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Focal cerebral ischemia activates neurovascular restorative dynamics in mouse brain

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TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Materials and methods
- *3.1. Animals*
- *3.2. Animal model of transient focal cerebral ischemia*
- *3.3. BrdU injections*
- *3.4. Immunohistochemistry*
- *3.5. Evaluation of functional revascularization*
- *3.6. Statistical analysis*

4. Results
- *4.1. Ischemic injury leads to neurogenesis in damaged brain.*
- *4.2. Ischemic injury activates angiogenesis and partial revascularization in the brain.*
- *4.3. White matter injury does not improve over time despite robust oligodendrogenesis after ischemic stroke.*

5. Discussion
6. Acknowledgements
7. References

1. ABSTRACT

Cerebral ischemia triggers regeneration of neural stem/progenitor cells (NSCs/NPCs), which are associated with neovascularization and white matter repair in the brain. This study analyzed the dynamics of neurogenesis, neovascularization, and white matter injury/repair after middle cerebral artery occlusion (MCAO) and elucidated their temporal association. Mice were subjected to MCAO for 60 minutes and sacrificed up to 28 days after reperfusion. Neurogenesis and angiogenesis, as measured by double staining of 5-bromo-2-deoxyuridine (BrdU) with DCX or tomato lectin, respectively, were substantially activated soon after ischemia and persisted for 4 weeks. Despite the moderate recovery of functional vessels in infarct margin from 7 days post-ischemia, a significant decrease in vascular density remained over time. Clusters of immature neurons localized proximal to angiogenic blood vessels beginning 14 days after ischemia, suggesting interplay between neurogenesis and revascularization. Progenitors of oligodendrocytes (NG2+) constitutively presented in the normal brain and proliferated soon after ischemia. However, axon damage and the loss of white matter integrity after ischemic stroke were almost irreversible, as revealed by sustained decreases of myelin basic protein (MBP) and neurofilament-200 expression.

2. INTRODUCTION

Stroke is a significant cause of morbidity and mortality worldwide. Approximately half of the survivors suffer from long-term sequelae, such as seizures and paralysis (1, 2). Recent discoveries have demonstrated that multiple regenerative processes, including neurogenesis (1), angiogenesis (2, 3), and oligodendrogenesis (4), could be triggered in adult brain after ischemic stroke, aimed toward neurovascular remodeling in peri-infarct areas during stroke recovery (5).

Neurogenesis, the production of new neurons, occurs continuously during adulthood and is one of the mechanisms for neuronal repair after cerebral ischemia. Studies with experimental animals and postmortem human brain tissue have reported increased neurogenesis within the adult brain after stroke (6, 7). Transgenic ablation of neurogenesis by neuronal precursor cell depletion has been shown to worsen stroke outcomes in mice (8), implying a direct and causal relationship between neurogenesis and functional recovery after stroke.

Although neurogenesis is an indispensable mechanism for neuronal repair, angiogenesis also serves a critical role in brain repair by restoring blood supply to the

affected tissue, thereby nourishing survived and newly generated cells. Increased angiogenesis containing newly born vascular endothelial cells, has been found in peri-infarct cortex after transient ischemia (9, 10). Histological measurement of vascular density showed a notable correlation between increased cerebral blood flow and increases in vascular density indicative of angiogenesis (11). The newly generated blood vessels could support neuronal regeneration in two ways. First, newly formed blood vessels may attract neural progenitor cells (NPCs) by producing trophic factors, including brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF), angiopoietin-2 (Ang2), and matrix metalloproteinases (MMPs), which promote the survival and/or differentiation of NPCs (12-14). Second, blood vessels may act as a physical scaffold to guide the migrating NPCs (14, 15). Accordingly, post-stroke patients with a high cerebral blood vessel density appear to make better progress and survive longer than patients with a low vascular density (12).

The interlinked neurogenesis and angiogenesis events could further interact with oligodendrocytes and astrocytes, creating a microenvironment that promotes myelination and neurite outgrowth within the brain (16). Immature oligodendrocyte proliferation has been found in areas surrounding the lateral ventricles, together with a delayed increase in the number of mature oligodendrocytes in peri-infarct areas, after ischemic injury (4). Previous research has documented that enhanced oligodendrogenesis by erythropoietin treatment attenuates white matter injury concurrently with increased neurogenesis after cerebral ischemia (17, 18), suggesting a possible interplay between white matter restoration, neurogenesis, and neurofunctional outcomes.

On the basis of the current knowledge of the significance of post-stroke neurogenesis, angiogenesis, and oligodendrogenesis, we find it conceivable that coordinating the interaction among these endogenous restorative events may lead to an effective neurological recovery after stroke. However, a comprehensive characterization of these events after stroke is still missing. In this study, we used a well-established murine model of transient focal cerebral ischemia to analyze the temporal profile of neurogenesis, neovascularization, and white matter injury/repair after stroke. Understanding the dynamics of these restorative responses would advance our knowledge of post-stroke recovery and open a new avenue for stroke treatment.

3. MATERIALS AND METHODS

3.1. Animals

Adult male C57B/L6 mice (25-28g, Shanghai SLAC Laboratory Animal Co., Ltd., Shanghai, China) were housed in the same animal care facility at a constant temperature (21 ± 1^0C) and with a 12-hr light/12-hr dark cycle. Food and water were available ad libitum. Animal care and surgical procedures were carried out under the protocols approved by IACUC at Fudan University in accordance with institutional guidelines. All efforts were made to minimize animal suffering and the number of animals used.

3.2. Animal model of transient focal cerebral ischemia

Transient focal cerebral ischemia was induced by middle cerebral artery occlusion (MCAO) (19). Briefly, mice were anesthetized with 1% isoflurane (Forane, Abbott, Abbott Park, Illinois, U.S.A.) in air. The carotid arteries were exposed and a 6-0 nylon monofilament coated with a silicone tip (0.16 to 0.18 cm diameter) was introduced into the external carotid artery and advanced 1.8 cm along the internal carotid artery until occluding the origin of the MCA. After 60 minutes, the filament was withdrawn to establish reperfusion and the wound was sutured. Rectal temperature was maintained at 37.0°C during and 30 min after surgery with a temperature-regulated heat lamp. The left femoral artery and vein were cannulated for blood sampling and fluid administration. Arterial blood gas was analyzed 15 minutes before and after induction of ischemia. To confirm the induction of ischemia and successful reperfusion, we evaluated changes in regional cerebral blood flow (rCBF) before, during, and after MCAO using laser Doppler flowmetry.

3.3. BrdU injections

To label newly generated cells, we injected the S-phase marker 5-bromo-2-deoxyuridine (BrdU) (50 mg/kg body weight, Sigma Labs, Inc., Santa Fe, NM, U.S.A.) intraperitoneally twice per day, 8 hours apart according to the indicated regimen (Figure 1A). Specifically, in the 1-day group, BrdU injections were separately given twice daily before and after surgery, and the animals were sacrificed 2 hours thereafter.

3.4. Immunohistochemistry

Animals were sacrificed on days 1, 3, 7, 14 or 28 of reperfusion (n=5 per group). Animals were deeply anesthetized with chloral hydrate (360mg/kg body weight, i.p.) before intracardiac perfusion with 0.9% saline followed by 4% paraformaldehyde. Brains were cryoprotected in 20% sucrose and then in 30% sucrose in phosphate-buffered saline. Immunohistochemistry staining was performed on 30μm free-floating sections in 24-well tissue culture plates. Sections for BrdU staining were pretreated with 1 N HCl for 1 hour at 37°C followed with 0.1 mol/L boric acid (pH 8.5) for 10 minutes at room temperature (14). After blocking with 10% goat serum albumin in phosphate-buffered saline for 1 hour, we incubated the sections with rat anti-BrdU (1:400; Abcam, Cambridge, MA, U.S.A. antibody at 4°C overnight. After washing, DyLightTM 594-conjugated goat anti-rat IgG (1:2000, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, U.S.A.) was incubated for 1 h at room temperature. Oligodendrocyte progenitor cells (OPCs) were visualized with NG2 (1:200; Cell Signaling, Danvers, MA, U.S.A.) immunostaining. Immature NPCs were visualized using anti-doublecortin (1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A.) immunostaining. Secondary IgG antibodies included AlexaFluor 488 (1:500; Molecular Probes, Eugene, OR, U.S.A.) and Cy3 (1:2000; Jackson Immunoresearch Laboratories, Inc., West Grove, PA, U.S.A.). Immunopositive cell densities were calculated as

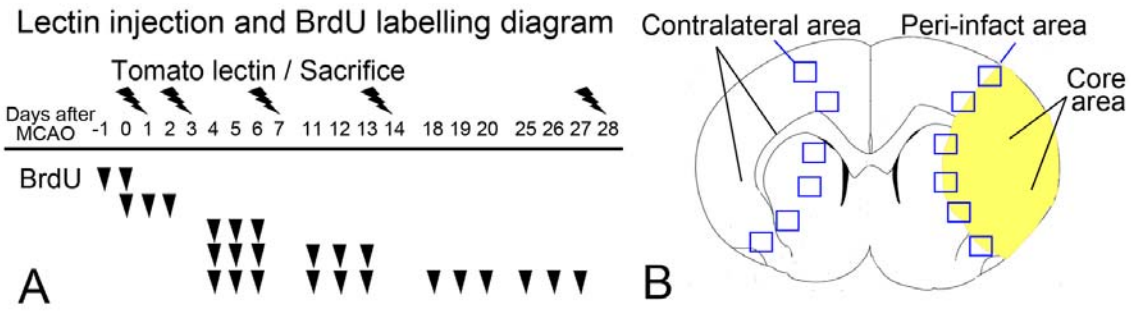

Figure 1. Experimental design for Brdu labeling and image capture. A, BrdU labeling diagram. B, Image analysis in 6 ipsilateral peri-infarct regions (green), ischemic core area (red) after MCAO, and the ischemic contralateral counterparts.

the number of cells in the designated areas (Figure 1B) divided by the area measured by the MCID image analysis system (Imaging Research, Inc., G.E. Healthcare Biosciences, Pittsburgh, PA, U.S.A.). To evaluate white matter injury, we stained coronal sections with myelin basic protein (MBP) (1:400; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A.) antibodies and images were digitized with a confocal microscope (Olympus Fluoview FV1000; Olympus). The mean intensity value of MBP staining was calculated in the corpus callosum (CC), cortex (Ctx), and striatum (Str) and expressed as the relative ratio of ipsilateral hemispheres vs contralateral hemispheres. Neurofilament 200 (1:200; Sigma Labs, Inc., Sante Fe, NM, U.S.A.) was stained in the adjacent section to reveal neuronal axons.

3.5. Evaluation of functional revascularization

Functional vessels were identified by transcardial administration (5 minutes before euthanasia) of biotinylated-lycopersicon esculentum lectin (tomato lectin, 1.25 mg/kg; Vector Laboratories, Burlingame, CA, U.S.A.), which labels endothelial cells in only perfused vessels. Image J software (National Institutes of Health, public domain, available at: http://rsb.info.nih.gov/nih-image/) was used for assessing functional revascularization after MCAO. Three parameters, total vascular number, total branch points, and total vascular length per mm^2, were measured. In addition, tomato lectin and BrdU were double labeled to identify newborn endothelial cells. Lectin and BrdU stainings were visualized with fluorescent-streptavidin (1:2000; Vector Laboratories, Burlingame, CA, U.S.A.) and DyLightTM594-conjugated secondary antibody (1:2000, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, U.S.A.). Vessels that showed double labels of tomato lectin and BrdU were counted in an area (590x432mm) from 6 ipsilateral regions on the infarct margin (Figure 1B), ischemia core, and ischemic contralateral area.

3.6. Statistical analysis

All values are expressed as mean ± standard error of the mean (20). Statistical comparisons among groups were performed with analysis of variance (ANOVA) followed by post hoc Fisher's probable least-squares difference (PLSD) tests. P-values less than 0.05 were considered statistically significant.

4. RESULTS

4.1. Ischemic injury leads to neurogenesis in damaged brain

Neurogenesis induced by ischemic injury in the adult brain starts with the proliferation of neural stem cells (NSCs) (21) and NPCs, followed by differentiation of NPCs and migration of neuroblasts to the ischemic boundary where neuroblasts mature into resident neurons (22). We observed a significant increase in the number of BrdU-incorporated Nestin+ NSCs at the subventricular zone (SVZ) and at the infarct border early after ischemia (data not shown). To specifically detect progenitor cells committed to neuronal differentiation, we performed double immunofluorescence staining of BrdU and DCX, a marker of migrating neuroblasts. In a normal adult brain or the contralateral hemisphere of the MCAO brain, we see very few cells immunoreactive for DCX or BrdU. Ischemia gave rise to a marked increase of DCX labeling in the ipsilateral Str beginning 14 days after insult (Figure 2A). Quantification of the number of DCX+ cells confirmed significant increases at late time points after ischemia in the ischemic Str compared with control animals, but was unaffected in the Str contralateral to the ischemic hemisphere (Figure 2B). In contrast to the late increase of DCX staining, the number of BrdU+ cells increased at 3 days after ischemia in both hemispheres with a much larger increment observed at the infarct border in the ischemic hemisphere, indicating an early increase in cell proliferation after ischemia. When we tried to co-localize DCX and BrdU, we found that the number of BrdU-labeled DCX+ cells greatly increased at 14 and 28 days in the ischemic Str, suggesting that newly generated NPCs successfully migrated into the injured Str beginning 14 days after MCAO and persisted for at least 28 days. It was noted that numerous BrdU-labeled cells did not express DCX, which may reflect active gliosis and an inflammatory response in the ischemic brain.

4.2. Ischemic injury activates angiogenesis and partial revascularization in the brain

The survival of newborn neurons depends on proper blood flow and energy and nutrient supply. To reveal functional revascularization after cerebral ischemia, we identified functional vessels by transcardial perfusion of Fluorescein isothiocyanate-conjugated tomato lectin, which

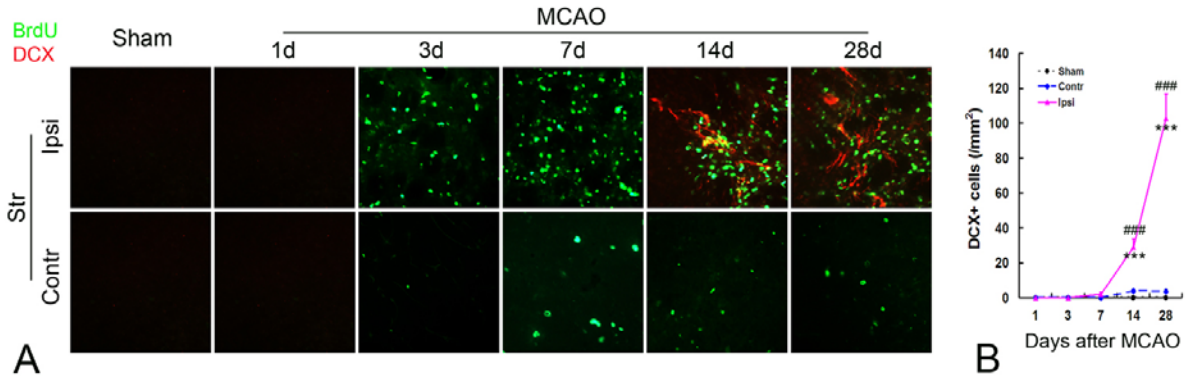

Figure 2. Migration of neuronal progenitor cells into striatum after MCAO. A, Representative images of BrdU (green) and DCX (red) immuno-fluorescence double-labeling in a peri-infarct region in the striatum (Str) or its contralateral counterpart of ischemic mice. Scale bar 100μm. B, Quantification of DCX+ cells, expressed as the number of DCX+ cells/mm2 in the peri-infarct region in the Str. Data are mean ± SE, n=5/group; ***p<0.001 vs. ipsilateral hemisphere of sham group. ###p<0.001 vs. contralateral hemisphere of ischemic group.

labels endothelial cells in only perfused vessels. Vascular density was evaluated by three-dimensional analysis at 1-28 days after MCAO in the peri-infarct regions of ischemic brain. All three vascular density parameters (total vascular surface area, total branch points, and total vascular length per mm^2) were dramatically decreased compared with those in the ischemic contralateral counterparts at day 1 after ischemia and were further impaired at day 3. Vascular density was partially recovered beginning at 7 days and further at 14 and 28 days after ischemia (Figure 3C and 3D). Despite the moderate increase in functional vessels from 7 days post-ischemia, significant differences in vascular density were observed between the infarct margin and the contralateral counterparts over the entire time course examined after MCAO, suggesting a sustained impairment in vascularization with very slow recovery in the peri-infarct regions after stroke. In the ischemic core regions, vascular density was significantly decreased beginning 1 day after ischemia without any recovery in the later time points (Figure 3A and 3B).

Double labeling for BrdU and intravascular tomato lectin was then performed to visualize endothelial cell proliferation and active angiogenesis after ischemia. We found that BrdU uptake in association with functional vessels increased in the peri-infarct regions beginning at 3 days, peaked at 7 days, and remained elevated until 28 days after ischemia (Figure 4). These data confirmed that ischemic stress could moderately stimulate functional neovascularization in the peri-infarct area.

To reveal the temporal and spatial association between newly born immature neurons and blood vessels, we performed tomato lectin and DCX double labeling. Similar as in Figure 3, we observed a significant increase in the number of DCX+ migrating neuroblasts in the peri-infarct areas of ipsilateral Str at 14 days and 28 days after ischemia. Interestingly, many of these DCX+ cells migrated in close proximity to lectin+ blood vessels (Figure 5), suggesting a possible interplay between post-stroke neurogenesis and revascularization.

4.3. White matter injury does not improve over time despite robust oligodendrogenesis after ischemic stroke

White matter injury is an important component in post-stroke brain damage (23). The presence of oligodendrocyte precursors (OPCs) in the mature brain may provide the opportunity for oligodendrocyte replenishment after injury. To characterize oligodendrogenesis after ischemic stroke, we examined progenitor cells committed to oligodendrocyte differentiation by BrdU and NG2 double staining. Our data demonstrated that NG2+ OPCs constitutively presented in the normal brain. Following MCAO, the number of BrdU-labeled NG2+ OPCs significantly increased from day 3 to day 28 in the CC, Ctx, and Str of ipsilateral hemispheres (Figure 6), suggesting that ischemic stress induces proliferation or repair of OPCs.

Using MBP as a marker of mature oligodendrocytes, we evaluated the extent of white matter injury in affected brain areas. Following MCAO, a decrease in the MBP immunoreactive density was observed in the Str beginning on day 1 and in the Ctx and CC beginning on day 3 (Figure 7A); this continued to day 28 post-ischemia. Similarly, the staining of neurofilament 200, a marker used to reveal neuronal axon recovery, showed a sustained decrease of immunoreactivity from day 1 to day 28 after MCAO (Figure 7B). These data suggest that despite the presence of OPC proliferation, severe impairment of the white matter integrity remained after ischemic stroke.

5. DISCUSSION

This study investigated the dynamics of neurogenesis, angiogenesis, and white matter injury over time after ischemic injury. Our observations found that 1) neurogenesis and angiogenesis were substantially activated soon after ischemic stroke and persisted for several weeks; 2) immature neuroblasts migrating in close proximity to functional blood vessels could be observed beginning at 14 days post-ischemia; 3) a significant decrease in vascular density maintained over time after MCAO despite the moderate recovery of functional vessels in infarct margin

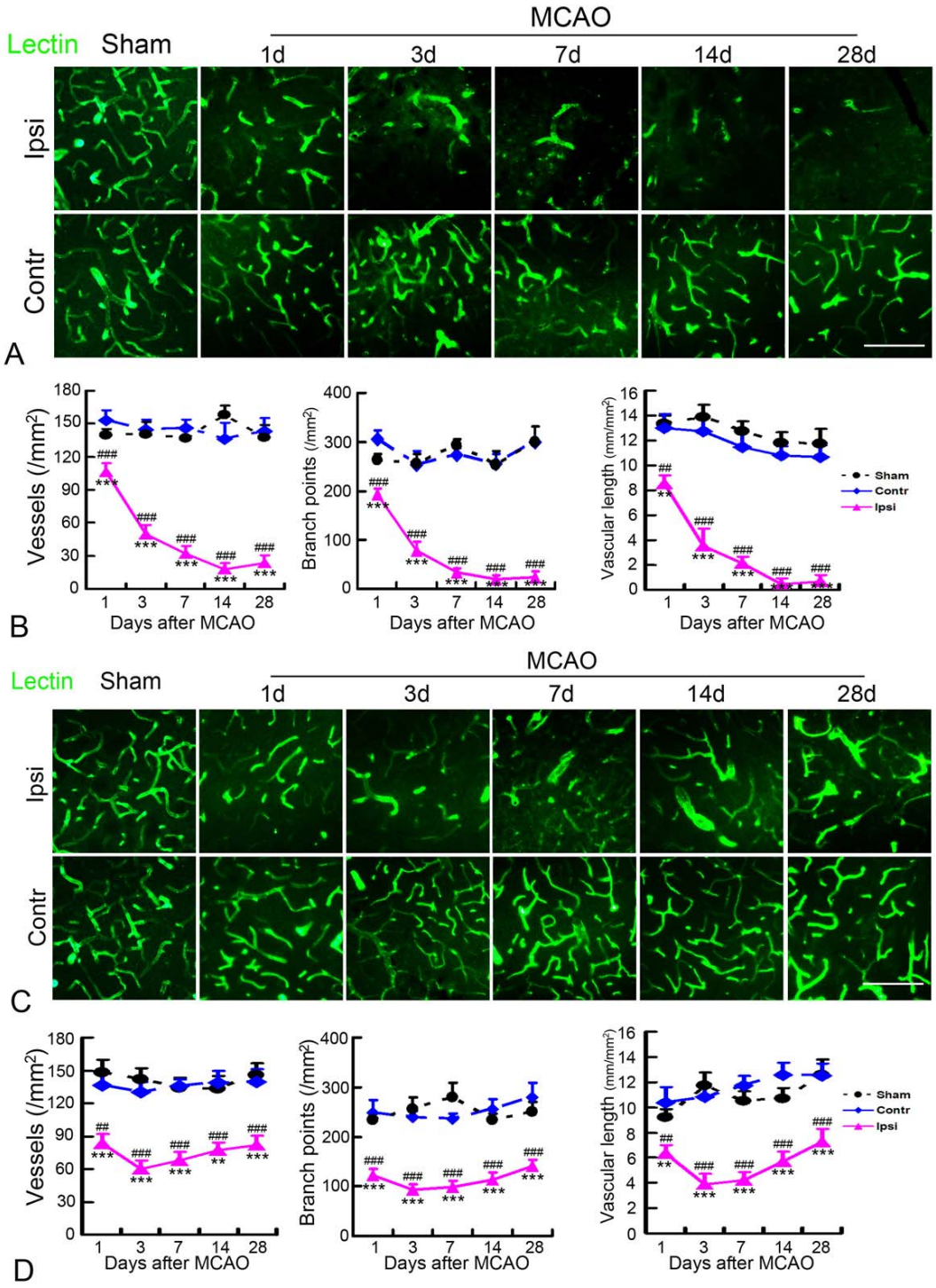

Figure 3. Impairment of functional vessels in peri-infarct regions and core areas after MCAO. A, Tomato lectin-stained cerebral vessels in the peri-infarct regions and contralateral counterparts at 1, 3, 7, 14 or 28 days after MCAO. Scale bar, 100μm. B, Quantification of functional vessels in peri-infarct regions and contralateral counterparts after MCAO. C, Tomato lectin-stained cerebral vessels in core areas and contralateral counterparts at 1, 3, 7, 14 or 28 days after MCAO. Scale bar, 100μm. D, Quantification of functional vessels in core areas and contralateral counterparts after MCAO. All three parameters, including total vascular surface area, total branch points, and total vascular length per mm2 were quantified. Data are mean ± SE, n=5/group; **p<0.01, ***p<0.001 vs. ipsilateral hemisphere of sham group. ##p<0.01, ###p<0.001 vs. contralateral hemisphere of ischemic group.

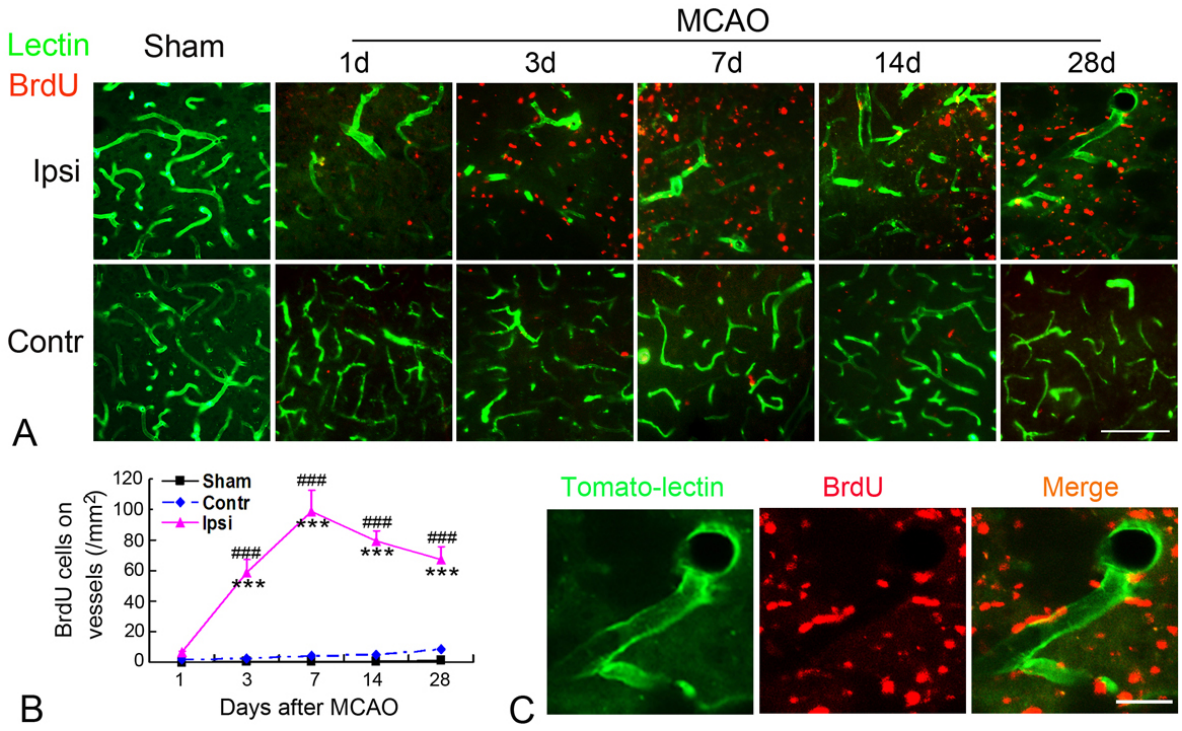

Figure 4. Temporal profile of neovascularization in peri-infarct regions after MCAO. A, Representative images of tomato lectin-stained vessels (green) and BrdU-labeled cells (red) in peri-infarct regions and contralateral counterparts at 1, 3, 7, 14 or 28 days after MCAO. Scale bar, 100μm. B, Quantification of BrdU-labeled cells associated with tomato lectin-stained vessels in peri-infarct regions and contralateral counterparts after MCAO. C, High magnification images of immunofluorescence double labeling with tomato lectin (green) and BrdU (red) at 28 days after MCAO. Scale bar, 20μm. Data are mean ± SE, n=5/group; ***p<0.001 vs. ipsilateral hemisphere of sham group. ###p<0.001 vs. contralateral hemisphere of ischemic group.

from 7 days post-ischemia; and 4) white matter injury after ischemic stroke is almost irreversible despite the sustained presence of newborn oligodendrocyte progenitors.

Accumulating research has emphasized the importance of neurogenesis in neurological recovery after stroke. An early increase of NSC/NPC proliferation in the SVZ has been well documented as an initiation of post-stroke neurogenesis (1). In agreement with previous reports, (6, 7) we further demonstrated here that newly born immature neurons are able to migrate into regions of peri-infarct Ctx and Str, which was manifested by the appearance of DCX+/Brdu+ cells in late (14 and 28 days) time points after stroke. Stroke-induced neurogenesis was also observed in the SVZ and ischemic boundary of adult human brains, even in elderly patients aged 60–87 years (24-26). Such increased neurogenesis in response to ischemic injuries should have created optimism for the possible regeneration of a stroke-damaged brain. However, disappointingly, the majority of newly generated neurons die soon after stroke. It has been reported that less than 20% of new neurons in the ischemic Str survive longer than 2 weeks (6), replacing only a small portion (about 0.2%) of the lost mature neurons. Another study showed that about 10% of the neuroblasts that migrate into the peri-infarct Ctx survive three months after stroke (27). In looking for factors that foster viable neuronal replacement, researchers have found that the creation of a favorable microenvironment with adequate blood supply and the stimulation of vascularization is important for the survival of new neurons. The association of newly born immature neurons and angiogenic blood vessels defines a neurovascular niche for post-stroke neurogenesis (27, 28). In line with this concept, it has been shown that within several months after the onset of brain ischemia, neuroblasts migrate along blood vessels toward the damage through an area exhibiting early vascular remodeling and a persistent increase of vessel density (28). Our current data support these reports and further show that clusters of migrating immature neurons localize proximal to angiogenic blood vessels within the peri-infart striatal tissue beginning at 14 days and persist until at least 4 weeks after stroke. Our results suggest that an effective blood supply should be accumulated in the peri-infart area within 2 weeks after ischemia to support the survival of these committed NPCs and promote their further differentiation.

However, our detailed time-course study on post-ischemic neovascularization revealed that although angiogenesis is activated in peri-infarct areas early after MCAO and maintains for several weeks, the functional revascularization in these regions delays until 7 days post-ischemia and progresses very slowly, resulting in a

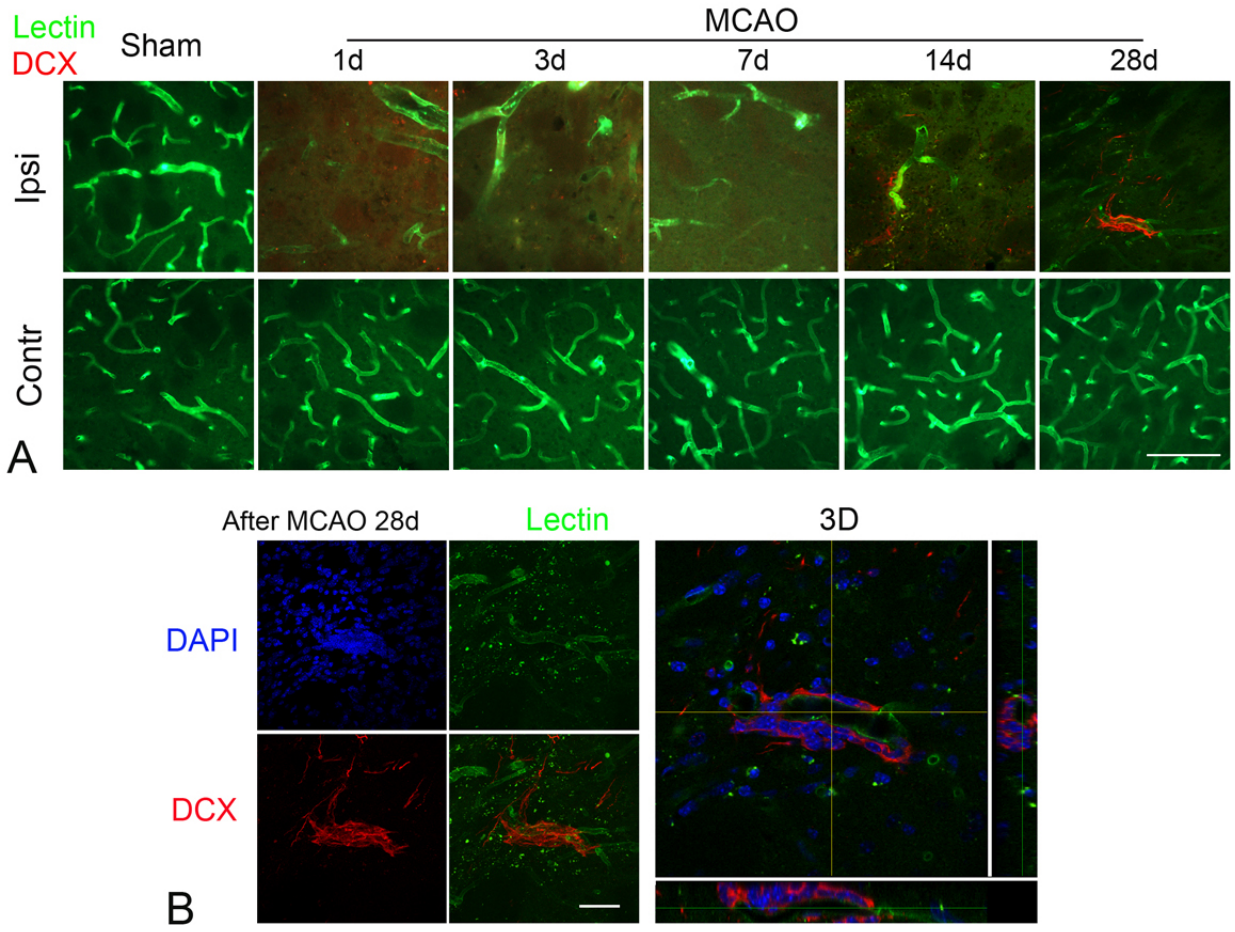

Figure 5. Close physical association between newly born immature neurons and blood vessels. A, Representative images of tomato lectin-stained vessels (green) and DCX-labeled immature neurons (red) in peri-infarct regions and contralateral counterparts of striatum (Str) at 1, 3, 7, 14 and 28 days after MCAO. Scale bar, 100μm. B, Three-dimensional confocal image of double labeled immunofluorescence for DCX (red) and tomato lectin (green) in peri-infarct regions of Str at 28 days after MCAO. Scale bar, 20μm.

sustained impairment in vascularization after stroke. In addition, the vascular density of ischemic core regions failed to recover by endogenous angiogenesis after ischemia. These results, on one hand, indicate the insufficiency of post-ischemia angiogenesis for brain repair but, on the other hand, suggest that vascular therapeutics geared toward enhancement of angiogenesis should best be given within 7 days after stroke.

To restore neurological function after cerebral ischemia, we suggest that a successful neuronal replacement should be followed by or accompanied by proper neurite outgrowth and remyelination. This study showed that the progenitors of oligodendrocytes constitutively present in the normal non-ischemic brain and proliferate actively soon after ischemic injury. Interestingly, despite such quick and prolonged responses from OPCs, axon damage as well as the severe loss of white matter integrity in affected brain areas showed very limited improvement over time. In support of our observation, such myelination failure, despite robust regeneration of pre-oligodendracytes, has also been reported in perinatal hypoxia/ischemia brain injury thought to be attributed to a persistent maturation arrest in pre-oligodendrocytes (29). Although the mechanism for this maturation arrest is far from clear, several signaling pathways might be involved. For example, the activation of the notch inhibitory signaling pathway, which restricts oligodendrocyte maturation (30), has been reported after cerebral ischemia (31, 32). Further study into the mechanism for myelination failure during stroke recovery is warranted, which could help identify potential targets for therapeutic intervention.

This study demonstrates that the regenerative processes, including neurogenesis, angiogenesis and oligodendrogenesis, start early after ischemic injury and persist for several weeks. The capacities of these endogenous responses, however, are not sufficient for successful brain repair. Nevertheless, the early regenerative efforts of the brain provide an opportunity for restorative therapies to enhance endogenous repair processes in ischemic brain tissue. Our recognition and characterization of the temporal connections among multiple endogenous

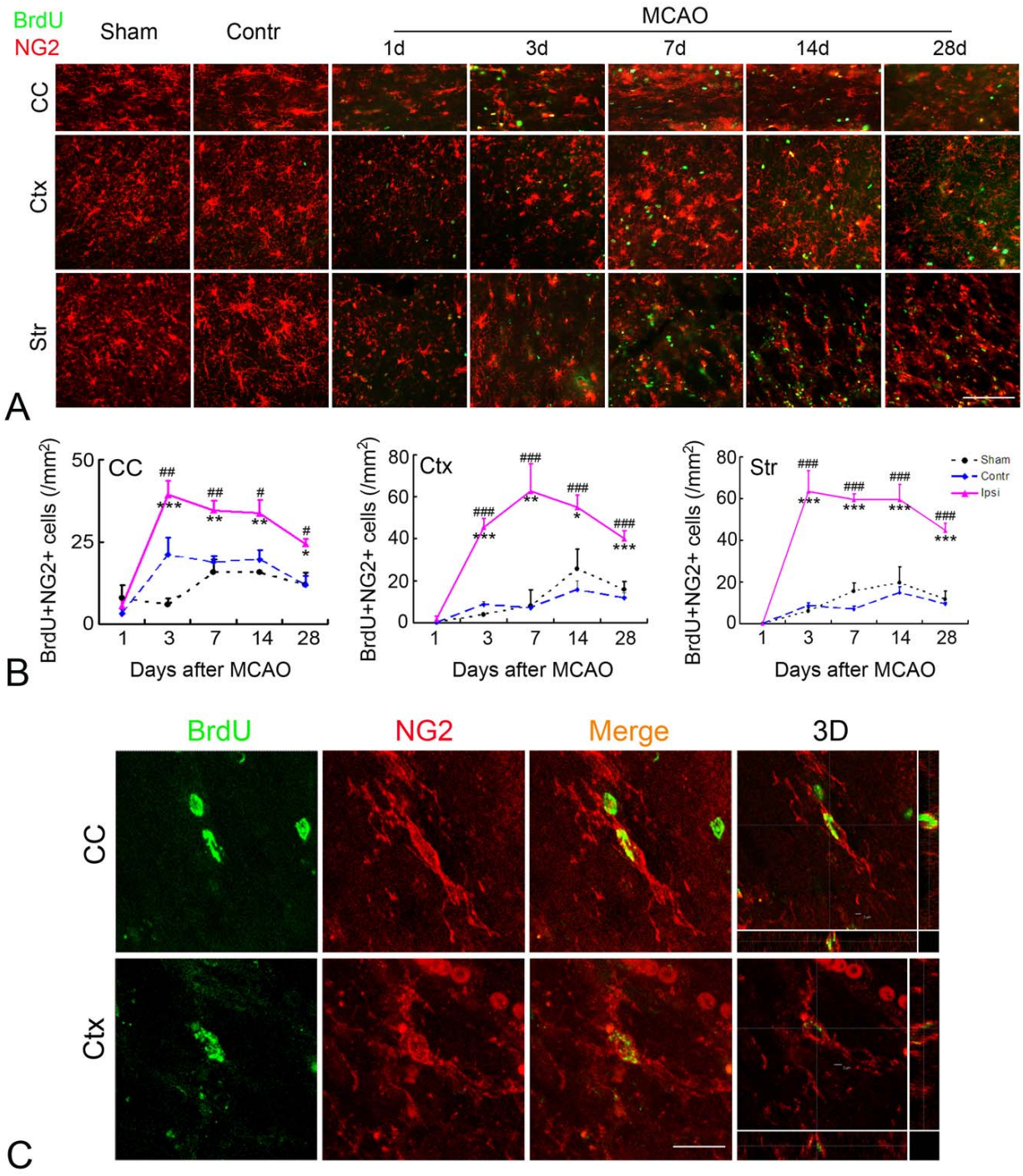

Figure 6. Proliferation of oligodendrocyte progenitor cells after MCAO. A, Upper panel: Immunostaining of NG2 (red) and BrdU (green) in the corpus callosum (CC), cortex (Ctx) and striatum (Str) of either the ipsilateral hemisphere of both ischemic and non-ischemic mice or the contralateral hemisphere of ischemic mice at 1, 3, 7, 14 or 28 days after MCAO. Scale bar 100μm. Lower panel: Quantification of NG2+/BrdU+ cells, expressed as the number of NG2+/BrdU+ cells/mm2 in the peri-infarct region and contralateral counterparts in the CC, Ctx, and Str. Data are mean ± SE, n=5/group; *p<0.05; **p<0.01; ***p<0.001 vs. ipsilateral hemisphere of sham group. #p<0.05, ##p<0.01, ###p<0.001 vs. contralateral hemisphere of ischemic group. B and C, High magnification images of immunofluorescence double labeling with NG2 (green) and BrdU (red) in the CC (B) and Ctx (C) after MCAO.

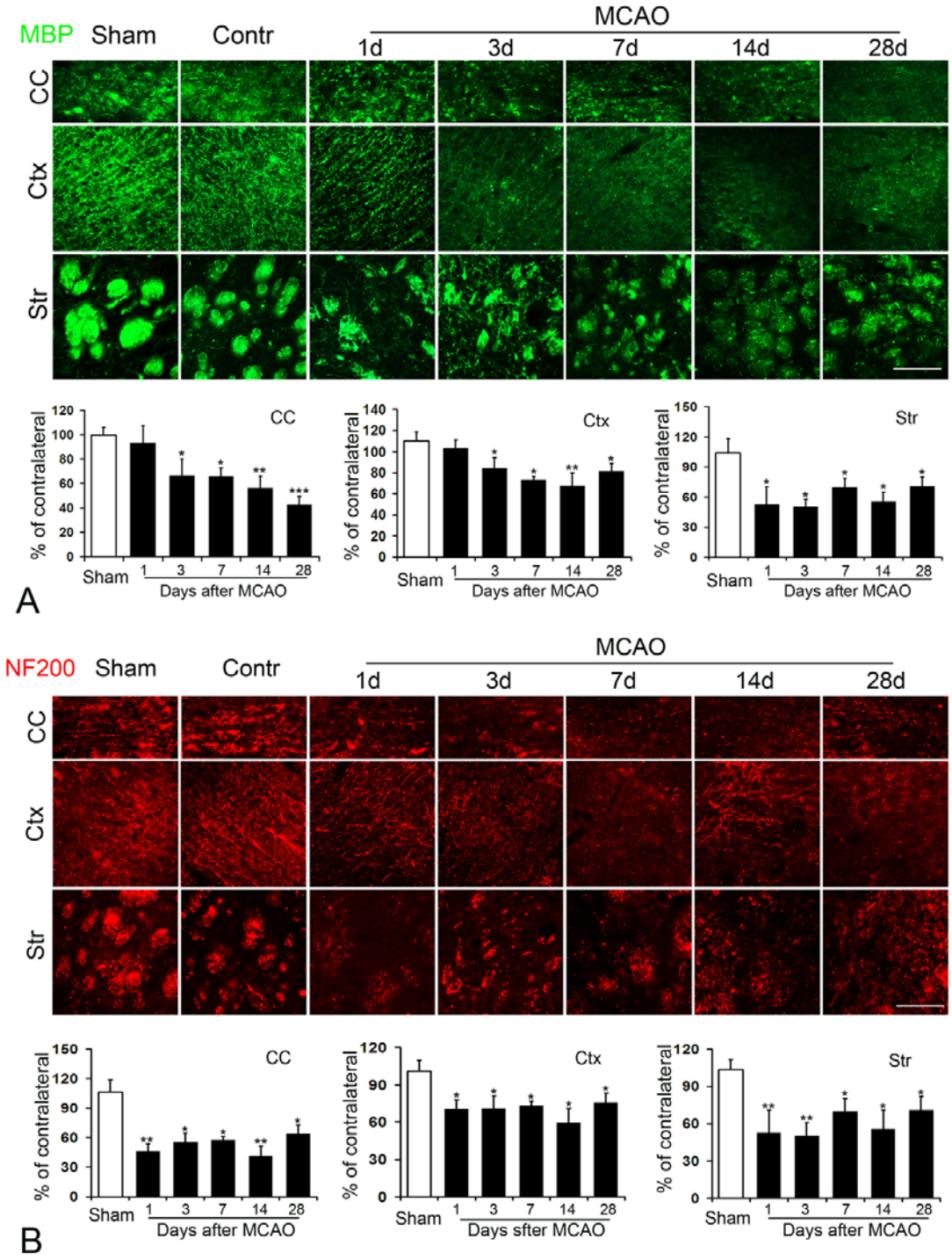

Figure 7. Temporal profile of white matter injury and axon damage after MCAO. A, Upper panel: Immunostaining of MBP in the corpus callosum (CC), cortex (Ctx) and striatum (Str) of either the ipsilateral hemisphere of both ischemic and non-ischemic mice or the contralateral hemisphere of ischemic mice at 1, 3, 7, 14 or 28 days after MCAO. Scale bar 100μm. Lower panel: Quantification of the MBP immunostaining intensity expressed as relative ratio of ipsilateral hemispheres vs contralateral hemispheres. B, Upper panel: Immunostaining of NF-200 in the CC, Ctx and Str of either the ipsilateral hemisphere of both ischemic and non-ischemic mice or the contralateral hemisphere of ischemic mice at 1, 3, 7, 14 or 28 days after MCAO. Scale bar 100μm. Lower panel: Quantification of the NF-200 immunostaining intensity expressed as relative ratio of ipsilateral hemispheres vs contralateral hemispheres. *p<0.05; **p<0.01; ***p<0.001 vs sham group.

restorative responses to stroke would be useful to direct the experimental stroke therapies toward establishing integrated neurovascular units in the injured brain and, thereby, achieving the functional recovery of the CNS.

6. ACKNOWLEDGEMENTS

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7. REFERENCES

1. Wiltrout, C., B. Lang, Y. Yan, R. J. Dempsey & R. Vemuganti: Repairing brain after stroke: a review on post-ischemic neurogenesis. *Neurochem Int*, 50, 1028-41(2007)

2. Palmer, T. D., A. R. Willhoite & F. H. Gage: Vascular niche for adult hippocampal neurogenesis. *J Comp Neurol*, 425, 479-94(2000)

3. Alvarez-Buylla, A. & D. A. Lim: For the long run: maintaining germinal niches in the adult brain. *Neuron*, 41, 683-6(2004)

4. Mandai, K., M. Matsumoto, K. Kitagawa, K. Matsushita, T. Ohtsuki, T. Mabuchi, D. R. Colman, T. Kamada & T. Yanagihara: Ischemic damage and subsequent proliferation of oligodendrocytes in focal cerebral ischemia. *Neuroscience*, 77, 849-61(1997)

5. Lo, E. H.: A new penumbra: transitioning from injury into repair after stroke. *Nat Med*, 14, 497-500(2008)

6. Arvidsson, A., T. Collin, D. Kirik, Z. Kokaia & O. Lindvall: Neuronal replacement from endogenous precursors in the adult brain after stroke. *Nat Med,* 8, 963-70(2002)

7. Parent, J. M., Z. S. Vexler, C. Gong, N. Derugin & D. M. Ferriero: Rat forebrain neurogenesis and striatal neuron replacement after focal stroke. *Ann Neurol*, 52, 802-13(2002)

8. Jin, K., X. Wang, L. Xie, X. O. Mao & D. A. Greenberg: Transgenic ablation of doublecortin-expressing cells suppresses adult neurogenesis and worsens stroke outcome in mice. *Proc Natl Acad Sci U S A,* 107, 7993-8(2010)

9. Zhang, Z. G., L. Zhang, Q. Jiang, R. Zhang, K. Davies, C. Powers, N. Bruggen & M. Chopp: VEGF enhances angiogenesis and promotes blood-brain barrier leakage in the ischemic brain. *J Clin Invest*, 106, 829-38(2000)

10. Zhang, Z. G., L. Zhang, W. Tsang, H. Soltanian-Zadeh, D. Morris, R. Zhang, A. Goussev, C. Powers, T. Yeich & M. Chopp: Correlation of VEGF and angiopoietin expression with disruption of blood-brain barrier and angiogenesis after focal cerebral ischemia. *J Cereb Blood Flow Metab*, 22, 379-92(2002)

11. Jiang, Q., Z. G. Zhang, G. L. Ding, L. Zhang, J. R. Ewing, L. Wang, R. Zhang, L. Li, M. Lu, H. Meng, A. S. Arbab, J. Hu, Q. J. Li, D. S. Pourabdollah Nejad, H. Athiraman & M. Chopp: Investigation of neural progenitor cell induced angiogenesis after embolic stroke in rat using MRI. *Neuroimage,* 28, 698-707(2005)

12. Teng, H., Z. G. Zhang, L. Wang, R. L. Zhang, L. Zhang, D. Morris, S. R. Gregg, Z. Wu, A. Jiang, M. Lu, B. V. Zlokovic & M. Chopp: Coupling of angiogenesis and neurogenesis in cultured endothelial cells and neural progenitor cells after stroke. *J Cereb Blood Flow Metab,* 28, 764-71(2008)

13. Leventhal, C., S. Rafii, D. Rafii, A. Shahar & S. A. Goldman: Endothelial trophic support of neuronal production and recruitment from the adult mammalian subependyma. *Mol Cell Neurosci*, 13, 450-64(1999)

14. Tavazoie, M., L. Van der Veken, V. Silva-Vargas, M. Louissaint, L. Colonna, B. Zaidi, J. M. Garcia-Verdugo & F. Doetsch: A specialized vascular niche for adult neural stem cells. *Cell Stem Cell*, 3, 279-88(2008)

15. Shen, Q., Y. Wang, E. Kokovay, G. Lin, S. M. Chuang, S. K. Goderie, B. Roysam & S. Temple: Adult SVZ stem cells lie in a vascular niche: a quantitative analysis of niche cell-cell interactions. *Cell Stem Cell*, 3, 289-300(2008)

16. Zhang, Z. G. & M. Chopp: Neurorestorative therapies for stroke: underlying mechanisms and translation to the clinic. *Lancet Neurol*, 8, 491-500(2009)

17. Iwai, M., R. A. Stetler, J. Xing, X. Hu, Y. Gao, W. Zhang, J. Chen & G. Cao: Enhanced oligodendrogenesis and recovery of neurological function by erythropoietin after neonatal hypoxic/ischemic brain injury. Stroke, 41, 1032-7(2010)

18. Zhang, L., M. Chopp, R. L. Zhang, L. Wang, J. Zhang, Y. Wang, Y. Toh, M. Santra, M. Lu & Z. G. Zhang: Erythropoietin amplifies stroke-induced oligodendrogenesis in the rat. PLoS One, 5, e11016

19. Chen, J., K. Jin, M. Chen, W. Pei, K. Kawaguchi, D. A. Greenberg & R. P. Simon: Early detection of DNA strand breaks in the brain after transient focal ischemia: implications for the role of DNA damage in apoptosis and neuronal cell death. J Neurochem, 69, 232-45(1997)

20. Thies, F., J. M. Garry, P. Yaqoob, K. Rerkasem, J. Williams, C. P. Shearman, P. J. Gallagher, P. C. Calder & R. F. Grimble: Association of n-3 polyunsaturated fatty acids with stability of atherosclerotic plaques: a randomised controlled trial. Lancet, 361, 477-85(2003)

21. Heller, A. R., T. Rossel, B. Gottschlich, O. Tiebel, M. Menschikowski, R. J. Litz, T. Zimmermann & T.

Koch: Omega-3 fatty acids improve liver and pancreas function in postoperative cancer patients. *Int J Cancer*, 111, 611-6(2004)

22. Iwai, M., K. Sato, N. Omori, I. Nagano, Y. Manabe, M. Shoji & K. Abe: Three steps of neural stem cells development in gerbil dentate gyrus after transient ischemia. *J Cereb Blood Flow Metab*, 22, 411-9(2002)

23. Pantoni, L., J. H. Garcia & J. A. Gutierrez: Cerebral white matter is highly vulnerable to ischemia. *Stroke,* 27, 1641-6; discussion 1647(1996)

24. Jin, K., X. Wang, L. Xie, X. O. Mao, W. Zhu, Y. Wang, J. Shen, Y. Mao, S. Banwait & D. A. Greenberg: Evidence for stroke-induced neurogenesis in the human brain. *Proc Natl Acad Sci U S A*, 103, 13198-202(2006)

25. Macas, J., C. Nern, K. H. Plate & S. Momma: Increased generation of neuronal progenitors after ischemic injury in the aged adult human forebrain. *J Neurosci*, 26, 13114-9(2006)

26. Minger, S. L., A. Ekonomou, E. M. Carta, A. Chinoy, R. H. Perry & C. G. Ballard: Endogenous neurogenesis in the human brain following cerebral infarction. *Regen Med*, 2, 69-74(2007)

27. Ohab, J. J., S. Fleming, A. Blesch & S. T. Carmichael: A neurovascular niche for neurogenesis after stroke. *J Neurosci,* 26, 13007-16(2006)

28. Thored, P., J. Wood, A. Arvidsson, J. Cammenga, Z. Kokaia & O. Lindvall: Long-term neuroblast migration along blood vessels in an area with transient angiogenesis and increased vascularization after stroke. *Stroke*, 38, 3032-9(2007)

29. Segovia, K. N., M. McClure, M. Moravec, N. L. Luo, Y. Wan, X. Gong, A. Riddle, A. Craig, J. Struve, L. S. Sherman & S. A. Back: Arrested oligodendrocyte lineage maturation in chronic perinatal white matter injury. *Ann Neurol,* 63, 520-30(2008)

30. John, G. R., S. L. Shankar, B. Shafit-Zagardo, A. Massimi, S. C. Lee, C. S. Raine & C. F. Brosnan: Multiple sclerosis: re-expression of a developmental pathway that restricts oligodendrocyte maturation. *Nat Med*, 8, 1115-21(2002)

31. Alberi, L., Z. Chi, S. D. Kadam, J. D. Mulholland, V. L. Dawson, N. Gaiano & A. M. Comi: Neonatal stroke in mice causes long-term changes in neuronal Notch-2 expression that may contribute to prolonged injury. *Stroke*, 41, S64-71(2010)

32. Arumugam, T. V., S. L. Chan, D. G. Jo, G. Yilmaz, S. C. Tang, A. Cheng, M. Gleichmann, E. Okun, V. D. Dixit, S. Chigurupati, M. R. Mughal, X. Ouyang, L. Miele, T. Magnus, S. Poosala, D. N. Granger & M. P. Mattson: Gamma secretase-mediated Notch signaling worsens brain damage and functional outcome in ischemic stroke. *Nat Med*, 12, 621-3(2006)

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