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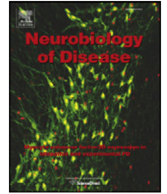

Neurogenesis is enhanced by stroke in multiple new stem cell niches along the ventricular system at sites of high BBB permeability

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ABSTRACT

Previous studies have established the subventricular (SVZ) and subgranular (SGZ) zones as sites of neurogenesis in the adult forebrain (Doetsch et al., 1999a; Doetsch, 2003a). Work from our laboratory further indicated that midline structures known as circumventricular organs (CVOs) also serve as adult neural stem cell (NSC) niches (Bennett et al., 2009, 2010). In the quiescent rat brain, NSC proliferation remains low in all of these sites. Therefore, we recently examined whether ischemic stroke injury (MCAO) or sustained intraventricular infusion of the mitogen bFGF could trigger an up-regulation in NSC proliferation, inducing neurogenesis and gliogenesis. Our data show that both stroke and bFGF induce a dramatic and long-lasting (14 day) rise in the proliferation (BrdU+) of nestin + Sox2 + GFAP+ NSCs capable of differentiating into Olig2+ glial progenitors, GFAP + nestin-astrocyte progenitors and Dcx+ neurons in the SVZ and CVOs. Moreover, because of the upsurge in NSC number, it was possible to detect for the first time several novel stem cell niches along the third (3V) and fourth (4V) ventricles. Importantly, a common feature of all brain niches was a rich vasculature with a blood–brain-barrier (BBB) that was highly permeable to systemically injected sodium fluorescein. These data indicate that stem cell niches are more extensive than once believed and exist at multiple sites along the entire ventricular system, consistent with the potential for widespread neurogenesis and gliogenesis in the adult brain, particularly after injury. We further suggest that because of their leaky BBB, stem cell niches are well-positioned to respond to systemic injury-related cues which may be important for stem-cell mediated brain repair.

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Introduction

It is now widely accepted that neural stem cells (NSCs) residing in the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the hippocampal dentate gyrus are capable of producing new neurons in the adult brain (Doetsch et al., 1999a,b; Doetsch, 2003a,b; Alvarez-Buylla and Lim, 2004; Lie et al., 2004; Mignone et al., 2004; Ming and Song, 2011).

In addition to these well-known sites of neurogenesis, we previously postulated that the circumventricular organs (CVOs) comprise a midline series of adult stem cell niches along the third and fourth ventricles (Bennett et al., 2009, 2010). CVO stem cells possess many of the same characteristics as SVZ neural stem cells (NSCs), including their subventricular location, their ability to proliferate and express stem cell markers like nestin, vimentin and Sox2 and their capacity to give rise to new neurons and glia in vitro, in vivo and after transplantation into the adult brain (Bennett et al., 2009, 2010). Moreover, similar to the SVZ, CVOs have permeable fenestrated capillaries and thus lack the endothelial blood–brain-barrier (BBB) present in the remainder of the brain (Johnson and Gross, 1993; Tavazoie et al., 2008). Consequently, the CVOs are able to sense and excrete factors from and into the blood, and are thus often called the "windows of the brain" (Gross and Weindl, 1987; Moyse et al., 2006; Joly et al., 2007).

In the quiescent brain, SVZ and CVO NSCs are present in relatively low numbers. Therefore, in this study, we sought to stimulate their proliferation and differentiation via ischemic injury (i.e. experimental stroke) or infusion with a NSC mitogen (bFGF). We will show that both ischemic stroke and bFGF produce a dramatic and long-lasting rise in the proliferation and differentiation of NSCs in the SVZ and CVOs but also in several other novel sites along the third (3V) and fourth (4V) ventricles not described previously. Importantly, all of these sites were also highly permeable to systemically injected sodium fluorescein, suggesting a possible systemic route for injury-related cues to signal niches of the need for new neurons and glia to repair the brain.

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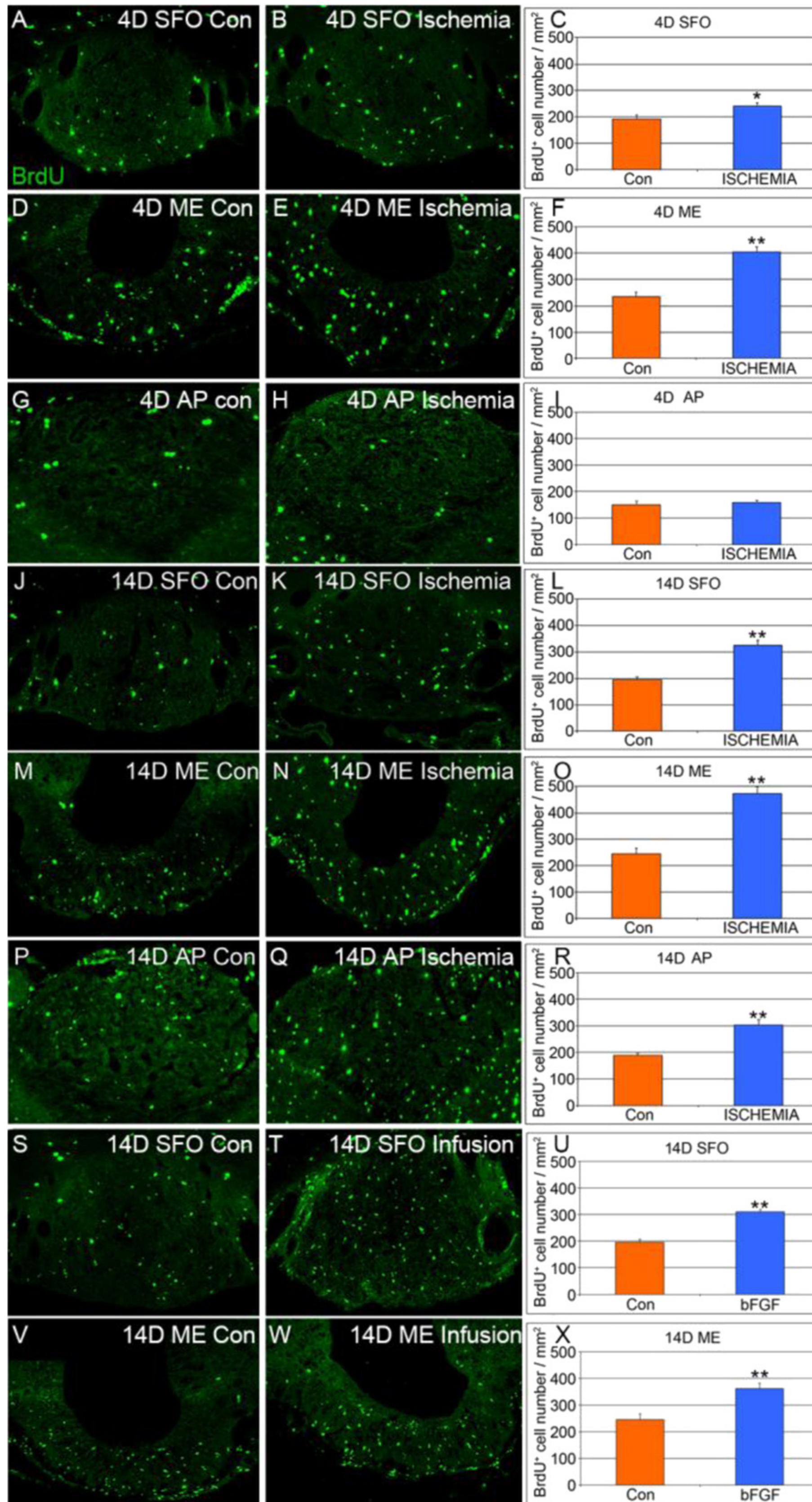


Fig. 1. Induction of cell proliferation in the CVO regions after MCAO and intraventricular bFGF infusion. Localization and quantification of BrdU-labeled cells in CVO regions: SFO (A–C), ME (D–F) and AP (G–I) at 4 days or in SFO (J–L), ME (M–O) and AP (P–R) at 14 days after MCAO or in SFO (S–U) and ME (V–X) 14 days after bFGF infusion as compared to control. Data is expressed as mean ± S.E.M. $^{*}p < 0.05$, $^{**}p < 0.01$.

Materials and methods

Animals

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 IACUC Committee of the Thomas Jefferson University. All surgery was performed under anesthesia, and all efforts were made to minimize suffering.

Antibodies and reagents

Bromodeoxyuridine (BrdU) was purchased from Fisher Scientific. Sodium Fluorescein was purchased from Pfaltz & Bauer, Inc. Basic fibroblast growth factor (bFGF) was purchased from (R&D Systems). The following primary antibodies were used in these experiments: rabbit anti-GFAP (DAKO, 1:1500), mouse anti-nestin (Millipore, 1:200), rabbit anti-doublecortin (Cell Signaling, 1:390), mouse anti-Sox2 (R&D Systems, 1:1000), rat monoclonal anti-BrdU (Accurate Chemical, 1:200), rabbit anti-Olig2 (Millipore, 1:500), rat anti-mouse CD31, mouse anti-rat CD-3 (BD Biosciences, 1:25). Rabbit anti-myeloperoxidase (MPO) (DAKO, 1:300) and rabbit anti-IBA-1 (WAKO, 1:600). All secondary antibodies were Alexa Fluor antibodies from Invitrogen including donkey anti-rabbit 405, 488 and 594, donkey anti-mouse 405, 488, 594 and 647, donkey anti-rat 488, 594 and 647.

BrdU administration

Adult male Sprague–Dawley rats weighing 275–300 g were administered BrdU (1 mg/ml) in their drinking water for one week, and then on the 7th day, the rats were subjected to middle cerebral artery occlusion (MCAO) or osmotic minipump implantation surgery. The rats received two BrdU injections (i.p., 100 mg/kg of body weight) 12 h apart, starting on the day of the operation and postoperative day 1 to day 3 for a total of 8 injections. On either postoperative day 4 or day 14, the rats were deeply anesthetized with Nembutal (70 mg/kg) and transcardially perfused with cold 4% paraformaldehyde.

Intraventricular bFGF infusion

bFGF was infused into the lateral ventricle using an Alzet osmotic minipump. Briefly, adult male Sprague-Dawley rats weighing 275–300 g were administered BrdU before and after surgery as described above. Rats were anesthetized and placed in a stereotaxic apparatus (David Kopf) with bregma and lambda in the same horizontal plane. A midline incision was made and a stainless steel cannula (28 gauge) was implanted in the lateral ventricle (reference to bregma: anteroposterior = −0.8 mm, lateral = +1.5 mm, depth = 3.5 mm) and connected to an osmotic minipump (model 2004, Alzet, Palo Alto, CA). The animals received recombinant human bFGF (50 μg/ml) or artificial cerebrospinal fluid (aCSF) (n = 3 for each group) at a flow rate of 0.25 μl/h, resulting in a delivery of 300 ng of bFGF per day for 14 d.

Focal ischemic stroke: MCAO

Adult male Sprague-Dawley rats weighing 275–300 g were anesthetized using SQ ketamine hydrochloride, xylazine and acepromazine maleate (60 mg/kg, 10 mg/kg, 5 mg/kg respectively). Body temperature was monitored with a rectal temperature probe, and maintained with a heating pad and/or a small fan to within 0.5 °C.

The rats were placed supine after anesthesia and MCAO was performed as previously reported (Goldmacher et al., 2013) and modified by Chen et al. (2013) and Wei et al. (2013). Briefly, the right common carotid (CCA) and external and internal carotid arteries (ECA, ICA) were exposed and the right ECA was ligated. The right CCA was ligated

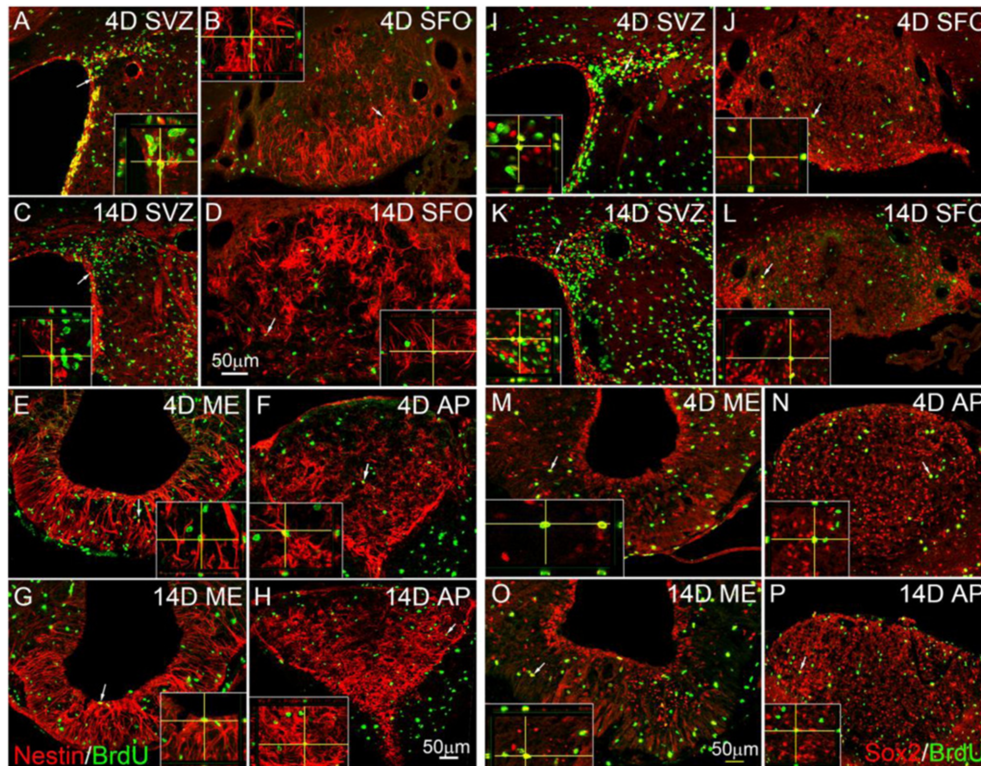


Fig. 2. Photomicrographs of dividing neural progenitor cells in the SVZ and CVO regions on days 4 and 14 after MCAO. (Left panel): Nestin and BrdU double-labeled cells can be found in the SVZ (A, C) and CVO regions: SFO (B, D), ME (E, G) and AP (F, H) at 4 (A, B, E, F) and 14 (C, D, G, H) days post-MCAO. (Right panel): Similarly, Sox2 and BrdU double-labeled cells can be found in the SVZ (I, K) and CVO regions: SFO (J, L), ME (M, O) and AP (N, P) at 4 (I, J, M, N) and 14 (K, L, O, P) days post-MCAO. Representative double-immunoreactive cells in each CVO region are indicated by arrows and shown at higher magnification (insets).

at the proximal end and right ICA blood flow was then blocked by clamping using a micro clip at its origin. A silicone rubber-coated nylon filament (diameter with coating is 0.39 mm, Doccol) was then inserted into the lumen of the CCA through a small opening. The clamp on the right ICA was then removed and the nylon filament was carefully advanced into the ICA until it obstructed the MCA. Two hours later, the nylon filament was removed and CCA was ligated to stop bleeding and allow reperfusion of the brain.

Viral reporter injections

Animals were anesthetized and placed in a Kopf stereotaxic frame. 2 μl of AAV-CAG-GFP expressing vector (Vector Biolabs, Philadelphia, PA) was stereotaxically injected into the right lateral ventricle near the SVZ (reference to bregma: anteroposterior = −0.8 mm, lateral = −1.5 mm, depth = 3.5 mm) at a rate of 0.2 μl/min via a 33 g needle connected to a 5 μl Hamilton syringe driven by a Harvard pump. Following infusion, the viral vector was permitted to diffuse away from the needle for 2 min before withdrawal. Animals were given an experimental stroke on the next day (>90% survival after both procedures) and sacrificed 14 days later.

Immunostaining

Animals were perfused with cold (4 °C) paraformaldehyde (4%). Brains were postfixed in 4% paraformaldehyde at 4 °C for 24–36 h and immersed in 30% sucrose solution at 4 °C, then embedded in OCT (Tissue-Tek, Sakura, Japan) before cutting with a cryostat (Microm HM505E). Coronal sections were cut at 20 μm (rat) or 30 μm (mouse) on a cryostat and collected onto slides. After antigen retrieval, sections were incubated with primary antibodies in blocking buffer containing 0.1% Triton X100 and 5% normal donkey serum (NDS) in 0.01 M phosphate-buffered saline (pH 7.4). Sections were incubated with primary antibodies for 48 h at 4 °C, washed and incubated with secondary antibodies for 2 h at room temperature in blocking buffer. The nuclear dye Hoechst 33258 or DAPI was added after secondary antibody incubation. Sections were then cover-slipped and examined, images were acquired using laser confocal microscopy (Olympus Fluoview).

For BrdU staining, tissue sections were treated with 2N HCl for 20 min at 30 °C. Sections were then washed with PBS followed by incubation for 10 min at room temperature with 0.1 M borate buffer (pH 8.5). Sections were then washed with PBS and processed for immunocytochemistry as above.

Sodium-fluorescein uptake into the brain

We assessed BBB permeability in normal rats (N = 3) and rats 3 h post-MCAO (N = 3) using the methods of Fabis et al. (2008) or Hawkins and Egleton (2006). Briefly, rats were anesthetized and perfused transcardially with oxygenated 0.9% normal saline followed by normal saline containing 1 g/L sodium fluorescein for 3–5 min (3 ml/min per hemisphere), followed by a 5 min washout with normal saline (Hawkins and Egleton, 2006). An incision of right atrium was made immediately preceding the delivery of solution to provide pressure relief. Alternately, 10% sodium fluorescein in normal saline (1.5 ml) was injected into peritoneal cavity as previously described (Fabis et al., 2008). The markers were allowed to circulate in the rats for 60 min before transcardial perfusion with 200 ml normal saline. Brains were quickly removed after perfusion, snap-frozen then stored at −80 °C until sectioning. Coronal sections (30 μm) were mounted onto slides and visualized in an Olympus confocal microscope.

Quantitative analysis

Every other coronal section (SFO and AP), or every fourth section (ME, bregma anteroposterior: from −2.20 to −3.30 mm) starting from the first rostral section of each CVO region was selected for immunostaining and quantitative analysis. All BrdU-positive nuclei in each specific CVO region (i.e. SFO, ME, AP) were counted at 200× magnification.

Statistical analysis

All data are presented as the mean ± SEM. The statistical significance of the mean was calculated by Student's *t*-test. A P-value < 0.05 was considered significant.

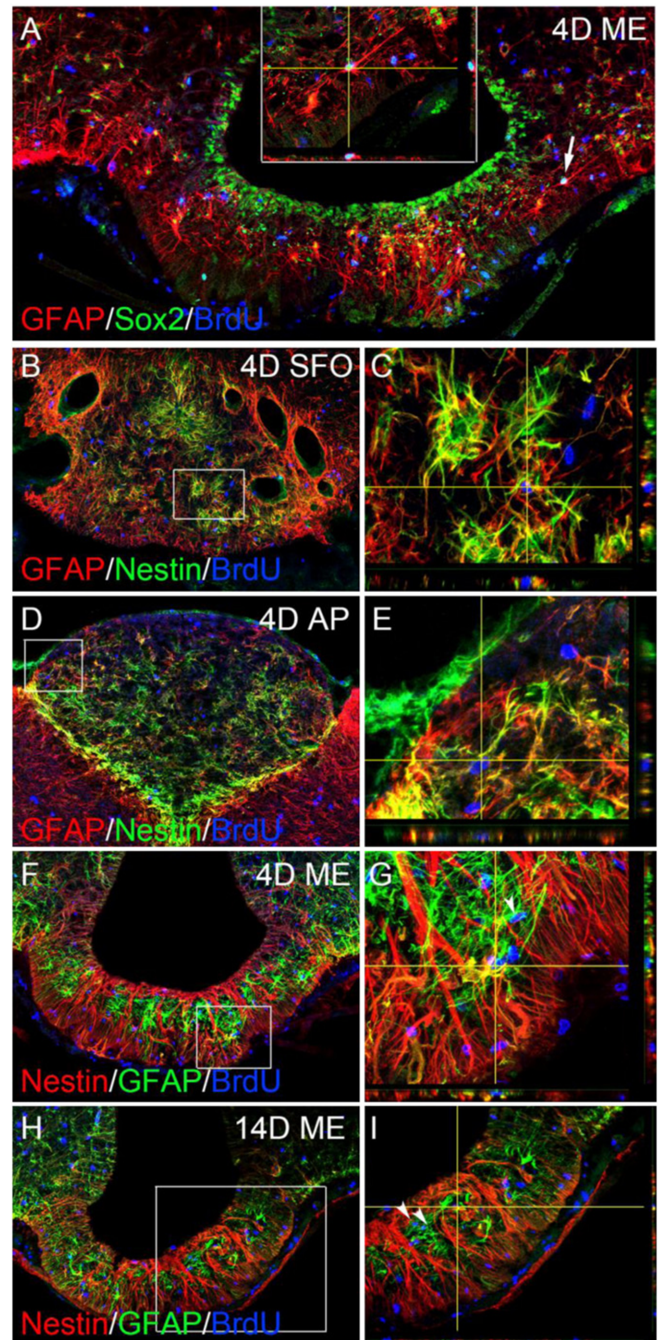


Fig. 3. Dividing cells have NSC identities in CVO regions on day 4 and day 14 after MCAO. A representative GFAP, Sox2 and BrdU triple-immunoreactive cell in the ME on day 4 after MCAO, indicating its identity as a NSC (A). Cell indicated in (A) by arrow shown at higher magnification (inset). In CVO regions (B, D, F and H), some BrdU+ cells express nestin and GFAP. Boxed areas are shown at higher magnification in C, E, G and I with representative triple-labeled cells indicated. Arrowheads indicate GFAP + BrdU + nestin-astrocyte progenitor cells.

Results

Ischemic injury and bFGF infusion induces a sustained proliferation of NSCs in SVZ and CVO niches

In these studies, we used control (unoperated; N = 3 in 4d group, N = 3 in 14d group) rats and rats (N = 4 in 4d group, N = 5 in 14d group) with moderate to severe ischemic damage after MCAO (Yang et al., 2010 as modified in Goldmacher et al. (2013)). All rats were administered BrdU as described in Materials and methods to assess cell proliferation in the SVZ and a variety of CVO regions (subfornical organ: SFO, median eminence: ME, area postrema: AP) at various times after experimental stroke. We found that by 4 days following MCAO, CVO niches (except for AP) exhibited a significant increase in the number of BrdU-labeled cells as compared to controls (Figs. 1A–I), similar in magnitude to that observed in the SVZ (not shown here). With time, this proliferative effect was enhanced and sustained such that the number of BrdU/Nestin, BrdU/Sox2, and BrdU/GFAP double positive cells was nearly doubled in all CVOs examined (Figs. 1J–R; Suppl. Fig. 1) by 14 days after stroke. This injury-induced effect was qualitatively and quantitatively similar to that observed when rats were intraventricularly infused with the stem cell mitogen bFGF for 14 days (Figs. 1S–X).

Besides stem cells, immune cells which are also highly proliferative can infiltrate the brain after ischemia (Gelderblom et al., 2009; Grønberg et al., 2013). To distinguish these cells from proliferating stem cells, brain sections were stained for the presence of blood-borne cells (CD-3 for T-cells, IBA-1 for macrophages and microglia and MPO for neutrophils). We found no BrdU+ cells in the niches that co-labeled with T-cell (Suppl. Fig. 2) or neutrophil (Suppl. Fig. 3) markers though some double-labeled cells were observed in the infarct core at 4 days post-MCAO. Only proliferating (BrdU + IBA-1+) macrophages/microglia were increased (Suppl. Fig. 4), contributing to the overall number of proliferating cells seen in niche and infarct sites on the ischemic side of the brain. Similar results were observed at 14 days post-MCAO (data not shown).

To assess whether these proliferating cells were indeed NSCs, we next double (Fig. 2) and triple (Fig. 3) labeled for BrdU and known phenotypic markers of stem cells, such as nestin, Sox2 and GFAP. Using confocal microscopy, we found that BrdU+ CVO cells from the SFO, ME and AP (Figs. 2B,D; E–H; J,L; M–P), similar to traditional stem cells of the SVZ (Figs. 2A,C,I,K), co-expressed the NSC markers GFAP, nestin and Sox2 at 4 and 14 days after MCAO (Figs. 2, 3).

Stroke induces cell proliferation in other novel niches along third and fourth ventricles

Importantly, besides the ME, proliferation of Sox + and nestin + BrdU-labeled NSCs was also greatly enhanced at other sites along the 3V but only on the side ipsilateral to the ischemia (Ipsi), as compared to the contralateral (Contra) uninjured side (Fig. 4). In fact, several distinct zones of proliferation were identified. The first zone was found along the upper wall of the third ventricle where dividing cells, like those in the SVZ and CVO stem cell niches, were subependymal in location and co-labeled for BrdU and NSC markers Sox2, nestin and GFAP (Figs. 4B,F).

Another highly proliferative cell population was observed at mid-3V level in ischemic rats. These BrdU+ cells could be distinguished

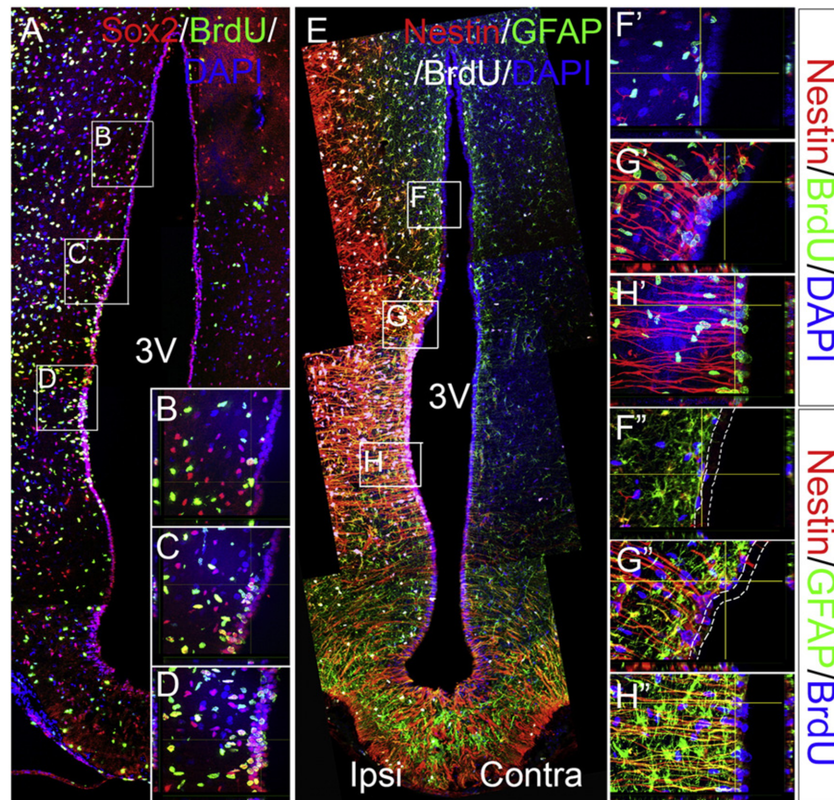


Fig. 4. Enhanced cell proliferation and distinctive cell morphology in three different zones of the third ventricle (3V) on day 4 after MCAO. Ipsi indicates the side ipsilateral and Contra the side contralateral to stroke. Along the 3V, many BrdU+ cells express the neural stem cell marker Sox2 (A). Boxed areas in A representing different 3V zones of proliferation (i.e. upper subependymal zone, middle transitional zone and lower ependymal zone) shown at higher magnification (B–D). Along the third ventricle, many BrdU+ cells express neural stem cell markers Nestin and GFAP (E). (F–H): Boxed areas in E representing different 3V zones showing triple-labeled cells from upper subependymal zone (F′–F″), middle transitional zone (G′–G″) and lower ependymal wall (H′–H″). Dashed line in F″ and G″ indicate the ependymal layer. Note distinct tanycyte morphology of dividing cells in H′–H″ compared to NSC in F′–F″ and the mixture of both cell types in G′–G″.

from other NSCs by their ependymal location and their long nestin + processes which did not always co-label for GFAP (Figs. 4D,H). These cells have been referred to as tanycytes (Robins et al., 2013) exhibited the greatest proliferation on the infarcted side (Figs. 4D,H). Interestingly, the region between zones B and D or F and H were transitional in nature, containing both dividing ependymal and subependymal cells (Figs. 4C,G).

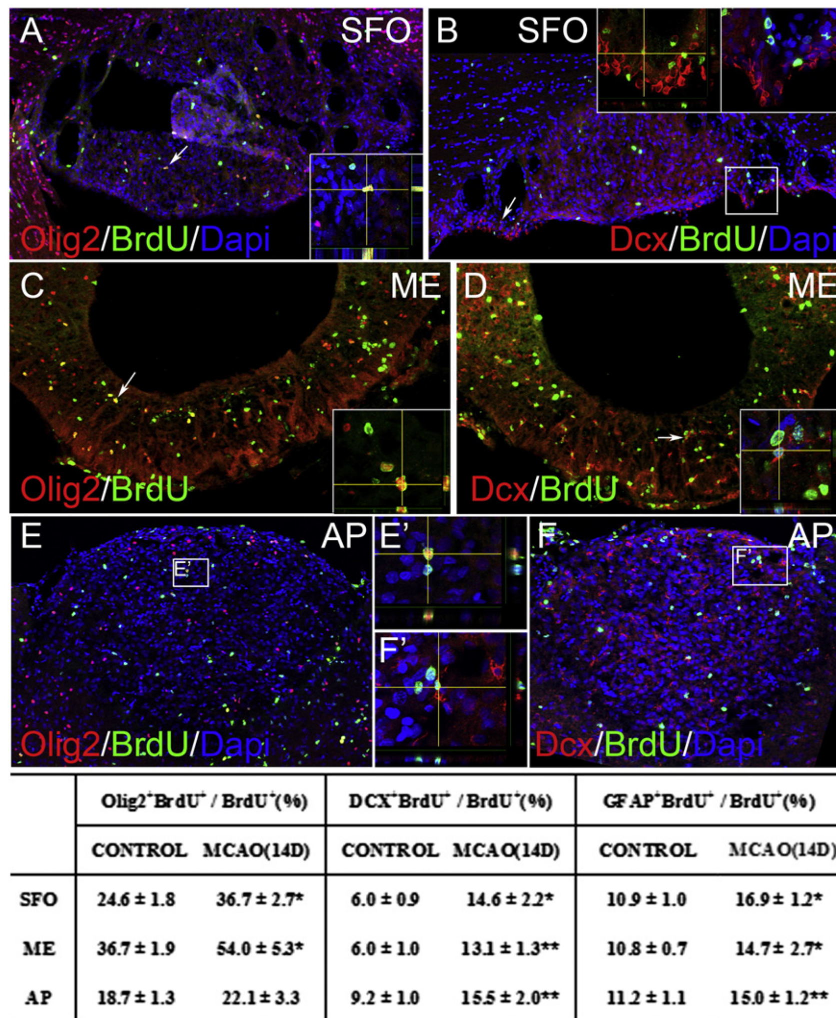
Likewise, we found novel sites of cell proliferation along the whole fourth ventricle but particularly at the lateral-most recesses. In the quiescent control brain, these regions contained very few Brdu + cells. However, by 14 days following the stroke, the number was enhanced (Figs. 6H–K).

To rule out the possibility that NSCs in these novel sites had migrated there from other more established niches like the SVZ, a viral vector (AAV-CAG-GFP) was injected into the right lateral ventricle to label the SVZ region a day prior to MCAO. Fourteen days later, we found no evidence of labeled cells in the 3V (Fig. 7) or 4V (data not shown) niches.

Proliferating CVO and 3V and 4V stem cells differentiate to express glial and neuronal markers

We next examined the potential of injury-induced CVO, 3V and 4V NSCs to develop down expected differentiation routes towards neurons, oligodendroglia and astrocytes. In these studies, BrdU was administered to rats for 4 days after MCAO and brains were examined immediately thereafter or 14 days post-MCAO. We found proliferating cells in the CVOs (Fig. 5) and in all 3V and 4V zones (Fig. 6) which co-labeled with the oligodendrocyte progenitor cell marker Olig2 at both 4 days and 14 days post-MCAO. These cells were significantly increased in number compared to controls (Fig. 5 Table). Since the astrocyte marker GFAP also marks NSCs, it is not ideal for tracking astrocyte differentiation unless simultaneously used with nestin (found in NSCs but not astrocytes). Indeed, using these two markers, we find GFAP + BrdU + nestin-astrocyte progenitor cells at 4 (Fig. 3G arrowhead) and 14 (Fig. 3I arrowheads) days post-MCAO in the CVOs as well as other niche sites (data not shown). Importantly, when neurogenesis was tracked using the early neuron marker doublecortin (Dcx), we also observed increased BrdU+/Dcx+ cells in CVOs (Fig. 5) and in 3V and 4V niches (Fig. 6) at 14 days post-MCAO. These cells likely withdrew from cell division 10 days earlier, at the end of BrdU treatment, before differentiation into postmitotic neurons. Interestingly, the proportion of differentiating neurons was significantly increased after MCAO compared to oligodendrocytes and astrocytes (Fig. 5 Table), representing a shift towards neurogenesis in the injured brain. Likewise, bFGF infusion also resulted in the differentiation of Dcx + neurons at these sites (Fig. 8).

Finally, in some cases BrdU + nestin + cells could be observed in a chain formation potentially migrating away from the 3V and ME niches



	$Olig2^{+}BrdU^{+}$ / $BrdU^{+}$(%)		$DCX^{+}BrdU^{+}$ / $BrdU^{+}$(%)		$GFAP^{+}BrdU^{+}$ / $BrdU^{+}$(%)	
	CONTROL	MCAO(14D)	CONTROL	MCAO(14D)	CONTROL	MCAO(14D)
SFO	24.6 ± 1.8	36.7 ± 2.7*	6.0 ± 0.9	14.6 ± 2.2*	10.9 ± 1.0	16.9 ± 1.2*
ME	36.7 ± 1.9	54.0 ± 5.3*	6.0 ± 1.0	13.1 ± 1.3**	10.8 ± 0.7	14.7 ± 2.7*
AP	18.7 ± 1.3	22.1 ± 3.3	9.2 ± 1.0	15.5 ± 2.0**	11.2 ± 1.1	15.0 ± 1.2**

Fig. 5. Differentiation of BrdU + cells into oligodendrocyte progenitors and neurons in CVO regions on day 14 after MCAO. BrdU + cells in the SFO (A, B) ME (C, D) and AP (E, F) differentiate into oligodendrocyte progenitors identified by staining for Olig2 (A, C, E). Newly generated (BrdU +) neurons were also identified by the neuronal marker Dcx (B, D, F). Higher magnification of labeled uni/bi-polar and stellate-shaped cells found in boxed areas and indicated by arrows are shown in insets. Table shows increases in the percentages of newly generated oligodendrocyte progenitors, astrocytes and neurons of total BrdU + proliferating cells as compared to controls in CVO regions. Data is expressed as mean ± S.E.M. *$p < 0.05$, **$p < 0.01$.

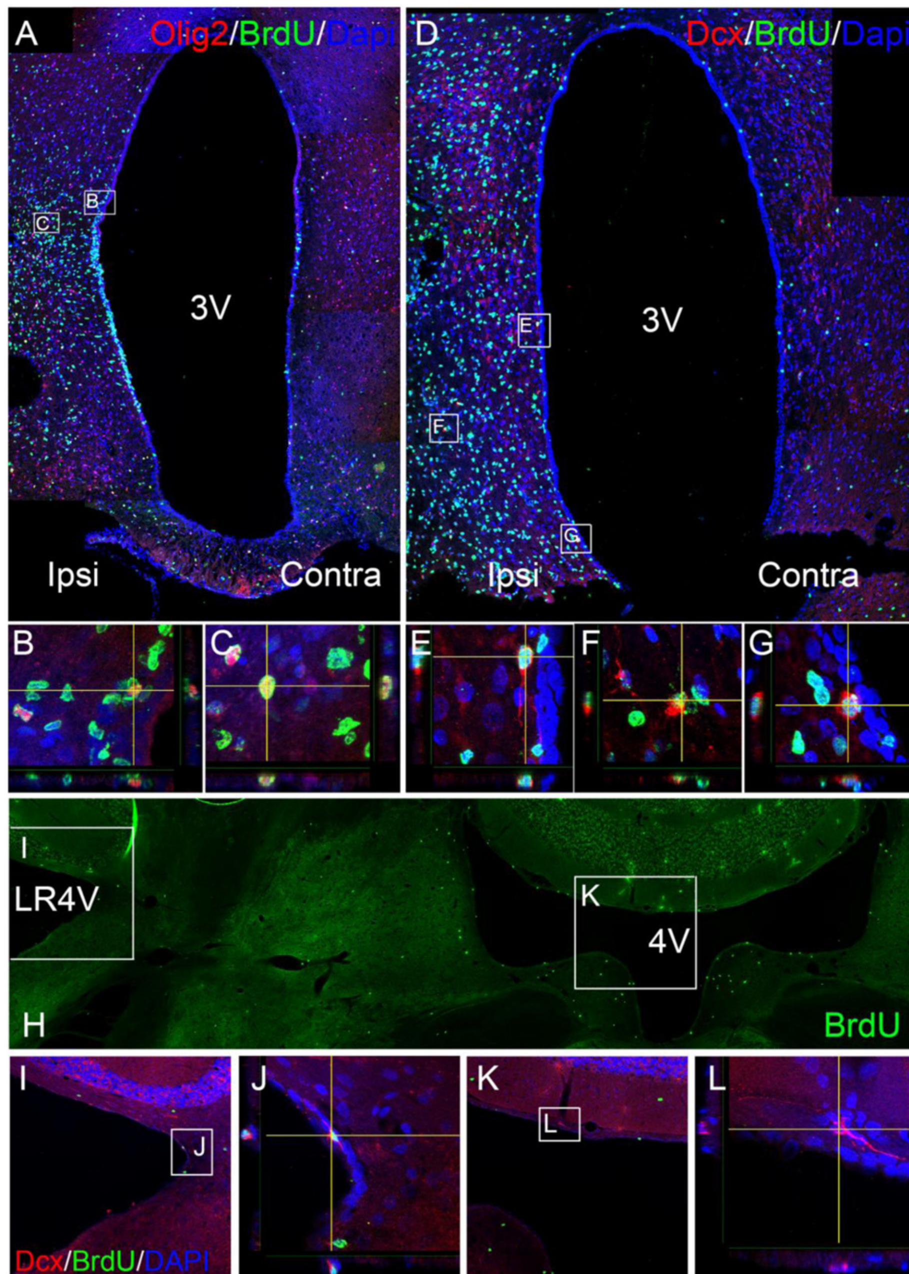


Fig. 6. Differentiation of BrdU+ cells into oligodendrocyte progenitors and neurons in 3V and 4V regions on day 14 after MCAO. Along the third ventricle, some BrdU+ cells express the oligodendrocyte progenitor marker (Olig2) on the side ipsilateral (Ipsi) to the stroke (A) as compared to the contralateral (Contra) side. The double-labeled cells in boxed areas in A are shown in higher magnification (B, C). Some newly generated (BrdU+) neurons were also identified by the neuronal marker Dcx (D). The double-labeled uni/bi-polar and stellate-shaped cells in the boxed areas in D are shown in higher magnification (E, F, G). Along fourth ventricle lateral recesses, BrdU+ cells can be found following stroke (H). These cells differentiated into Dcx+ neurons by 14 days post-MCAO (I–J). Some BrdU-Dcx+ neurons can also be found at the same time (K–L). Boxed regions are shown in higher magnification.

(Suppl. Fig. 5) to the infarct core where differentiated stem cell progeny (BrdU + Dcx+, BrdU + GFAP+ and BrdU + Olig2 cells) can be seen (Suppl. Fig. 5).

Increased BBB leakage in all brain stem cell niches after stroke

The SVZ and CVOs are highly vascularized brain regions (Figs. 9A–E), containing fenestrated capillaries associated with a permeable BBB (Johnson and Gross, 1993; Tavazoie et al., 2008). We wondered whether BBB leakiness was common to all stem cell niches, even novel sites in the 3V and 4V, and whether permeability was increased in these locations following disruption of the BBB due to stroke. To examine these issues, we injected sodium-fluorescein into control and MCAO rats and examined all presumptive brain NSC niches. Consistent with their fenestrated capillaries, we found fluorescein leakage into all stem cell niches in the control brain, including the SVZ, CVOs, 3V and 4V (Figs. 9F,H,J,L,N). Following ischemic injury, increased leakage of fluorescein was seen at the stroke site (Fig. 9G) and apparently in all niche regions (Figs. 9I,K,O arrows). In fact, because fluorescence intensity was so great, it was necessary to reduce exposure time for all MCAO images. Nonetheless, we found that the sites of stroke-enhanced fluorescence paralleled stroke-enhanced cell proliferation/neurogenesis. Thus, both parameters were increased in the SVZ and 3V, particularly on the ipsilateral side where BBB compromise was likely greatest as a result of the nearby stroke (Abo-Ramadan et al., 2009; Engelhardt and Liebner, 2014). In the brainstem, at a distance from the stroke site, we observed increased fluorescence throughout the 4V similar to 4V sites of neurogenesis (Fig. 9M). Taken together, these data indicate a positive

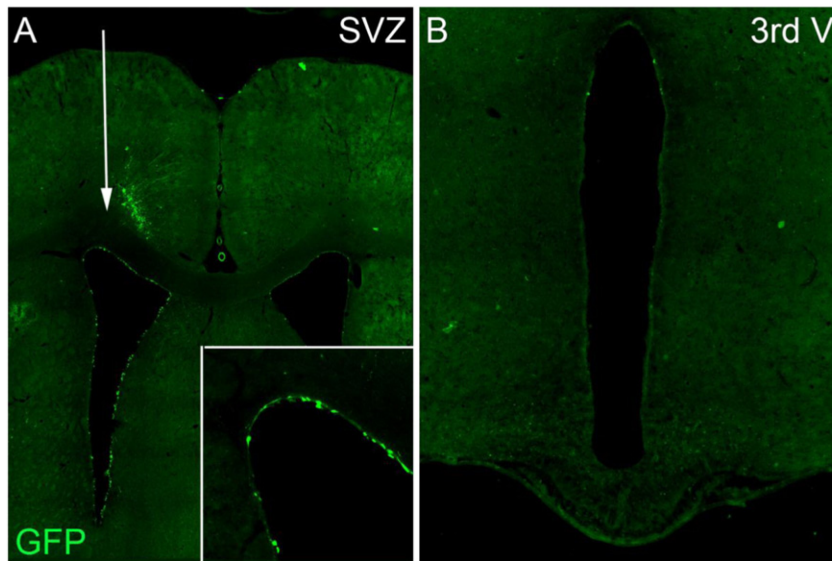


Fig. 7. Expression of AAV-CAG-GFP in NSCs 14 days after injection of virus into the lateral ventricle near the SVZ region and MCAO. In low power view (A), please note GFP+ cells (see inset) in SVZ and immediately adjacent to the injection site. In contrast, GFP+ cells were not observed in the 3V and ME niches (B).

correlation between stem cell proliferation and the degree of BBB leakiness in the adult brain, both of which are amplified after ischemic injury.

Discussion

Although for many years, the brain was believed to be a static structure, in the past several decades it has become widely accepted that two zones of neurogenesis exist, both in the adult forebrain. Thus, throughout life, NSCs found in the SVZ of the anterolateral ventricle and the SGZ of the hippocampal dentate gyrus respectively give rise to new olfactory interneurons and hippocampal granule neurons (Rakic, 1985a,b; Doetsch et al., 1999b; Doetsch, 2003a,b; Alvarez-Buylla and Lim, 2004; Lie et al., 2004; Mignone et al., 2004). Several years ago, our laboratory further showed that an additional series of adult stem cell niches called the CVOs exist along the ventricular midline (Bennett et al., 2009). As with the SVZ and SGZ, CVO cells stained for the classic NSC markers GFAP, nestin and vimentin and divided to give rise to new neurons and glia in the adult brain. Moreover, following their heterotopic transplantation, CVO-derived NSCs had the potential

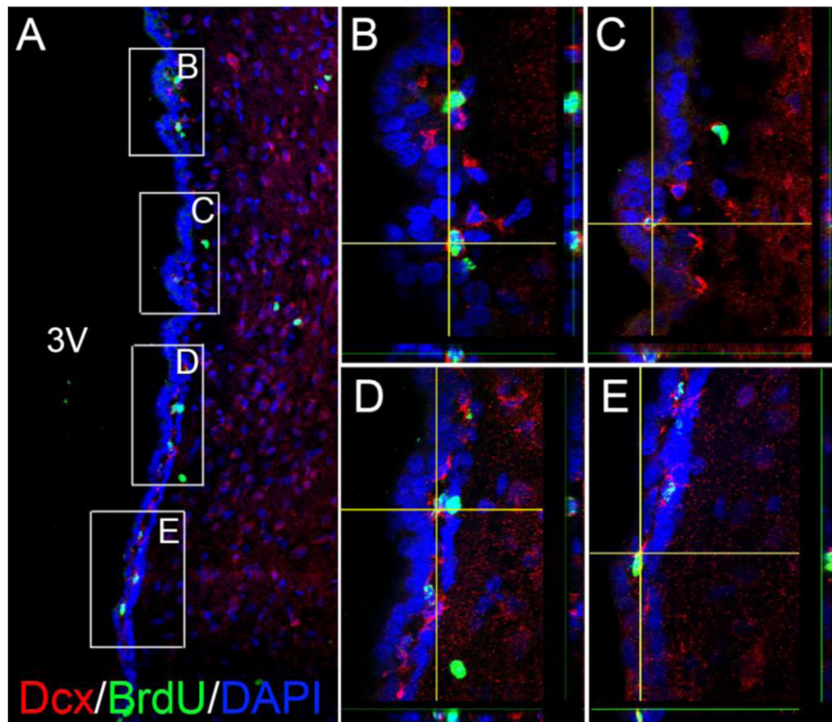


Fig. 8. Neurogenesis along the third ventricle 14 days after intraventricular bFGF infusion. BrdU+ cells with neuronal identities observed in the 3V after staining with Dcx (A). The boxed areas from (A) show double-labeled cells in two different zones of 3V wall at higher magnification (B–C: upper subependymal zone, D–E: transitional zone).

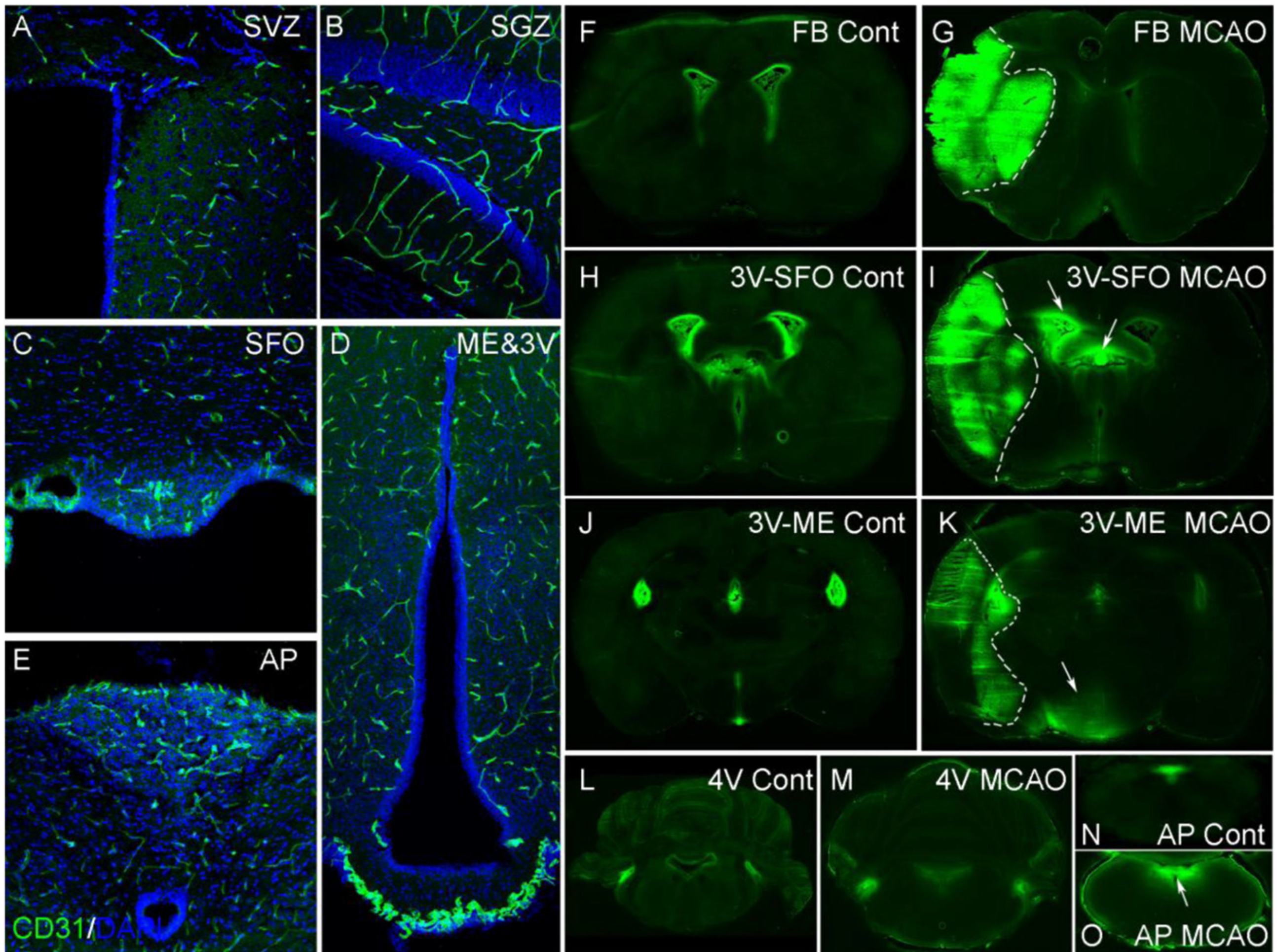


Fig. 9. The stem cell niches in adult brain are highly vascularized and increased blood–brain-barrier permeability is observed after stroke. Abundant CD31 immunoreactive blood vessels present in SVZ, SGZ, SFO, ME and AP regions (A–E). Note the absence of CD31 immunoreactive blood vessels in the center of ME indicating its unique vasculature profile of the CVOs (D). Sodium-fluorescein labels almost all stem cell niches in the normal brain, including the SVZ, CVOs (F, H, J, L and N). Note fluorescence particularly in novel 3V and 4V (lateral recesses) niches (J, L, M). Three hours after MCAO, sodium-fluorescein labeling was found in the infarcted regions (cerebral cortex and striatum) (G, I) as well as expanded areas surrounding CVOs, 3V and 4V niches (I, K, M and O). Fluorescence was so bright that all MCAO images were reduced in intensity. Arrows indicate enhanced spread of fluorescein in CVO regions. Dashed lines indicate fluorescein leakage due to stroke infarct.

to integrate into the rostral migratory stream and differentiate into mature neurons in the olfactory bulb, similar to SVZ-derived NSCs (Bennett et al., 2010).

In this study, we examined neurogenesis/gliogenesis in the SVZ and CVO sites, both in the quiescent brain where NSC proliferation remains quite low and following ischemic injury where NSCs are thought to play a role in repair (Temple, 2001; Shen et al., 2004; Barkho et al., 2006). Indeed, we found a dramatic and sustained rise in NSC proliferation in the SVZ and CVOs after focal stroke. These findings are consistent with several recent reports showing similar increases in the SVZ and SGZ of the MCAO-lesioned rat (Zhang et al., 2001, 2007; Thored et al., 2006; Wang et al., 2011; Sanin et al., 2013), and significantly, in ischemic patients (Sanin et al., 2013). Importantly, the increase in proliferating cells could not be attributed to the infiltration of proliferating immune cells (T-cell, neutrophil) after stroke although there was a rise in dividing IBA-1+ (nestin negative) macrophages/microglia on the ischemic side.

Of particular significance, this study further revealed unexpected collections of dividing (BrdU+) stem cells at other sites along the ventricular system, in structures unrelated to the SVZ and CVO niches. These sites, which had not been detected previously, became apparent as a result of the enhanced proliferation seen after stroke or bFGF stimulation. Of particular note were the groups of BrdU+ cells seen along the third ventricle. These cells fell into two distinct classes, one of which was subependymal and stained positively for nestin, GFAP and Sox2. This novel cell group was similar in location and phenotypic characterization to NSCs found in the SVZ and CVOs. The second group of cells was composed of ependymal cells with long processes projecting away from the ventricular wall. These cells known as tanycytes have been described previously both in the quiescent brain (Chiasson et al., 1999; Spassky et al., 2005) and after injury (Lindvall and Kokaia, 2008; Carlén et al., 2009; Barnabé-Heider et al., 2010; Robins et al., 2013) or other inductive cues (Sundholm-Peters et al., 2004; Xu et al., 2005; Zhang et al., 2007; Migaud et al., 2010; Lee et al., 2012). Lying between these two groups of cells, we noted a transitional zone composed of a mixture of dividing subependymal and ependymal cells.

In addition, proliferating BrdU+ cells were also observed along the lining of the fourth ventricle, particularly at the lateral recesses, near the aperture connecting the ventricular and subarachnoid spaces. These sites likely had escaped previous discovery due to their low number in the uninjured brain but stroke-enhanced cell proliferation enabled their detection here. The fact that we found no evidence of virally (AAV-CAG-GFP) labeled SVZ NSCs in 3V or 4V 14 days after MCAO suggests that stem cells in these regions did not migrate there from other areas of high stem cell proliferation (i.e. SVZ). Instead, the 3V and 4V are likely novel niches for stem cell production.

Consistent with this notion of widespread ventricular niches are earlier studies demonstrating that cells isolated from the adult lateral, third and fourth ventricles (sites of CVO, 3V and 4V niches) generate

neurospheres in culture (Reynolds and Weiss, 1992; Weiss et al., 1996; Chouaf-Lakhdar et al., 2003; Bauer et al., 2005; Charrier et al., 2006; Itokazu et al., 2006). The induction of stem cells was mimicked in all these sites by intraventricular infusion of the cell mitogen bFGF. Taken together, these findings suggest that stem cell niches are present at multiple sites along the entire ventricular system, not only in the forebrain as once thought, and that stem cells in these niches are increased dramatically following injury or bFGF.

Significantly, in all the stem cell niches, we found BrdU + labeled Dcx + neurons, Olig2 + oligodendrocytes and GFAP + nestin-astrocyte progenitors 14 days after MCAO injury and bFGF infusion. Proportionally, there was a shift towards neurogenesis even in niches which predominantly give rise to glia in the absence of injury (Bennett et al., 2009). Finally, in some cases, clusters of cells appeared to migrate in a chain away from these niches, much like the rostral migratory stream leaving the SVZ (Pencea et al., 2001). And indeed, differentiated stem cell progeny (BrdU + Dcx +, BrdU + GFAP + and BrdU + Olig2 cells) were observed in the infarct region, although their niche of origin could not be determined. Regardless of where these cells derive from, our results are consistent with widespread and greatly amplified neurogenesis and gliogenesis in the adult brain after injury, suggesting a potential role for these cells in brain repair.

However, just how these cells are signaled to proliferate and differentiate after injury remains unknown. The fact that MCAO inductive cues must traverse long distances from the site of injury to reach niches even in the lower brainstem raises the prospect of a systemic route of communication. As shown here and elsewhere (Johnson and Gross, 1993; Shen et al., 2008; Tavazoie et al., 2008), the CVOs and SVZ indeed contain a rich vasculature of fenestrated capillaries, ideally suited for the delivery of systemic signals. Consistent with this finding, we found leakage of injected sodium fluorescein at all of the sites in which induced proliferation and neurogenesis/gliogenesis had been observed, including SVZ, CVO, 3V and 4V niches. Moreover, following stroke, which further disrupts BBB integrity and function (Abo-Ramadan et al., 2009; Engelhardt and Liebner, 2014), we observed greater spread of fluorescein at these sites. Together, these data suggest that blood-borne cues could signal even distant stem cell niches (Slevin et al., 2000; Zhang et al., 2000; Offner et al., 2006; Li et al., 2013), communicating with them through fenestrated capillaries made more leaky after stroke. However, the fact that ICV infusion of bFGF which does not reduce BBB function (Murakami et al., 2008; Huang et al., 2012) also induces proliferation suggests that CSF cues may also signal stem cell niches after injury. Finally, local factors, such as VEGF and TNF-α, which have been shown to be important modulators of neurogenesis in the SVZ after stroke (Kovács et al., 1996; Thored et al., 2006; Iosif et al., 2008; Lee et al., 2008; Kokaia et al., 2012) may also play a key role in CVO and 3/4V niches.

The precise source and identity of these cues and the molecular pathways via which they signal adult stem cell niches to increase proliferation, neurogenesis and gliogenesis are important new lines of investigation. Additionally, in the future, it will be key to show which niches give rise to neurons and glia after injury and that these newly minted neurons and glia deriving from these niches indeed develop into functional neurons, astrocytes and oligodendrocytes capable of repairing injury in the adult brain.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nbd.2014.11.016.

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