3C, 3D, 3G) 14 days after the initiation of VEGF treatment. The vast majority of these BrdU+ cells were Nestin (a marker of NSC) positive, indicating that $VEGF_{165}$ infusion, similar to MCAO, enhances proliferation in the SVZ 14 days later. In contrast, in the SGZ, the only known brain stem cell niche without a leaky BBB, there was no obvious difference in the numbers of BrdU+ cells found between $VEGF_{165}$ and saline groups (Fig. 3I).

To assess the level of neurogenesis, we next labeled brain sections for DCX, a marker for neuroblasts. We found that there were more DCX+ cells 14 days after $VEGF_{165}$ infusion in the SVZ ($p < .05$; Fig. 3H), indicating enhanced neurogenesis after $VEGF_{165}$ infusion. Again, there was no significant difference in the numbers of DCX+ cells in SGZ in the $VEGF_{165}$ infusion group compared to the saline infusion group (Fig. 3J). In order to assess angiogenesis after VEGF infusion, collagen IV was used for staining. No obvious differences in vessels were noted in SVZ and adjacent striatum between saline and VEGF infusion groups (Fig. 3E, 3F). This could be because of overall low concentrations of VEGF in the relative large peripheral blood pool.

VEGF Increases the Notch Ligand DLL4 in Cells of the Vasculature

Once bound, the VEGFR2 receptor is known to activate downstream Notch signaling through DLL4 ligand-dependent and -independent pathways [62, 83, 91]. Therefore, in our next study, we tested whether VEGF and DLL4 levels rose in the brain in a coordinated fashion after stroke. We found that indeed the rise in VEGF levels in the infarcted hemisphere (Supporting Information Fig. S1) was temporally correlated with a significant but transient increase in DLL4 mRNA levels at 24 hours not seen at 3, 7, and 14 days after MCAO (Fig. 4A).

Similarly, elevating the levels of VEGF or causing hypoxia increased the mRNA levels of DLL4 in ECs in culture (Fig. 4B–4D). We found that the addition of 100 ng/ml recombinant murine $VEGF_{165}$ to a low growth factor media (Fig. 4D) significantly induced DLL4 mRNA levels 6.5 fold in bEnd.3 ECs in culture. As the rapid upregulation of VEGF expression in ECs after hypoxia has been extensively investigated and reported in the past [87, 88, 92], we did not repeat the same experiments to measure

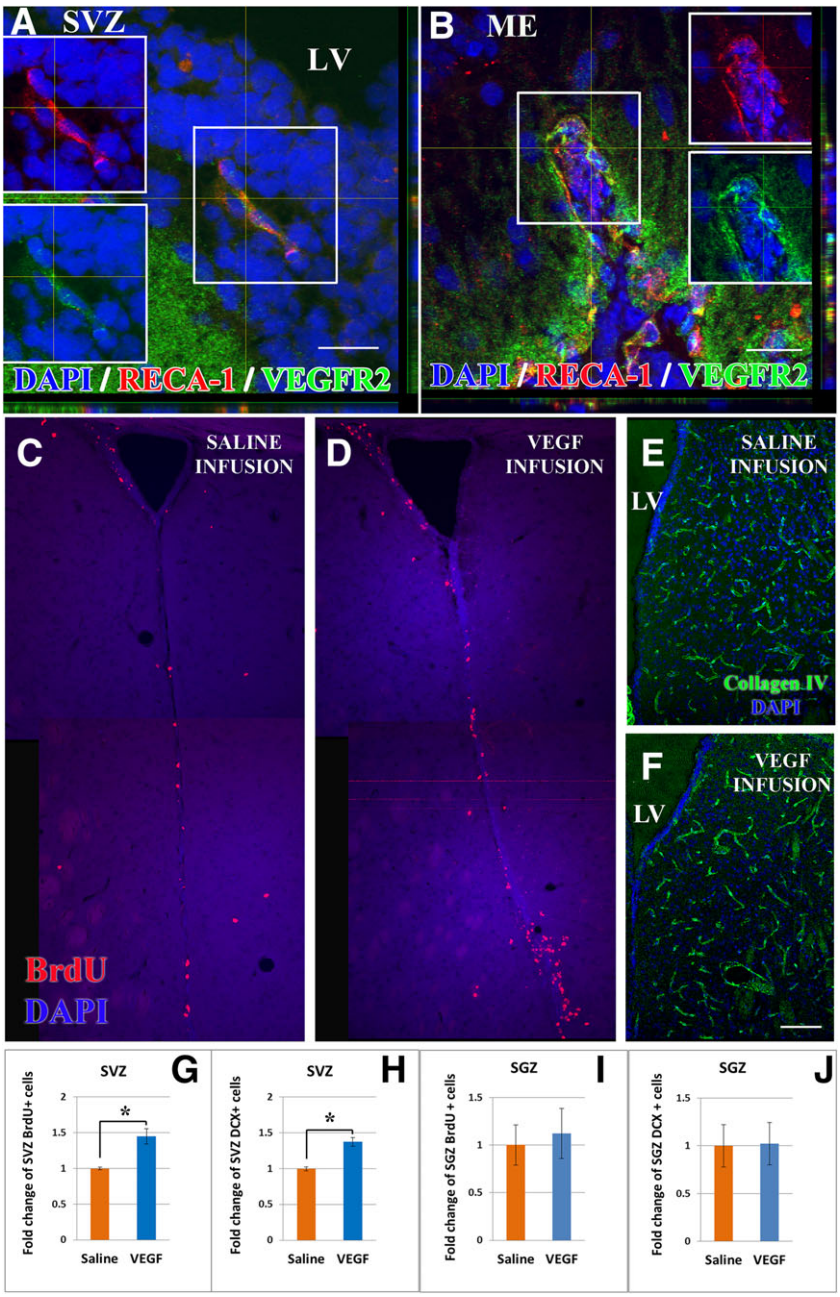

Figure 3. Increase in neural stem cell proliferation and neurogenesis in the SVZ but not SGZ with VEGF infusion in vivo. **(A, B):** confocal images of blood vessels showed RECA-1+ ECs colabeled with VEGFR2 in SVZ and ME. After continuous VEGF165 infusion, neural stem cell proliferation (BrdU+) and neurogenesis (DCX+) were found increased in the SVZ **(C, D, G, H)**, but not in the SGZ **(I, J)**. Meanwhile, angiogenesis after VEGF infusion was examined and collagen IV was used to reveal angiogenesis in mouse brain. **(E, F):** No obvious differences in vessels were noted in SVZ and adjacent striatum between saline and VEGF infusion groups. Data are expressed as mean ± SEM; *$p < .05$. Scale bars: 20 μm in **a** and **B**, 100 μm in **E** and **F**. Abbreviations: BrdU, bromodeoxyuridine; DAPI, 4′,6-diamidino-2-phenylindole; LV, lateral ventricle; ME, median eminence; SGZ, subgranular zone; SVZ, subventricular zone; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

VEGF levels after hypoxia. We exposed the bEnd.3 EC cells to hypoxia which should induce the upregulation of VEGF according to those reports. When bEnd.3 cells were subjected to OGD for 2.5 hours followed by continued hypoxic conditions (i.e., 5% O_2) for 24 or 48 hours, there was a significant 2.5 fold and 5.5 fold, respective, increase in DLL4 mRNA levels ($p < .01$; Fig. 4B), mimicking the significant rise of DLL4 levels in the infarcted brain after MCAO (Fig. 4A).

Upregulated DLL4 in Cells of Vasculature Leads to Increased Notch Signaling in NSCs

To further investigate in which cells DLL4 expression level increased after stroke, we examined blood vessels in the SVZ niche and nearby penumbra area and found that DLL4 colocalized with laminin+ blood vessels (Fig. 5A1–5A4), CD31+ ECs (Fig. 5B1–5B4), and PDGFRβ+ pericytes (Fig. 5C1–5C4). As microglia activation is prominent after stroke, we also stained IB4+

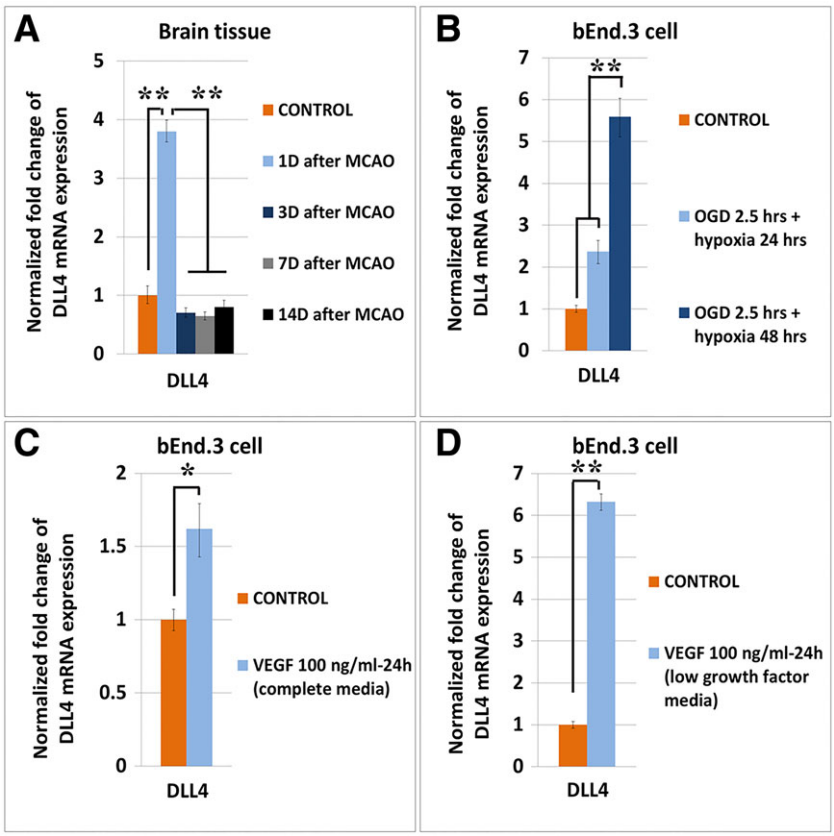

Figure 4. Exogenous VEGF or OGD increase DLL4 expression in endothelial cells (ECs) in culture or brain after MCAO. **(A):** DLL4 levels are increased in infarcted hemisphere compared to normal brain 1 day after MCAO. Levels return to baseline by 3–14 days post-MCAO. **(B):** DLL4 expression in ECs under OGD conditions for 24 or 48 hours as compared to normal conditions. **(C, D):** DLL4 expression in bEnd.3 ECs grown with 100 ng/ml VEGF in complete serum containing media **(C)** or low growth factor media **(D)** was compared to untreated cultures. Data are expressed as mean ± SEM; $*p < .05$, $**p < .01$. Abbreviations: MCAO, middle cerebral artery occlusion; OGD, oxygen–glucose deprivation; VEGF, vascular endothelial growth factor.

microglia but observed almost no colabeling of DLL4+ cells and IB4+ microglia (Fig. 5D1–5D4). In control group, DLL4 staining was found very low in vascular cells (data not shown). Thus, several cell types present in the vasculature, including ECs and pericytes, express higher levels of the Notch ligand DLL4 after stroke.

Next, we assessed the temporal change and pattern of DLL4 expression in the SVZ region. In control rats, a limited number of Notch receptor intracellular domain positive (activated intracellular domain of the Notch receptor) (NICD+) cells were found in the SVZ with low DLL4 level (Fig. 6A). On days 3 and 7, after MCAO, DLL4 expression in the SVZ was much higher than other selected time points and by day 14 post-MCAO, few NICD-labeled cells were found in the SVZ (Fig. 6B–6F). Notably, NICD+ cells could be found next to DLL4+ cells (Fig. 6A–6E). Thus, the temporal change of DLL4 protein level is partially consistent with the changes of mRNA levels, particularly when the lag in protein translation is taken into consideration. Another important stem cell niche in brain, the ME, showed intense staining of DLL4 in the control and MCAO groups, with no evident difference among the groups (Supporting Information Fig. S3).

We also stained the sections for Jagged1, another important Notch ligand in angiogenesis. In control rats, there was weak staining of Jagged1, mainly in the ependymal cells which increased from day 3 to day 7. Most interestingly, Jagged1 expression is exclusively limited to the ependymal cells as compared with DLL4 expression in the SVZ on day 3 after MCAO (Supporting Information Fig. S4).

Concomitant with the initiation of Notch signaling in vascular cells, we found that adjacent BrdU+ cells expressed the activated NICD (Fig. 6). Many NICD+ cells also colabeled for BrdU (Fig. 6G–6I; arrows) as might be expected in proliferating BrdU+ cells in the niche after stroke. Once the cascade was initiated, NSCs further signaled neighboring NSCs through subsequent DLL4/NICD signaling to proliferate (Fig. 6J–6L) and differentiate into new neurons.

We further examined the lineage of the activated NICD/BrdU cells. Indeed, some triple labeled Nestin/NICD/BrdU cells and EGFR/NICD/BrdU cells were found in SVZ (Fig. 7A–7E and Supporting Information Fig. S5). Because of the timeframe required for NSC differentiation into neuroblasts, we examined SVZ after 14 days of MCAO. Some DCX+ neuroblasts adjacent to the SVZ were found colabeled with NICD, indicative of their downstream participation in the signaling pathway (Fig. 7F). As the importance of the communication of vasculature and stem cells in brain stem cell niches has been extensively evaluated recently, we used collagen IV to examine the angiogenesis after stroke and found in infarction sites. Active angiogenesis was found from day 1 to 14 days after MCAO, peaking on day 7 (Supporting Information Fig. S6). All these data demonstrate that the cells of the vasculature working through Notch signaling regulate the activities of NSCs in the niche to drive neurogenesis.

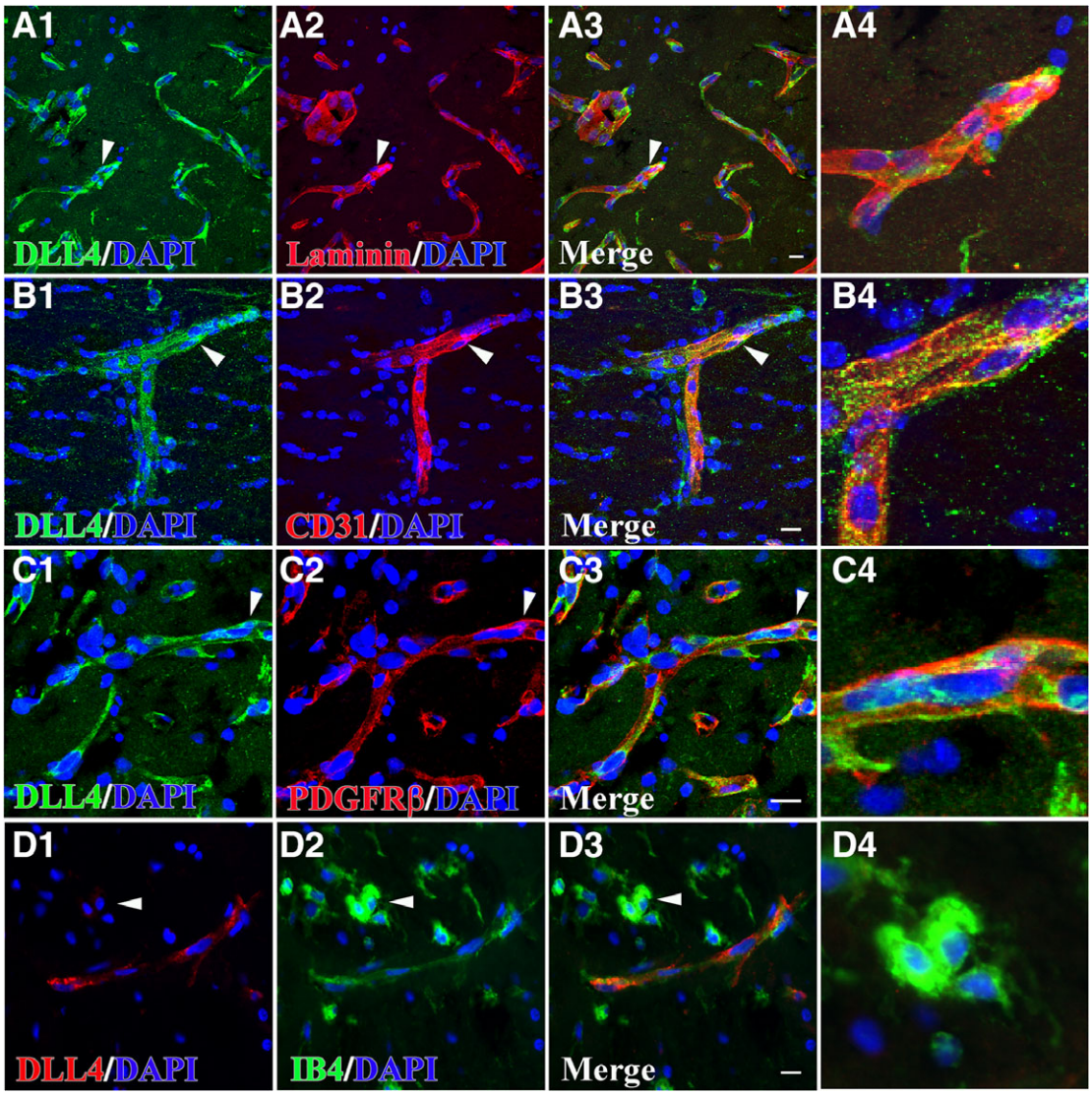

Figure 5. DLL4 is upregulated in vascular cells after stroke. DLL4 signals were found in laminin+ vessels **(A1–A4)** or CD31 vessels/endothelial cells (ECs) **(B1–B4)** (arrowheads: DLL4+ ECs) or PDGFRβ+ pericytes **(C1–C4)** (arrowheads: DLL4+ pericytes) but not in IB4+ microglia **(D1–D4)** (arrowheads: DLL4− microglia). Colabeled cells (arrowheads) are enlarged and shown in right column. Scale bars: 10 μm. Abbreviation: DAPI, 4′,6-diamidino-2-phenylindole.

Discussion

The term "vascular niche" was first coined to reflect the fact that NSCs proliferate and differentiate into neurons immediately adjacent to the vasculature of normal or injured brain [14, 15, 93–96]. However, the fundamental importance of this relationship and the mechanisms/signaling pathways underlying it remained an enigma. The findings of this study demonstrate that stem cell niches such as the SVZ and CVOs are unique in the intact brain insomuch as the BBB is both structurally and functionally incomplete. Structurally, the absence of glial end feet or pericytes on blood vessels in the niche allows stem cells and their processes to directly abut ECs and other cells of the vasculature, a configuration not seen elsewhere in the brain [14, 94, 96]. Additionally, niche blood vessels are characterized by an absence of the usual tight junctions seen between ECs [14]. Moreover, in the case of one of the CVO niches, the ME, capillaries are fenestrated where parenchyma is partitioned from blood by a mere membrane. At the functional level, these unique morphological features are consistent with a leaky BBB, allowing stem cells unfettered access to circulating factors normally prevented entrance into the CNS [54–57]. The one exception is the SGZ, which unlike other brain niches does not lie adjacent to a ventricle and does not possess a leaky BBB in the normal brain.

Stroke notoriously causes further disruption in BBB integrity, especially in the region of the infarction [97]. Based on the results from other groups [96, 98, 99] as well as regarding ours angiogenesis and neurogenesis, the observed dynamic changes in neurogenesis and angiogenesis are closely correlated. Similarly, our previous studies showed that the BBB in all stem cell niches also becomes more porous after stroke [13]. As infarction does not cause direct damage to niches, particularly SGZ in MCAO model which is far from the infarct, this effect is likely mediated by circulating factors leaking through the BBB [19]. That cells of the SGZ niche are capable of responding to circulating factors is further suggested by the presence of fine NSC processes which ensheath local vessels [58, 59]. Moreover, in the

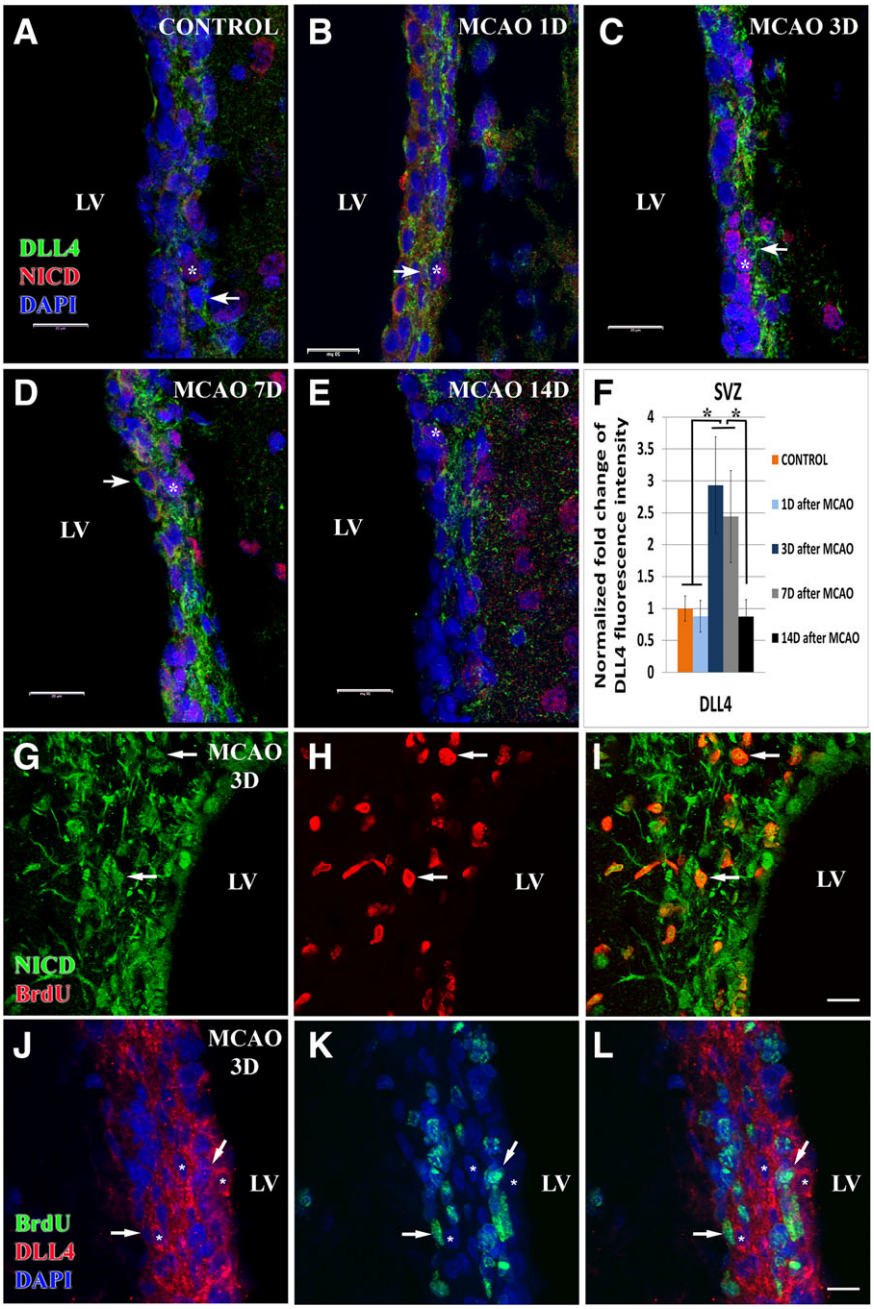

Figure 6. The activated NICD and DLL4 signals are increased in BrdU+ dividing neural stem cells of the subventricular zone (SVZ) after stroke. Temporal changes of DLL4 and activated Notch1 (NICD) expression in the SVZ. **(A):** In control rat, limited NICD+ cells were detected in the SVZ. Notably, one NICD+ cell (asterisk) was found next to a DLL4+ cell (arrow). **(B–D):** From day 1 to day 7, post-MCAO, more NICD+ cells were found in the SVZ, and some NICD+ cells (asterisks) were next to DLL4+ cells (arrows). **(E):** By day 14 post-MCAO, few NICD-labeled cells were found in the SVZ. **(F):** On day 3 and day 7, after MCAO, DLL4 expression in the SVZ was much higher than other selected time points by the quantification of relative fluorescence intensity. **(G–I):** Some BrdU+ cells colabeled with NICD (arrows). **(J–L):** At the same time point, BrdU+ cells (arrows) were found adjacent to DLL4+ cells (asterisks) in the SVZ. Data are expressed as mean ± SEM; $*p < .05$, $**p < .01$. Scale bars: 10 μm. Abbreviations: BrdU, bromodeoxyuridine; DAPI, 4′,6-diamidino-2-phenylindole; LV, lateral ventricle; MCAO, middle cerebral artery occlusion; NICD, notch receptor intracellular domain.

current study, we observed pinocytotic vesicles in niche ECs at the EM level, reflecting a state of hyperpermeability soon after MCAO. This heightened permeability was further substantiated by the leakage of FITC-dextran (40 kDa) from SVZ blood vessels into brain parenchyma 1 day after stroke.

A further consequence of stroke is a dramatic change in the composition of the blood, particularly with respect to circulating growth factors and cytokines [23–26, 39–59]. One critically important growth factor is VEGF which rises significantly in blood after MCAO [24]. As we show that the BBB is permeable to substances the size of VEGF (39 kDa) after stroke, it is not surprising then that we found a transient but significant spike in VEGF levels in the infarcted hemisphere 1 day after MCAO.

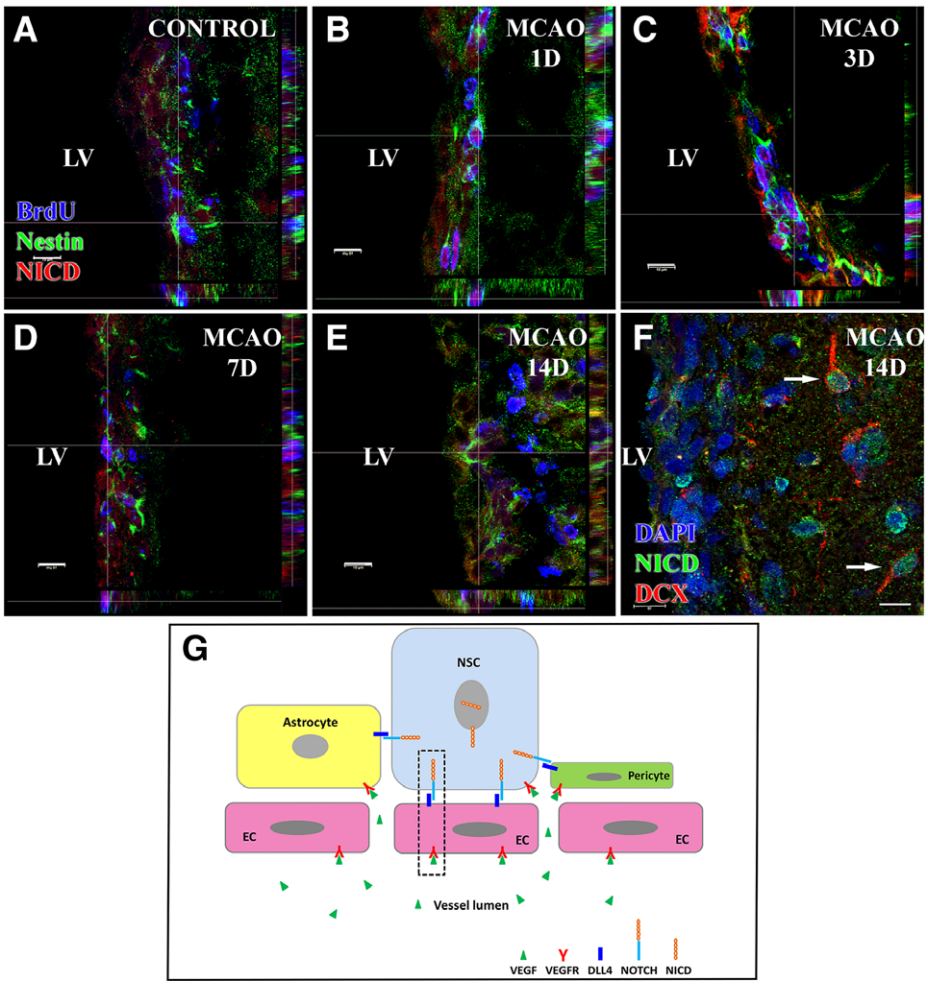

Figure 7. Subventricular zone (SVZ) neural stem cell activation and proliferation by using Notch signaling after stroke. **(A):** In control group, the NICD staining was relatively low, with some dividing NSCs (Nestin+ and BrdU+) in the SVZ. **(B–D):** After MCAO, more nestin+ NSCs were triple labeled with activated Notch1, NICD, and cell proliferation maker, BrdU. **(E):** On day 14, post-MCAO, few NICD+ Nestin+ BrdU+ triple labeled NSCs were found in the SVZ. **(F):** After 14 days, post-MCAO, some DCX+ neuroblasts were found colabeled with NICD in SVZ (arrows). Scale bars: 10 μm. **(G):** Proposed schematic picture of circulating VEGF and DLL4 pathway in the stem cell niche. Direct (VEGF to NSC) or indirect (VEGF to vasculature cell or astrocytes to NSC) routes are involved in regulation of NSC activity by circulating factors via compromised blood-brain barrier function in the already leaky stem cell niche. Noticeably in the highlighted box, systemic factors such as VEGF induce expression of the Notch ligand DLL4 on EC (here as an example for indirect pathway) through the VEGFR, and activation of subsequent DLL4-Notch signaling pathway from EC to NSC. Abbreviations: BrdU, bromodeoxyuridine; DAPI, 4′,6-diamidino-2-phenylindole; EC, endothelial cell; LV, lateral ventricle; MCAO, middle cerebral artery occlusion; NICD, notch receptor intracellular domain; NSC, neural stem cell; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

Until the current study, almost nothing was known about downstream consequences of increasing circulating VEGF and its leakage into the permeable stem cell niche. While a plethora of previous studies have shown that upregulation of VEGF signaling is associated with enhanced brain angiogenesis and neurogenesis, most of these effects were attributed to cues secreted locally by cells in the niche [23, 32, 39, 60, 61]. In addition to the local cues, we show here that the continual in vivo infusion of exogenous $VEGF_{165}$ into the peripheral circulation also leads to increased NSC proliferation and neuronal differentiation in the intact SVZ. We further show that blood vessels in the SVZ contain ECs which label for the VEGFR2 receptor, consistent with an ability to bind and respond to circulating VEGF. Consistent with previous reports [89, 90], VEGFR2 is not readily expressed by NSCs in the SVZ in vivo in normal animals. Moreover, further examination of VEGFR2 in brain sections from MCAO groups from day 1 to day 14 did not support the notion of upregulation of VEGFR2 expression on NSCs after MCAO. Taken together, these results indicate that circulating $VEGF_{165}$ may have a limited direct effect on NSCs without the presence of its major receptor, VEGFR2. In contrast, the SGZ does not respond to infused $VEGF_{165}$ with enhanced neurogenesis, likely as a result of its impermeable BBB. However, after stroke, our previous studies show that even the BBB in SGZ which is not directly damaged by MCAO becomes leaky to circulating factors and induces stem cell proliferation and differentiation [19]. Together, these results suggest that amplified systemic VEGF is capable of driving stem cell activities in niches lacking a complete BBB in a fashion analogous to stroke where NSCs access to increased circulating levels of VEGF through a compromised BBB impacts neurogenesis.

The molecular mechanism via which amplified systemic VEGF mediates these profound effects on the stem cell niche is not yet understood. Once VEGF binds its receptor, it is known to activate

downstream Notch signaling through DLL4 ligand-dependent and -independent pathways in cells [16, 62, 64–68, 78–84]. It is not clear in these studies that in which cells the increase in DLL4 was taking place. Likewise, we found a parallel and concurrent rise in VEGF levels and DLL4 expression, both at the transcriptional (mRNA levels in brain tissue) and protein (DLL4 immunostaining in ECs, pericytes) levels after stroke. However, a similar DLL4 upregulation was observed when ECs were grown in vitro in media containing high VEGF levels or when cells were grown under conditions that mimic stroke (OGD). Although we do not know whether this latter effect is also mediated by VEGF, it raises the possibility that ECs intrinsically increase VEGF signaling after stroke to produce autocrine or paracrine effects on DLL4 expression. Indeed, there are many previous reports that support this possibility [24, 60, 61, 88, 100]. Taken together, these findings suggest that both hypoxia in stroke and the increased levels of circulating VEGF caused by stroke dramatically amplify DLL4 signaling in cells of the vasculature by VEGFR even in the intact BBB. However, the leaky BBB after stroke most likely provides better access for sampling of those factors by pericytes, astrocytes, and NSCs. Interestingly, the ME, a circumventricular stem cell niche, has very high DLL4 expression even under normal conditions, suggesting a continual activation of Notch signaling in the ME because of its higher permeability (via fenestrated capillaries) compared to the SVZ. Concomitant with the initiation of Notch signaling in ECs (and pericytes) in the niche, we found that adjacent NSCs expressed the activated NICD. Further staining of brain sections with DLL4 and NICD revealed NICD+ cells oftentimes adjacent to DLL4+ cells in the SVZ, suggesting that cell-cell contact may be critical in the activation of the DLL4-Notch signaling pathway. These data indicate that the cells of the vasculature working through Notch signaling regulate the activities of NSCs in the niche to drive neurogenesis and the leaky BBB facilitates and augments these processes. Indeed, research now shows that dynamic oscillation in Hes1 expression in neural progenitors plays a critical role in maintenance of neural progenitors by mutual activation of Notch signaling [101]. Further study of activation of DLL4 Notch signaling pathway among stem cell themselves is warranted to better understand the types of cells involved and their distinctive functions and regulation in this process. One possibility raised by our studies is that the transient increase of VEGF binding to VEGFR2+ vascular cells initiates subsequent events, the subsequent neurogenesis is then fine-tuned at their own pace, wherein stem cell oscillation plays a more important role. To reveal the lineage of the activated NICD/BrdU cells, we found some triple labeled Nestin/NICD/BrdU cells and EGFR/NICD/BrdU cells in SVZ. These data suggest that Notch signaling pathway is activated in those neural progenitors which are undergoing active neurogenesis and that the activated Notch signaling pathway may be required in neuronal differentiation as indicated by NICD+/DCX+ neuroblasts.

Of further potential significance is a recent study showing that another Notch ligand, Jagged1, is important in maintaining stem cell quiescence in the SVZ [16]. Intriguingly, in angiogenesis, Notch ligands DLL4 and Jagged1 have opposing effects [102]. According to our results, the Jagged1 immunostaining is exclusively found in ependymal cells, indicating the distinctive function compared to DLL4 which is mainly expressed in the SVZ region. The role of these ligands in neurogenesis and whether Jagged1 and DLL4 play a similar reciprocal role in regulating NSC quiescence and activation respectively remains to be determined.

Conclusion

In summary, the findings of the present study establish that stroke, which further compromises BBB function in the already leaky stem cell niche, enhances access to systemic factors, including high levels of VEGF. Working through the VEGFR2 found on ECs (and pericytes), the growth factor induces expression of the Notch ligand DLL4, a finding mirrored in culture and in vivo by exogenous VEGF or stroke/OGD (schematic pathway highlighted in the box of Fig. 7). Because of the intimate contact that uniquely exists between cells of the vasculature and NSCs in brain niches, this up-surge in DLL4 leads to enhanced Notch signaling in neighboring stem cells, inducing their proliferation and differentiation into neurons. This is the first demonstration that cells of the vasculature can regulate the activities of stem cells in brain niches when triggered by systemic stroke-induced factors.

Acknowledgments

We acknowledge the excellent support of Bodil Tuma for EM and Aurelie Ky for animal caring. This research was supported by the Joseph and Marie Field Foundation to R.R. and L.I.

Author Contributions

R.L.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; J.C.: collection and/or assembly of data, data analysis and interpretation, manuscript writing; L.K., R.I.: collection and/or assembly of data, data analysis and interpretation, provision of study material or patients; R.R.: conception and design, financial support, administrative support; L.I.: conception and design, manuscript writing, financial support, administrative support, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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