Effects of Aging on Neural Stem/Progenitor Cells and Oligodendrocyte Precursor Cells After Focal Cerebral Ischemia in Spontaneously Hypertensive Rats

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Aging and vascular comorbidities such as hypertension comprise critical cofactors that influence how the brain responds to stroke. Ischemic stress induces neurogenesis and oligodendrogenesis in younger brains. However, it remains unclear whether these compensatory mechanisms can be maintained even under pathologically hypertensive and aged states. To clarify the age-related remodeling capacity after stroke under hypertensive conditions, we assessed infarct volume, behavioral outcomes, and surrogate markers of neurogenesis and oligodendrogenesis in acute and subacute phases after transient focal cerebral ischemia in 3- and 12-month-old spontaneously hypertensive rats (SHRs). Hematoxylin and eosin staining showed that 3- and 12-month-old SHRs exhibited similar infarction volumes at both 3 and 14 days after focal cerebral ischemia. However, recovery of behavioral deficits (neurological score assessment and adhesive removal test) was significantly less in 12-month-old SHRs compared to 3-month-old SHRs. Concomitantly, numbers of nestin+ neural stem/ progenitor cells (NSPCs) near the infarct border area or subventricular zone in 12-month-old SHRs were lower than 3-month-old SHRs at day 3. Similarly, numbers of PDGFR-α+ oligodendrocyte precursor cells (OPCs) in the corpus callosum were lower in 12-month-old SHRs at day 3. Lower levels of NSPC and OPC numbers were accompanied by lower expression levels of phosphorylated CREB. By day 14 postischemia, NSPC and OPC numbers in 12-month-old SHRs recovered to similar levels as in 3-month-old SHRs, but the numbers of proliferating NSPCs (Ki-67+nestin+ cells) and proliferating OPCs (Ki-67+PDGFR-α+ cells) remained lower in the older brains even at day 14. Taken together, these findings suggest that aging may also decrease poststroke compensatory responses for neurogenesis and oligodendrogenesis even under hypertensive conditions.

Key words: Neural stem cells; Oligodendrocyte precursor cells (OPCs); Middle cerebral artery occlusion; Stroke; Spontaneously hypertensive rat (SHR); Aging

INTRODUCTION

Brain pathophysiology is influenced by a dynamic balance between deleterious and beneficial responses to the initial insult (22). Stroke and brain injury trigger a wide spectrum of neurovascular perturbations, glial activation, and secondary neuroinflammation that may all amplify neuronal cell death cascades, but, at the same time, many endogenous neuroprotective responses such as neurogenesis and oligodendrogenesis may also be activated (25).

Although the adult brain retains capabilities for regeneration and recovery, aging may significantly dampen these endogenous protective mechanisms. In particular, the capacity for neurogenesis appears to diminish with age. This is primarily due to a general reduction in neuronal precursor cell proliferation because of age-related alterations in the cellular microenvironment (2,18,21,35). Similarly, oligodendrogenesis and white matter homeostasis may also be affected by white matter senescence. In healthy, young adult brains, myelin-forming mature oligodendrocytes in white matter can be newly generated from their precursor cells [oligodendrocyte precursor cells (OPCs)]. After white matter injury, OPCs rapidly

proliferate and migrate to fill the demyelinated area, differentiate into mature oligodendrocytes, and restore myelin sheaths (15,24,31). However, myelin density, along with cognitive function, spontaneously declines with increasing age both in humans and rodents (9,30), indicating that the capacity for oligodendrogenesis may be compromised by white matter senescence.

Aging is a major risk factor in stroke and other forms of cerebrovascular disease (16,19). The risk of stroke doubles every decade after age 55 (23), and older patients show less functional recovery after stroke when compared with younger patients (4). In rodent models of stroke, it has been confirmed that aging worsens brain damage and suppresses brain remodeling/repair after ischemic damage (28,32). However, aging is not the only cofactor involved. Vascular comorbidities such as hypertension are also known to be major contributors to stroke risk and stroke injury (8,34,37), but it is unknown how aging may affect stroke-induced compensatory responses under hypertensive conditions. In this study, therefore, we asked how aging may affect neural/stem progenitor cell (NSPC) and OPC responses after stroke in spontaneously hypertensive rats (SHRs).

MATERIALS AND METHODS

All experiments were performed following an institutionally approved protocol in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and NIH Guidelines on Ethical Animal Use and Rigor. All experiments and procedures were conducted following a Massachusetts General Hospital (MGH) Institutional Animal Care and Use Committee (IACUC)-approved protocol. These IACUC approval protocols and studies were all in compliance with the MGH ethics review procedure and conducted in compliance with GlaxoSmithKline policy on care, welfare, and treatment of laboratory animals. All experimental animals were sacrificed by decapitation under deep isoflurane anesthesia, according to the IACUC-approved protocols. All procedures and analyses were performed following standard requirements for experimental design including allocation concealment, randomization, blinding, and determining statistical power.

Animal Model

Twenty-three 3-month-old and 24 12-month-old male SHRs (Charles River Laboratories Wilmington, MA, USA) were used in this study. Rats were anesthetized with isoflurane (1–1.5%; Baxter, Deerfield, IL, USA) in a 30%/70% mixture of oxygen/nitrous oxide, with the anesthetic dose titrated to maintain spontaneous respiration. Rectal temperatures were monitored and maintained at $37 \pm 0.5°C$ with a thermostat-controlled heating pad. All rats underwent middle cerebral artery occlusion

(MCAO). The standard intraluminal method was used (26). Briefly, a ventral midline incision was made on the neck, and the common carotid artery (CCA), extra carotid artery (ECA), and internal carotid artery (ICA) were exposed. The superior thyroid artery and occipital artery branching from the ECA was coagulated and cut. The pterygopalatine artery (PPA) branching from the ICA was ligated using 4-0 silk. The ECA was ligated using 4-0 silk, and the CCA and ICA were closed temporarily. The ECA was cut, and a filament was placed through the ICA to the bifurcation of the ICA and the middle cerebral artery (MCA), thus inducing focal cerebral ischemia. At 75 min after induction of ischemia, the filament was withdrawn for reperfusion, and the ECA was ligated using 4-0 silk. Cerebral blood flow (CBF) was monitored by laser Doppler flowmetry (LDF) during surgery to evaluate the severity of ischemia. As expected, LDF dropped below 30% of baseline (100%) for the duration of the focal cerebral ischemia. At 3 or 14 days after MCAO, rats were sacrificed while deeply anesthetized by 5% isoflurane, using intracardiac perfusion with ice-cold saline. Brains were removed and snap frozen. Coronal sections of 20 μM thickness were cut on a cryostat (Microm HM 505 E; GMI Inc., Ramsey MN, USA) at −20°C and collected on glass slides, then stored at −80°C for future use.

Behavioral Tests

Two behavioral tests were performed at 28 days after MCAO by a blinded investigator, who also trained the animals before surgery. The modified Neurological Severity Score (NSS) reflects neurological deficits after stroke using a graded scale from 0 to 18 (normal: 0, maximal neurological deficit: 18). The NSS test is a comprehensive test that assesses motor and sensory function, stability using a beam balance test, abnormal reflexes, and abnormal movements (6). The adhesive removal test also assesses somatosensory function (5). In this test, a rat is first accommodated to a testing box and then removed gently from the box in order to apply adhesive tape to the wrist of each forelimb with equal pressure. The rat is then placed back in the box, and the time required for the rat to react to the adhesive tape is recorded. The maximal time was set to 120 s.

Hematoxylin and Eosin Staining and Evaluation of Infarction

Coronal sections were selected and processed for hematoxylin and eosin (H&E) staining (Thermo Fisher Scientific, Cambridge, MA, USA). Briefly, sections were removed from −80°C and thawed. The fresh frozen sections were fixed with 4% paraformaldehyde (PFA; Wako, Osaka, Japan) for 15 min, then rinsed with warm running water for 1 min. Sections were stained with hematoxylin

for 5 min to stain the nuclei and rinsed for 3 min with water. Next, sections were stained with eosin for 3 min and followed by rinsing for 1 min. Afterward, the sections were dehydrated with 70% ethanol, 100% ethanol, and 100% ethanol (in respective order) for 10–15 s each. Following that, the sections were cleared using xylene. Sections were then mounted with a cover slide with Permount. Infarct volume was calculated as previously reported (12). Briefly, the sections were scanned, and the infarct area in each section was calculated by subtracting the noninfarcted area of the ipsilateral side from the area of the contralateral side with ImageJ analysis software (NIH, Bethesda, MD, USA). Infarct areas on each section were summed and multiplied by section thickness to give the total infarct volume.

Immunohistochemistry

Coronal sections (2 mm anterior to bregma) were selected and processed for immunohistochemistry. Staining was done as previously described (12). Briefly, sections were removed from −80°C and thawed. The fresh frozen sections were fixed with 4% PFA for 15 min. After being further washed three times in PBS containing 0.1% Triton X-100, they were incubated with 10% Block ACE (AbD Serotec) in phosphate-buffered saline (PBS) (Boston BioProducts, Ashland, MA, USA) for 1 h at room temperature. Then sections were incubated in PBS/0.3% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA) solution containing primary antibodies: anti-Ki-67 (proliferative cell marker, 1:100; Abcam, Cambridge, UK) and anti-PDGFR-α (OPC marker, 1:100; Santa Cruz Biotechnology, Dallas, TX, USA) at 4°C overnight. After washing with PBS three times, they were incubated with secondary antibodies: fluorescein (FITC) AffiniPure F(ab′)$_2$ fragment donkey anti-mouse/goat IgG (H+L), rhodamine (TRITC) AffiniPure F(ab′)$_2$ fragment donkey anti-rabbit/goat IgG (H+L), Alexa Fluor® 647 AffiniPure donkey anti-rabbit IgG (H+L) (1:200; Jackson Immunoresearch Laboratories, West Grove, PA, USA) for 1 h at room temperature. Similarly, sections were stained with anti-Ki-67 and anti-nestin antibodies (NSPC marker, 1:100; Abcam). Then the sections were washed three times and covered with VECTASHIELD mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA). Stained sections were analyzed with a fluorescence microscope (Eclipse Ti-5; Nikon, Tokyo, Japan).

Western Blot Analysis

Tissue samples of brain sections were prepared in Pro-PREP™ Protein Extraction Solution (Boca Scientific, Boca Raton, FL, USA). Samples were heated with equal volumes of sample buffer containing 2× SDS sample buffer and 2-mercaptoethanol (2-ME) (Sigma-Aldrich) at 95°C for 5 min. Then each sample was loaded onto 4–20% Tris-glycine gels. After electrophoresis and transferring to polyvinylidene difluoride membranes (Novex, Waltham, MA, USA), the membranes were blocked in 0.2% I-Block™ regent (Applied Biosystems, Carlsbad, CA, USA) for 1 h at room temperature. Then they were incubated overnight with primary antibodies: anti-nestin (1:1,000; Abcam), anti-PDGFR-α (1:1,000; Santa Cruz Biotechnology), anti-phosphorylated cyclic adenosine monophosphate (cAMP) response element-binding (pCREB, 1:1,000; Millipore, Darmstadt, Germany), CREB antibody (1:1,000; Millipore), brain-derived neurotrophic factor (BDNF) antibody (1:1,000; Applied Biological Materials, Richmond, BC, Canada), and anti-β-actin (1:5,000; Sigma-Aldrich) at 4°C, followed by incubation with peroxidase-conjugated secondary antibodies (Amersham ECL HRP-conjugated, 1:2,000; GE HealthCare, UK). Signals were visualized by enhanced chemiluminescence (Amersham ECL Western Blotting Detection Reagent; GE Healthcare, Wilkes-Barre, PA, USA). Optical density was assessed using ImageJ analysis software.

Measurements of Cell Area and Cell Counting

Nestin signaling in the peri-infarct area, ipsilateral subventricular zone (SVZ) area, contralateral cortex, and SVZ was analyzed by quantifying the stained areas using ImageJ analysis software. To obtain data as to cell numbers from immunostained brain sections, an investigator blinded to the experimental groups counted the number of PDGFR-α, double positive stained cells in peri-infarct area for Ki-67 and nestin and double positive stained cells in the ipsilateral corpus callosum for Ki-67 and PDGFR-α.

Statistical Analysis

Pearson's chi-squared test was used for mortality rate. For other data comparisons, a Mann-Whitney U-test was used to determine any significant differences between 3-month-old and 12-month-old groups. All data are expressed as mean ± SD. A value of $p < 0.05$ was considered statistically significant.

RESULTS

We subjected 3-month-old and 12-month-old SHRs to transient 75 min of MCAO followed by reperfusion. We performed surgery on 23 rats in the 3-month-old group and 24 rats in the 12-month-old group. Of the 47 rats, no animals died in the 3-month-old group during the recovery phase, but three rats died in the 12-month old group. However, there was no significant difference in the mortality rate between 3- and 12-month-old groups ($p = 0.08$). Laser Doppler measurement confirmed that there was no difference in regional cerebral blood flow before and after arterial occlusions (data not shown). Standard H&E

staining demonstrated that both 3- and 12-month-old SHRs exhibited similar infarct volumes at days 3 and 14 post-ischemia (3-month-old at day 3: 173 ± 47 mm^3 of $N=5$, 12-month-old at day 3: 182 ± 30 mm^3 of $N=5$, 3-month-old at day 14: 222 ± 29 mm^3 of $N=5$, 12-month-old at day 14: 228 ± 67 mm^3 of $N=5$). In addition, up to day 14, there was no significant difference in cerebral hemorrhage transformation between 3- and 12-month old groups (1 out of 10 rats in 3-month old group, 2 out of 10 rats in 12-month old group). However, there were differences in terms of neurological recovery over time. At day 28 post-ischemia, NSS scores in 12-month-old SHRs were higher (i.e., worse behavioral deficits) compared to 3-month-old SHRs (Fig. 1A). In addition, in the adhesive removal test, the time of initial contact to tape removal was also longer in 12-month-old SHRs (Fig. 1B).

Since there were apparent differences in stroke recovery between the two groups, we next asked whether differences in cellular compensatory responses were also detectable. We assessed surrogate markers of stroke-induced neurogenesis and oligodendrogenesis by immunostaining for NSPCs and OPCs. Anti-nestin antibody was used as a marker for NSPCs and anti-PDGF-R-α for OPCs. At both acute and subacute times, nestin$^+$ areas in the contralateral side were similar between 3-month- and 12-month-old SHRs (3-month-old at day 3: $1.9 \pm 1.1\%$, 12-month-old at day 3: $1.1 \pm 0.6\%$, 3-month-old at day 14: $1.9 \pm 1.0\%$, 12-month-old at day 14: $1.5 \pm 0.7\%$). In addition, numbers of PDGFR-α^+ cells in the corpus callosum of the contralateral side were also not different between 3-month-old and 12-month-old SHRs (3-month-old at day 3: 180 ± 21 cells/mm^2, 12-month-old at day 3: 176 ± 23 cells/mm^2, 3-month-old at day 14: 183 ± 17 cells/mm^2, 12-month-old at day: 186 ± 18 cells/mm^2).

However, ipsilateral responses were different. At day 3 postischemia, the areas of nestin$^+$ NSPCs in peri-infarct cortex and around the SVZ region were larger in 3-month-old SHRs compared to 12-month-old SHRs (Fig. 2). Similarly, 3-month-old SHRs showed a larger number of PDGFR-α^+ OPCs in the corpus callosum of the ipsilateral ischemic side (Fig. 3). Western blot analysis of dissected brain homogenates demonstrated that expression levels of nestin and PDGFR-α were higher in brain samples from 3-month-old SHRs (Fig. 4), although there were no significant differences in expressions of nestin and PDGFR-α in contralateral sides (Supplementary Fig. S1; available at http://www.nmr.mgh.harvard.edu/~etm/supplement/liang-et-al-2016/). Notably, levels of phospho-CREB expression were also higher in the 3-month-old SHR brains (Fig. 5 and Supplementary Fig. S1, available at http://www.nmr.mgh.harvard.edu/~etm/supplement/liang-et-al-2016/), indicating that age-related decline in acute postischemic neurogenesis and oligodendrogenesis

may be, at least partly, related to the downregulation of CREB signaling.

Next we investigated the subacute phase of neurogenesis and oligodendrogenesis at day 14 after cerebral ischemia. There were no significant differences between 3-month-old versus 12-month-old SHRs in terms of NSPC and OPC numbers (NSPCs in peri-infarct area: 3-month-old $4.2 \pm 0.9\%$, 12-month-old $3.3 \pm 1.4\%$, OPCs in corpus callosum: 3-month-old 271 ± 36 cells/mm^2, 12-month-old 262 ± 20 cells/mm^2). However, the number of proliferating NSPCs (Ki-67$^+$/nestin$^+$ double positive cells) and proliferating OPCs (Ki-67$^+$/PDGFR-α^+ cells) were higher in 3-month-old SHR brains (Fig. 6), suggesting that even in the subacute phase after stroke, NSPCs/OPCs may tend to proliferate as a compensatory
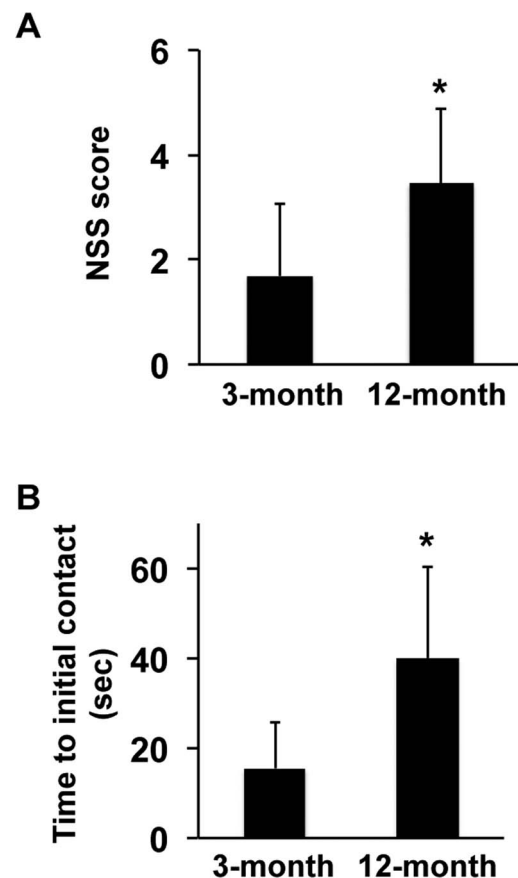

Figure 1. Three-month-old SHRs showed better neurological outcomes at day 28 after MCAO. (A) Although there was no difference in the NSS score before MCAO, 3-month-old SHRs exhibited lower NSS scores (e.g., better neurological outcome) at day 28 after MCAO. Values are expressed as mean$\pm$SD of $N=13$ for 3-month-old and $N=11$ for 12-month-old SHRs. *$p < 0.05$. (B) Although there was no difference in the time to initial contact with adhesive tape before MCAO, 3-month-old SHRs exhibited better scores on the adhesive test at day 28 after MCAO. Values are expressed as mean$\pm$SD of $N=13$ for 3-month-old and $N=11$ for 12-month-old SHRs. *$p < 0.05$.

response, and younger SHRs may continue to maintain a higher proliferation of NSPCs and OPCs during prolonged recovery postischemia.

DISCUSSION

Neuronal and oligodendrocyte loss is one of the most important and common features in neurological diseases such as stroke. It is now recognized that in parallel with ongoing pathophysiology, endogenous mechanisms are also triggered to compensate for lost brain cells. In the context of compensatory brain repair mechanisms, NSPCs and OPCs play critical roles because they constitute the major types of immature cells in adult brain and are activated after injury to differentiate into neurons or oligodendrocytes. However, aged brains tend to slowly lose these endogenous repair systems. Past studies extensively examined the mechanisms of age-related decline in neurogenesis and oligodendrogenesis, but most of those studies used normal, nondiseased animal models. In contrast, stroke tends to occur in patients with ongoing vascular comorbidities such as hypertension. Our current study confirmed that in SHRs, aging dampens stroke-induced neurogenesis and oligodendrogenesis. Therefore, our findings provide an additional insight into how aging
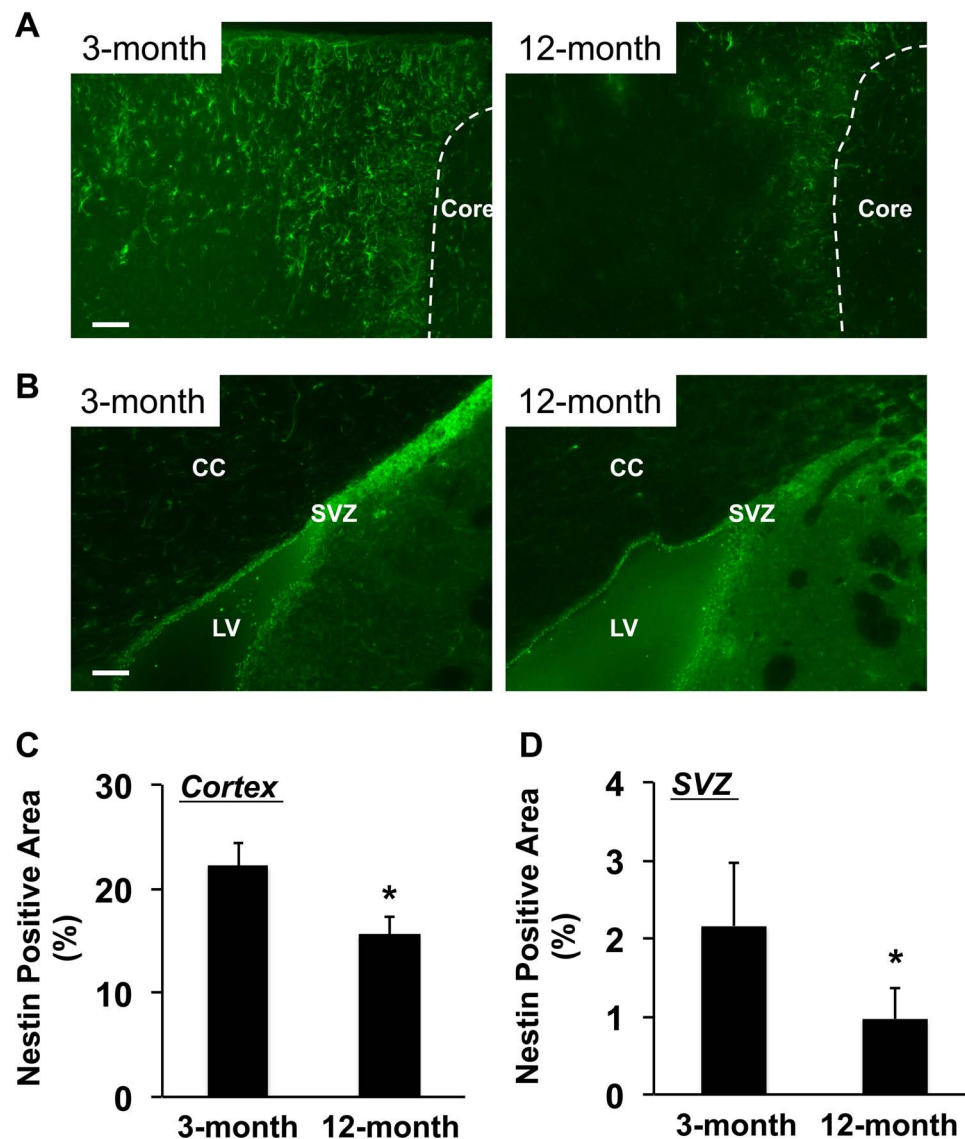

Figure 2. Number of NSPCs was larger in 3-month-old SHRs at day 3 after MCAO. (A) Representative pictures for staining of anti-nestin antibody (a marker for NSPCs) in the peri-infarct area in cortex. SHR brains were removed at day 3 after MCAO. (B) Representative pictures for staining of anti-nestin antibody around SVZ region. SHR brains were removed at day 3 after MCAO. CC, corpus callosum; LV, left ventricle; SVZ, subventricular zone. (C, D) At day 3 after MCAO, nestin⁺ areas were significantly smaller in 12-month-old SHRs in both peri-infarct region and SVZ area. Values are expressed as mean±SD of $N=5$. *$p<0.05$. Scale bars: 100 μm.

affects brain remodeling after injury within the context of a common ischemic injury.

In most CNS diseases, the extent of loss of neurons and oligodendrocytes will likely impact the disease phenotype. Therefore, enhancement of endogenous neurogenesis and oligodendrogenesis could potentially boost functional recovery by replacing damaged neurons and oligodendrocytes (38). Although several difficult hurdles remain in this approach, stem cell therapy may provide an avenue to enhance endogenous compensatory neurogenesis and oligodendrogenesis. Cell-based therapy is now relatively well accepted as a valid therapeutic strategy for repairing damaged organs, including the brain. In preclinical studies using CNS disease models, stem cell treatment was shown to be protective for the damaged brain. Notably, transplanted cells may not support the brain by direct cell replacement only but also protect brain cells indirectly by secreting trophic factors (3,14,27). In addition, recent studies suggest that transplanted cells can create a "biobridge" that fills the gap between the neurogenic niche and the site of the brain injury (11,33). These studies support the idea that the efficacy of cell-based therapy depends, in part, on the rate of compensatory

neurogenesis and oligodendrogenesis. In this regard, our current study may be useful in describing the age-related decline in stroke-induced neurogenesis and oligodendrogenesis under hypertensive conditions.

In this study, we used an anti-nestin antibody to identify NSPCs and an anti-PDGFR-α antibody to identify OPCs. Although nestin and PDGFR-α are widely used as markers for NSPCs and OPCs, respectively, many cell marker proteins are shared between several kinds of mature/immature cells in the CNS. For example, after ischemic stroke, activated astrocytes also express nestin (10,20,29). In addition, PDGFR-α+ NSPCs can generate both neurons and oligodendrocytes (17). In fact, our pilot experiments for triple staining of nestin/glial fibrillary acidic protein (GFAP)/Ki-67 detected several nestin/GFAP double positive cells in the cortex (Supplementary Fig. S2, available at http://www.nmr.mgh.harvard.edu/~etm/supplement/liang-et-al-2016/), but in the penumbral region (e.g., near the core edge) on day 3 after MCAO, most nestin+ cells were negative for GFAP (Supplementary Fig. S2, available at http://www.nmr.mgh.harvard.edu/~etm/supplement/liang-et-al-2016/). More importantly, 3-month-old SHRs exhibited
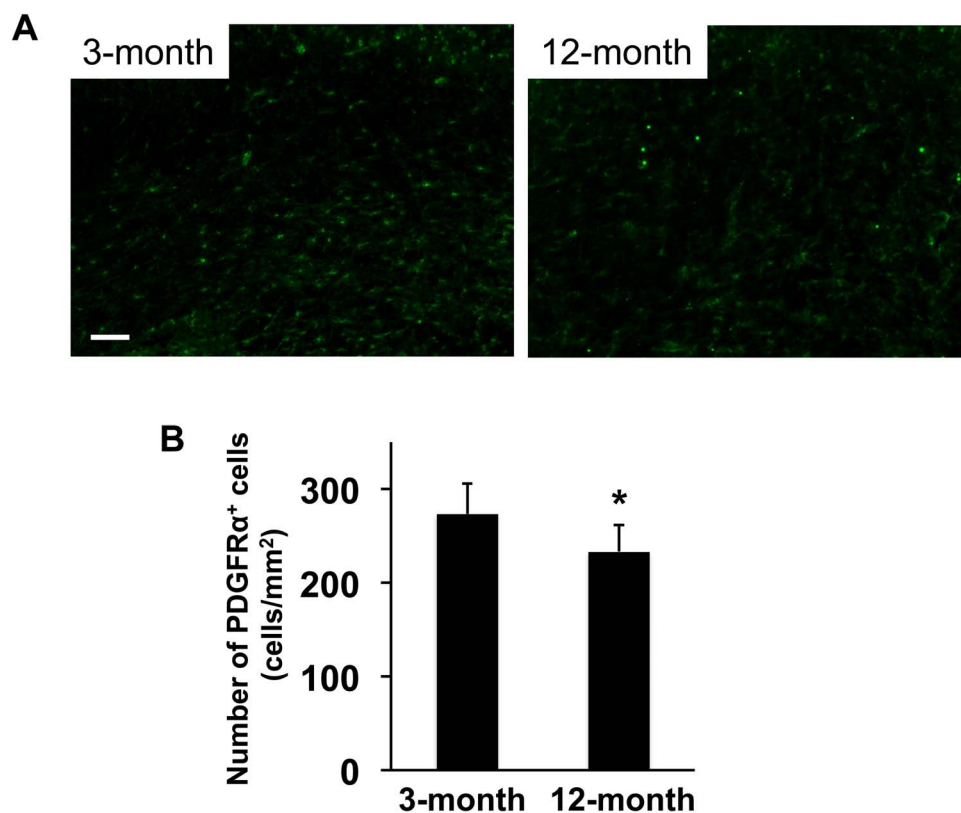

Figure 3. Number of OPCs was larger in 3-month-old SHRs at day 3 after MCAO. (A) Representative pictures for staining of anti-PDGFR-α antibody (a marker for OPCs) in the corpus callosum. SHR brains were removed at day 3 after MCAO. (B) At day 3 after MCAO, PDGFR-α+ cells were significantly smaller in 12-month-old SHRs in the corpus callosum. Values are expressed as mean±SD of $N=5$. $*p<0.05$. Scale bar: 100 μm.

a larger number of nestin[+]/Ki-67^{+}/GFAP^{−} cells compared to 12-month-old animals (Supplementary Fig. S2, available at http://www.nmr.mgh.harvard.edu/~etm/supplement/liang-et-al-2016/), further supporting our conclusion that aging may dampen neurogenesis in SHRs after stroke. Another pilot experiment of nestin/PDGFR-α/Ki-67 triple staining showed that most PDGFR-α signals did not overlap with nestin signals in the corpus callopus region (Supplementary Fig. S3, available at http://www.nmr.mgh.harvard.edu/~etm/supplement/liang-et-al-2016/), indicating that PDGFR-α may be a marker that is specific for OPCs rather than NSPCs, at least in corpus callosum. Nevertheless, we should be careful with these cell markers, since cells may change their protein production profiles in unexpected ways, especially after injury. Furthermore, since NSPCs may express GFAP under some conditions (7), future studies need to carefully identify NSPCs (and OPCs) with other cell marker antibodies.

Our current study demonstrates that similar to normotensive animals, aging may suppress regenerative responses after brain injury in hypertensive rats. However, we did not examine how hypertensive conditions affect the cerebrovascular system and neurological outcomes after stroke in SHRs. SHRs have normal systolic blood pressure by 4–8 weeks of age, after which hypertension rapidly develops (especially between 12 and 14 weeks) (1). After that, there may be no substantial differences in blood pressure between 3- and 12-month-old SHRs (1). However, it still remains to be elucidated how chronic hypertension affects the cerebrovascular system. In general, despite elevation of total peripheral resistance in hypertensive conditions, resting cerebrovascular resistance and cerebral blood flow
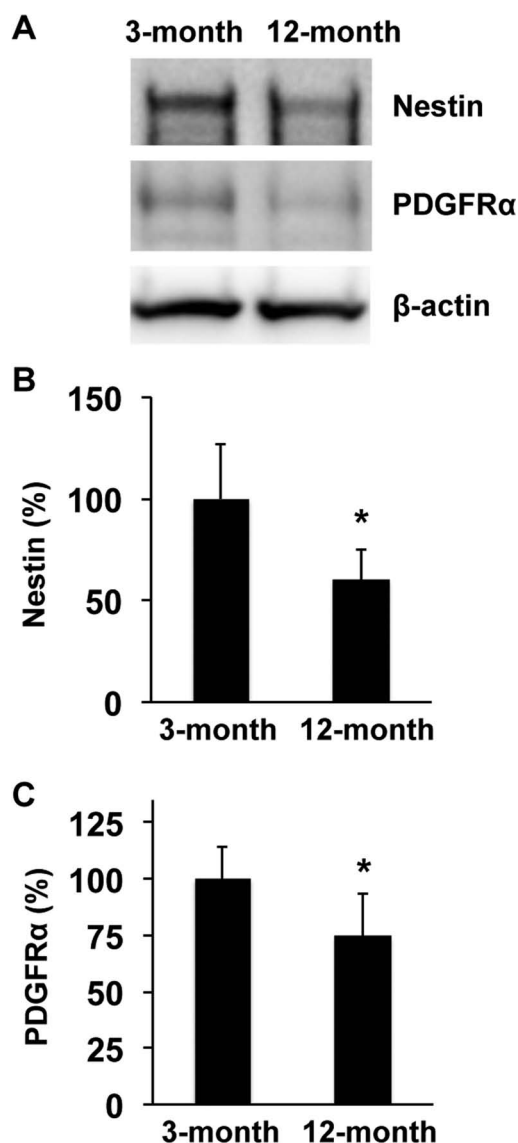

Figure 4. Nestin and PDGFR-α expression were higher in 3-month-old SHRs at day 3 after MCAO. (A) Representative images for Western blotting of anti-nestin, anti-PDGFR-α, or anti-β-actin antibodies. Brain samples from the ischemic side at day 3 after MCAO were subjected to Western blot analysis. β-Actin was used as a loading control. (B, C) Levels of nestin and PDGFR-α expression in 3-month-old SHRs were higher at day 3 after MCAO. Values are expressed as mean ± SD of $N = 5$. *$p < 0.05$.
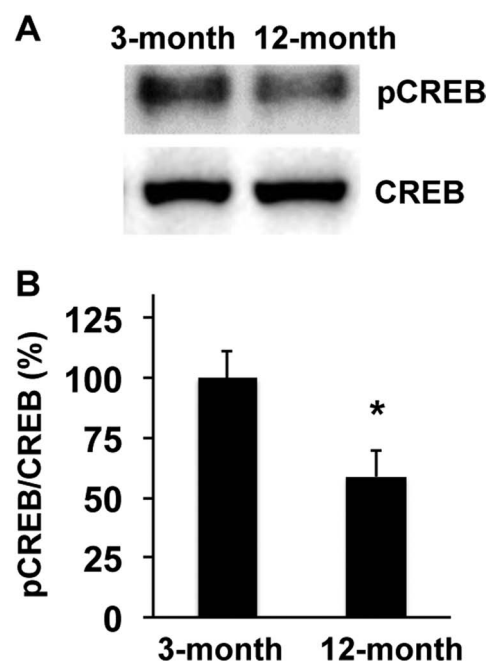

Figure 5. Phosphorylation level of CREB was higher in 3-month-old SHRs at day 3 after MCAO. (A) Representative images for Western blotting of anti-pCREB antibody. Brain samples from the ischemic side at day 3 after MCAO were subjected to Western blot analysis. Total CREB was used as a baseline control. (B, C) Levels of pCREB in 3-month-old SHRs were higher at day 3 after MCAO. Values are expressed as mean ± SD of $N = 5$. *$p < 0.05$.

(CBF) would remain at normal levels due to cerebral auto-regulation (36). Chronic hypertension induces an upward shift in the autoregulation curve, and therefore, CBF would be more susceptible to pressure-associated fluctuations in aged SHRs than younger animals (13). Our current study did not detect clear differences in regional CBF during MCAO between 3- and 12-month-old animals. Nonetheless, it is possible that during the recovery phase, CBF (and the cerebrovascular system itself) in the 12-month-old group may be abnormal and affect neurological outcomes in a deleterious way after stroke. Therefore, future studies need to carefully examine the cerebrovascular systems of young and aged SHRs to understand pathophysiological mechanisms of stroke under hypertensive conditions.

Taken together, our initial findings here suggest that in SHRs, aging dampens the compensatory responses in neuronal and oligodendrocyte repair after injury, which may result in worse neurological recovery. However,
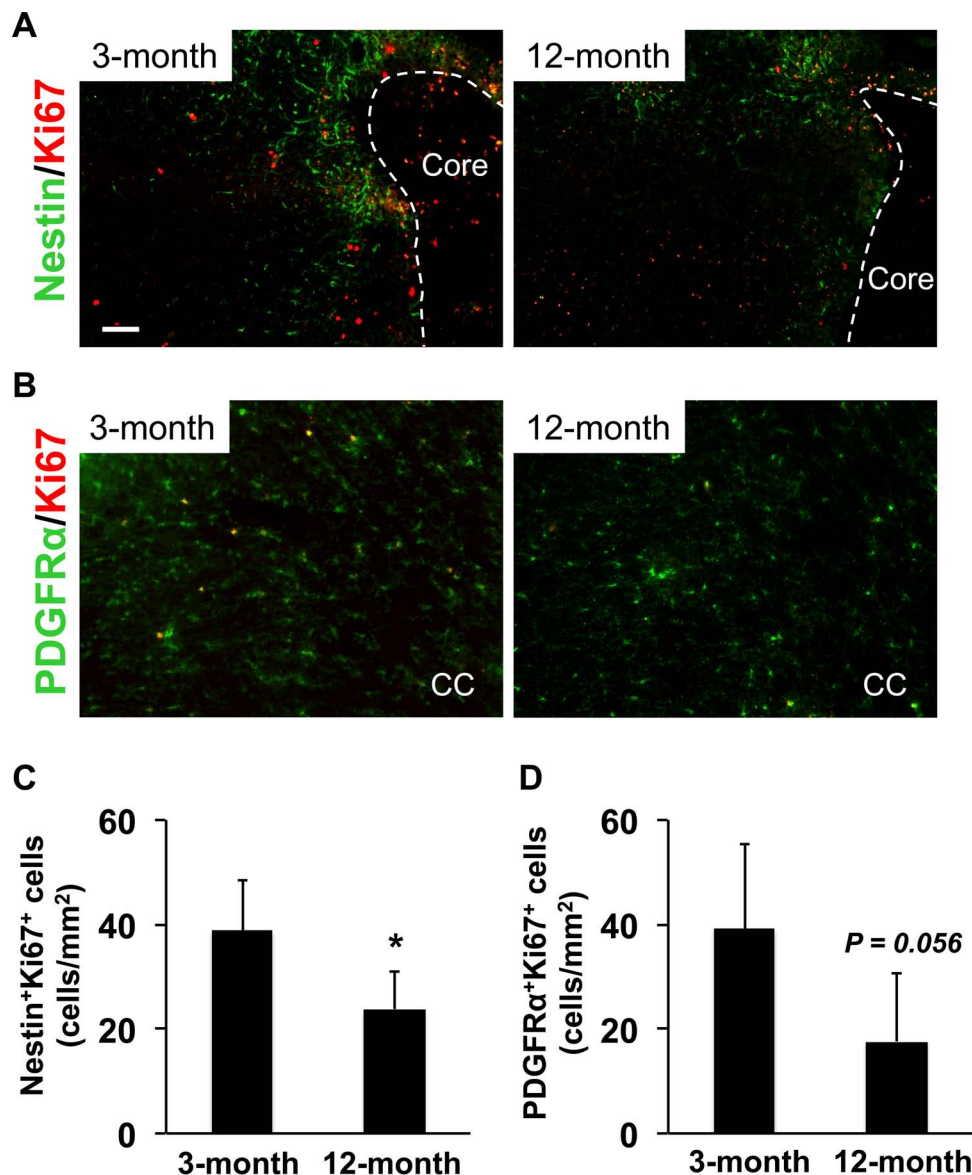

Figure 6. Numbers of proliferating NSPCs and OPCs were larger in 3-month-old SHRs at day 14 after MCAO. (A) Representative pictures for double staining of anti-nestin and anti-Ki-67 (a marker for proliferating cells) antibodies in the peri-infarct area in the cortex. SHR brains were removed at day 14 after MCAO. (B) Representative pictures for double staining of anti-PDGFR-α and anti-Ki-67 antibodies in corpus callosum. SHR brains were removed at day 14 after MCAO. (C, D) At day 14 after MCAO, the number of proliferating NSPCs (nestin$^+$/Ki-67$^+$ cells) was significantly larger in 3-month-old SHRs. As for proliferating OPCs (PDGFR-α$^+$/Ki-67$^+$ cells), although the p value was not less than 0.05, there was a trend that 3-month-old SHRs showed a larger number of proliferating OPCs at day 14 after MCAO. Values are expressed as mean ± SD of $N=5$. *$p<0.05$. Scale bar: 100 μm.

there are several important caveats and limitations to keep in mind. First, we did not compare the rate of stroke-induced neurogenesis/oligodendrogenesis in SHRs with that in normotensive rats. Whether hypertensive conditions confound the age-related decline in compensatory mechanisms should be examined in future studies. Second, we examined histological outcomes on days 3 and 14 and assessed neurological outcomes on day 28. Therefore, we could not analyze correlations between neurological and histological outcomes. In future studies, we need to carefully investigate how histological changes would affect neurological behaviors. Third, we assessed only proliferation of NSPCs and OPCs as an index for neurogenesis and oligodendrogenesis, but for successful brain repair, proliferating NSPCs and OPCs must differentiate into functional neurons and oligodendrocytes (25). Further studies into this subject are required to understand the effects of aging on stroke-induced neurogenesis and oligodendrogenesis. Fourth, although we showed that downregulation of CREB activation in 12-month-old SHRs may be associated with age-related decline in the proliferation of NSPCs and OPCs, the precise underlying mechanisms are mostly unknown. Our pilot study indicated that most cells with a pCREB signal did not overlap with nestin or PDGFR-α signals (Supplementary Fig. S4, available at http://www.nmr.mgh.harvard.edu/~etm/supplement/liang-et-al-2016/), raising the possibility that CREB activity in neighboring cells may upregulate trophic factor production, such as BDNF, to support NSPC and OPC function. In fact, the expression level of BDNF in 3-month-old SHRs was higher than that in 12-month old animals after stroke (Supplementary Fig. S4, available at http://www.nmr.mgh.harvard.edu/~etm/supplement/liang-et-al-2016/). How CREB signaling mediates neurogenesis and oligodendrogenesis after stroke would be an important research topic in this field. Finally, our study did not examine the mechanisms through which compensatory neurogenesis/oligodendrogenesis can help neurological recovery. In our model systems, although infarct volumes assessed by H&E staining at days 3 and 14 were the same between 3-month-old and 12-month-old SHRs, stroke-induced proliferation in NSPCs/OPCs was larger in younger animals, and at the later time point, younger animals showed better neurological scores in NSS and adhesive removal tests. However, we only assessed neurological function by two behavioral tests at one time point. Multiple behavioral tests at multiple time points would be needed to further investigate the relationship between compensatory neurogenesis/oligodendrogenesis and neurological/cognitive recovery.

In summary, our current study described an age-related decline in stroke-induced regenerative responses in NSPCs and OPCs in a hypertensive animal model of focal cerebral ischemia. In addition to aging, hypertension is also a major risk factor for stroke. Further investigations into the mechanisms that mediate the interaction between aging and hypertension are warranted in order to pursue therapeutic approaches for boosting endogenous repair mechanisms in stroke-damaged brains.

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