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Notch1 Signaling Modulates Neuronal Progenitor Activity in the Subventricular Zone in Response to Aging and Focal Ischemia

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Abstract

Neurogenesis diminishes with aging and ischemia-induced neurogenesis also occurs, but reduced in aged brain. Currently, the cellular and molecular pathways mediating these effects remain largely unknown. Our previous study has shown that Notch1 signaling regulates neurogenesis in subventricular zone (SVZ) of young-adult brain after focal ischemia, but whether a similar effect occurs in aged normal and ischemic animals is unknown. Here, we used normal and ischemic aged rat brains to investigate whether Notch1 signaling was involved in the reduction of neurogenesis in response to aging and modulates neurogenesis in aged brains after focal ischemia. By Western blot, we found that Notch1 and Jagged1 expression in the SVZ of aged brain was significantly reduced compared with young-adult brain. Consistently, the activated form of Notch1 (Notch intracellular domain; NICD) expression was also declined. Immunohistochemistry confirmed that expression and activation of Notch1 signaling in the SVZ of aged brain were reduced. Double or triple immunostaining showed that Notch1 was mainly expressed in DCX-positive cells, whereas Jagged1 was predominantly expressed in astroglial cells in the SVZ of normal aged rat brain. In addition, disruption or activation of Notch1 signaling altered the number of proliferating cells labeled by bromodeoxyuridine (BrdU) and doublecortin (DCX) in the SVZ of aged brain. Moreover, ischemia-induced cell proliferation in the SVZ of aged brain was enhanced by activating the Notch1 pathway, and was suppressed by inhibiting the Notch1 signaling. Reduced infarct volume and improved motor deficits were also observed in Notch1 activator-treated aged ischemic rats. Our data suggest that Notch1 signaling modulates the SVZ neurogenesis in aged brain in normal and ischemic conditions.

Keywords

Notch1 signaling pathway; aged rat brain; neurogenesis; focal cerebral ischemia

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INTRODUCTION

The treatment of ischemic stroke remains an intimidating mission because few therapeutic strategies have proven to be effective. Systemic thrombolysis with intravenous tissue plasminogen activator (tPA) is the only proven treatment to improve clinical outcome of patients with acute ischemic stroke (Brott & Bogousslavsky 2000; Adams *et al.* 2007). But because of an increased risk of hemorrhage beyond 3 hours post-stroke, only certain stroke patients (1-2%) can benefit from tPA. Therefore, new therapies need to be found that protect and repair the damaged brain after stroke. Regenerative cell-based therapies offer long-term hope for many patients with stroke, as neural stem/progenitor cells (NSCs) may be possible for dead or injured neural cells to be replaced after acute stroke (Kondziolka *et al.* 2000; Kelly *et al.* 2004; Jin *et al.* 2005). As stroke in humans usually afflicts the elderly (Arnold 1981; Ramirez-Lassepas 1998; Popa-Wagner *et al.* 2007; Popa-Wagner *et al.* 2011), it is important to know whether stem cells can be a potential therapy for stroke in aged models of stroke (Popa-Wagner *et al.* 2010).

NSCs persist in the subventricular zone (SVZ) of the lateral ventricle and in the subgranular zone (SGZ) of dentate gyrus throughout adulthood in mammals (McDermott & Lantos 1991; Eriksson *et al.* 1998; Jin *et al.* 2001; Yoshimura *et al.* 2001). There is substantial evidence for increased proliferation of NSCs in the adult brain after brain injuries. In the young-adult animal, stroke induces the proliferation of endogenous NSCs located in the SVZ (focal ischemia) (Jin *et al.* 2001) and in the dentate SGZ (global ischemia) (Liu *et al.* 1998). The newborn cells can migrate into the damaged brain regions (Jin *et al.* 2003b), and express phenotypic markers of mature neurons (NeuN, MAP-2) and region-specific mature neuronal markers like dopamine (Arvidsson *et al.* 2002; Parent *et al.* 2002), and form synapses (Yamashita *et al.* 2006). Evidence for functional neuronal replacement in the damaged brain regions has been reported from a model of global cerebral ischemia that affects the hippocampus predominantly. Infusion of epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF-2) led to regeneration of hippocampal CA1 neurons derived from the dentate SGZ, which integrated into the existing brain circuitry and were thought to have contributed to ameliorating neurological deficits (Nakatomi *et al.* 2002). Despite an age-related reduction in basal SVZ proliferation (Troppe *et al.* 1997; Jin *et al.* 2003a; Maslov *et al.* 2004; Luo *et al.* 2006), we found that NSCs in the SVZ of aged rats (Jin *et al.* 2004) and in the ischemic penumbra region of human (Jin *et al.* 2006) retained the capacity for proliferation and lesion-directed migration in response to cerebral ischemia, although the response was less robust than in younger animals. In addition, Yagita *et al.* (Yagita *et al.* 2001) reported increased proliferation, but reduced survival of new neurons after global ischemia in middle-aged rats in comparison to young adult rats. Darsalia *et al.* (Darsalia *et al.* 2005) stated that postischemic neurogenesis in the SGZ of 15-months-old rats was reduced, but neurogenesis in the SVZ was increased. We found that conditional ablation of neurogenesis in the SVZ of young-adult and middle-aged mice increased infarct size and exacerbated postischemic sensorimotor behavioral deficits (Jin *et al.* 2010). These findings suggest that ischemia-induced neurogenesis is critical for functional recovery after stroke and may be used as the new therapeutic strategy for stroke. However, the cellular and molecular pathways mediating these effects in aged animals remain largely unknown.

Previous studies show that Notch signaling is a fundamental pathway controlling cell fate acquisition and plays critical roles during maintenance, proliferation, and differentiation of NSCs in developing brain (Artavanis-Tsakonas *et al.* 1999). Recent evidence shows that Notch1 signaling is conserved in the regulation of adult neurogenesis. First, the Notch1 signaling molecules are expressed in cells of the SVZ and the SGZ of postnatal brain (Stump *et al.* 2002; Chojnacki *et al.* 2003a). Second, expression of the Notch1 ligands, Jagged1 and Delta1, in neurogenic brain regions decreases with aging (Givogri *et al.* 2006). Third,

disruption of Notch1 signaling interferes with the maintenance and proliferation of NSCs (Chojnacki *et al.* 2003a; Wang *et al.* 2009a). Our previous study showed that Notch1 and its downstream targets were expressed in SVZ cells, and that the number of BrdU-positive (proliferating) cells in the normal adult SVZ was significantly altered after inhibiting or activating the Notch1 pathway (Wang *et al.* 2009b). In addition, we found that Notch1 signaling in the SVZ was activated after focal ischemia and that ischemia-induced cell proliferation in the SVZ could be blocked by inhibiting the Notch1 pathway in young-adult brain. However, whether Notch1 signaling activity contributes, directly or indirectly, to the age-dependent decline in neurogenesis, including that following stroke, remains largely unknown.

The present study was undertaken to examine the effect of Notch1 signaling on neurogenesis in the SVZ of the normal and ischemic aged rat brain *in vivo*. We found that the Notch1 and Jagged1 expression was decreased with aging, along with reduced activation level of Notch1 signaling. Double or triple immunostaining showed that Notch1 and Notch1 activated form Notch1(Notch intracellular domain; NICD) were predominantly localized to doublecortin (DCX)-positive cells, whereas Jagged1 was mainly expressed in astrocytes, in the SVZ of aged rat brain. Disruption or activation of Notch1 signaling altered the number of proliferating cells in the SVZ of aged brain. In addition, ischemia-induced cell proliferation in the SVZ of aged brain was enhanced by activating the Notch1 pathway and was suppressed by inhibiting the Notch1 signaling. Reduced infarct volume and improved motor deficits were also observed in Notch1 activator-treated aged ischemic rats. Our data suggest that Notch1 signaling modulates the SVZ neurogenesis in aged brain in normal and ischemic conditions.

MATERIALS AND METHODS

Ethics Statement

All animal procedures were approved by Institutional Animal Care and Use Committee of Buck Institute of Research on Aging and University of North Texas Health Science Center, and conducted according to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, and every effort was made to minimize suffering and to reduce the number of animals used.

Focal cerebral ischemia

Aged (20-24 months old) male Fisher 344 rats were obtained from the National Institute of Aging rodent colony and were anesthetized with 2.0% isoflurane in 70% N₂O/30% O₂. Permanent distal middle cerebral artery occlusion (dMCAO) was performed as described previously (Nawashiro *et al.* 1997; Won *et al.* 2006). Briefly, a 2-cm incision was made under the surgical microscope between the left orbit and tragus, the temporal muscle was retracted laterally. A small burr hole (2mm diameter) was made with a high-speed microdrill through the outer surface of the semitranslucent skull just over the visibly identified middle cerebral artery (MCA) at the level of the inferior cerebral vein. Saline was applied to the area throughout the procedure to prevent heat injury. The inner layer of the skull and the dura were removed with fine forceps and left dMCAO was performed by electrocoagulation (by means of a small-vessel cauterizer) without damaging the brain surface. If the brain surface was visibly damaged or if the MCA bled owing to incomplete artery occlusion/coagulation, the animal was killed and not used. The temporal muscle was repositioned and the skin was closed. After surgery, the rats were placed in a cage under an infrared heating lamp until they recovered from anesthesia. Rectal temperature was maintained at 37.0±0.5°C using a thermostat-controlled heating pad (Harvard Apparatus, Holliston, MA,

USA). Sham-operated rats underwent identical surgery except that the MCA was not occluded.

Administration of Notch signaling activator and inhibitor

Male Fisher 344 rats (N=8-12 per group) were anesthetized and implanted with an osmotic minipump (Alzet 1003D; Alza Corporation, CA, USA) as described previously (Wang *et al.* 2009b). Briefly, the cannula was placed into the left lateral ventricle, 1.6 mm lateral to the midline, 0.8 mm posterior to the bregma and 4.0 mm deep into the pial surface. Notch1 activator and inhibitor were prepared as described previously (Wang *et al.* 2009b). For Notch1 activator, Notch1-activating antibody (clone 8G10; Millipore, Temecula, CA, USA) 10 µg/ml was dissolved in the artificial cerebrospinal fluid (aCSF; Harvard apparatus, Holliston, MA, USA) at a 1:4 dilution. For Notch1 inhibitor, Jagged1-Fc (10 µg; R&D systems, Minneapolis, MN, USA) was incubated for 1 hr on ice with anti-human Fc antibody (5 µg; Sigma-Aldrich, St Louis, MO, USA) at a 2:1 ratio and the final concentration of Jagged1-Fc was 50 µg/ml. Each rat was infused for 3 days with 1 µl/hr of either (1) Notch1-activating antibody, (2) the Jagged1-Fc-anti-Fc complex, or (3) aCSF alone (vehicle). BrdU (50mg/kg; Sigma-Aldrich) was dissolved in saline and given intraperitoneally, twice daily for 3 days at 8 hr intervals, and rats were killed on day 4.

Immunohistochemistry

Brain tissues were postfixed in paraformaldehyde overnight, embedded in paraffin, and then cut into 6-µm sections, which were deparaffinized with xylene and rehydrated with ethanol, followed by antigen retrieval using the antigen unmasking solution (Vector Laboratories, CA, USA) according to the manufacturer's instructions. Immunohistochemistry was performed as described previously (Wang *et al.* 2009b). In brief, endogenous peroxidase activity was blocked by incubation in 3% H₂O₂ for 30 min at room temperature. After several washes with PBS, the sections were incubated in blocking solution (10% goat or rabbit serum and 0.3% Triton X-100 in PBS) for 1 hr at room temperature. Primary antibodies used were (1) rabbit polyclonal anti-Notch1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:50); (2) rabbit polyclonal anti-NICD (Abcam, Cambridge, MA, USA; 1:200); (3) goat polyclonal anti-Jagged1 (Santa Cruz Biotechnology; 1:50). Primary antibodies were incubated in PBS with 0.3% Triton X-100 and 1% bovine serum albumin (BSA) at 4°C overnight. Sections were then washed with PBS and incubated for 1 hr at room temperature with biotinylated goat anti-rabbit or rabbit anti-goat antibody (1:200). Avidin-biotin complex (Vector Elite; Vector Laboratories) and a diaminobenzidine (DAB; Vector Laboratories) were used to obtain a visible reaction product. Processing was stopped with H₂O and sections were dehydrated through graded alcohols, cleared in xylene, and coverslipped in permanent mounting medium (Vector Laboratories). Sections were examined and photographed with a Nikon microscope and the Nikon DS-Fi1 color camera (Nikon, Melville, NY, USA). Controls included omitting the primary or secondary antibody.

Double- or Triple-label immunostaining

Double or triple immunostaining was performed on brain sections as previously described (Wang *et al.* 2009b). The primary antibodies used, in addition to those listed above, were mouse monoclonal anti-BrdU (Sigma-Aldrich; 1:500), goat or rabbit polyclonal anti-DCX (Santa Cruz Biotechnology; 1:50), and mouse monoclonal anti-GFAP (Sigma-Aldrich; 1:500). The secondary antibodies were Alexa Fluor 488-, 594-, or 647- conjugated donkey anti-mouse, anti-goat, or anti-rabbit IgG (1:200; Invitrogen, Grand Island, NY, USA). To detect BrdU-labeled cells in brain sections, sections were incubated in 2 mol/L of HCl at 37°C for 30 min, and rinsed in 0.1 mol/L of boric acid (pH 8.5) and PBS. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) using the Prolong Gold antifade reagent (Invitrogen). Fluorescence signals were detected with the Nikon inverted Ti-U

microscope and the Nikon DS-Qi MC camera (Nikon), and images were acquired using the Nikon Imaging System (Nikon). Selected images were recorded using an LSM 510 NLO Confocal Scanning System mounted on an Axiovert 200 inverted microscope equipped with a two-photon Chameleon laser. Controls included omitting either the primary or secondary antibody.

Cell counting

BrdU-, DCX-positive and double-labeled cells in the SVZ, along the lateral walls of the lateral ventricles (beginning at 1.18 mm anterior to bregma), were counted in five to seven 6- μ m paraffin coronal sections per animal (n=3-4 per group), spaced 120 μ m apart, by an observer blind to experimental condition using the Nikon inverted Ti-U microscope in fluorescein isothiocyanate (FITC) and Texas (Tx) Red channels with a 40 x objective. An LSM 510 NLO Confocal Scanning System was also used to record the z-stack images to confirm colocalization of DCX and BrdU-labeled cells in the SVZ. Results were expressed as the average number of BrdU- and DCX-positive cells in SVZ.

Western Blotting

Western blotting was conducted as previously described (Wang *et al.* 2009b). The SVZ was dissected from both young-adult (3 months old) and aged (24 months old) Fisher 344 rat brains as previously described (Ortega *et al.* 2011). Rats were transcardially perfused with PBS. The brains were removed and placed in a coronal brain matrix, and cut into 1-mm sequential sections throughout the site of the SVZ. The specimens were collected in PBS and the SVZs were dissected from the surrounding tissue with a Zeiss V8 dissecting microscope. Cell lysates were extracted in PBS containing 0.05% Nonidet P-40 (Roche, South San Francisco, CA, USA), 0.25% sodium deoxycholate, 50mmol/L Tris-HCl (pH 8.5), 100 mmol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid (EDTA, pH 8.0), 1 mg/mL aprotinin, and 100 mg/mL phenylmethylsulfonyl fluoride. Protein (50 μ g) was boiled at 100°C in the SDS sample buffer for 5min, electrophoresed on SDS/12% PAGE gels, and transferred into polyvinylidene difluoride membranes, which were incubated overnight at 4°C using one of the following primary antibodies: (1) rabbit polyclonal anti-Notch1 (Santa Cruz Biotechnology; 1:50), (2) rabbit polyclonal anti-NICD (Abcam; 1:500), (3) goat polyclonal anti-Jagged1 (Santa Cruz Biotechnology; 1:500), or (4) mouse monoclonal anti-actin (Oncogene Science, Cambridge, MA, USA; 1:20,000). Membranes were washed with PBS/0.1% Tween 20, incubated at room temperature for 60 min with horseradish peroxidase conjugated anti-mouse, anti-rabbit, or anti-goat secondary antibody (Santa Cruz Biotechnology; 1:3,000), and thereafter washed three times for 15 min with PBS/Tween 20. Peroxidase activity was visualized by chemiluminescence (NEN Life Science Products, Boston, MA, USA). Antibodies were removed with stripping buffer (100 mmol/L 2-mercaptoethanol/2% SDS/62.5 mmol/L Tris-HCl, pH 6.7) at 50°C for 30 min, followed by washing with PBS/Tween 20, and membranes were reprobbed. Densitometry measurements were normalized to actin.

Neurobehavioral testing

Rats (N=8 per group) underwent neurobehavioral tests to evaluate functional outcome. Animals were trained prior to dMCAO and deficits were assessed 72 hr thereafter. The investigator performing the tests was blinded to the experimental condition.

Ladder rung walking test—The ladder rung walking test is sensitive for quantifying skilled locomotion. The degree of motor dysfunction after MCAO was measured by counting the number of foot-faults of the impaired limbs per round, as described previously (Sun *et al.* 2012). Baseline and postoperative testing sessions consisted of three traverses

across the ladder. An error was scored for any foot slip or misstep. The number of errors of the affected forelimb and hindlimb in each trial was counted. The mean number of errors in three traverses was calculated.

Limb placing test—The limb placing test was used to assess sensorimotor dysfunction (De Ryck *et al.* 1989). Rats were evaluated for the ability to place the limbs on a tabletop surface in response to visual, tactile, or proprioceptive input, as described previously (Sun *et al.* 2012). Each of seven tasks was scored by a blinded observer as: 0, no placing; 1, incomplete or delayed placing; or 2, complete, immediate placing. Forelimb and hindlimb scores were averaged for each animal (normal score, 14)

Elevated body swing test (EBST)—The elevated body swing test was conducted to evaluate asymmetric motor behavior. Rats were held by the tail and raised ~10 cm above the testing surface (Borlongan & Sanberg 1995). The initial direction of body swing which was turning of the upper body >10° to either side was recorded in 10 trials, observed over 5 min. The number of turns in each (left or right) direction was recorded, and the percentage of left turns was calculated.

Cylinder Test—Forelimb use bias was analyzed by observing the rat's movements over 3-minute intervals in a transparent, 18-cm-wide, 30-cm-high poly (methyl methacrylate) cylinder. A mirror behind the cylinder made it possible to observe and record forelimb movements when the rat was facing away from the examiner. After an episode of rearing and wall exploration, a landing was scored for the first limb to contact the wall or for both limbs if they made simultaneous contact. Percentage use of the impaired limb was calculated.

Infarct volume

Rats were deeply anesthetized and decapitated 72hr after dMCAO. Brains were removed and 50- μ m coronal sections (400- μ m apart) were prepared and stained with crysel violet. Stained sections (7 per rat, 4 rats per group) were scanned on a desktop scanner and infarct area was measured by a blinded observer using Image J software, and areas were multiplied by the distance between sections to obtain the respective volumes. Infarct volumes were expressed as a percentage of the volume of the structures in the control hemispheres, as described previously (Swanson *et al.* 1990).

Statistical Analysis

Quantitative results were expressed as the mean $\pm$ SEM. The statistical significance of difference between means was evaluated using Student *t*-test or one-way analysis of variance (ANOVA), followed by Newman-Keuls *post hoc* multiple comparison tests. $P < 0.05$ was regarded as statistically significant.

RESULTS

Our previous study has shown that Notch1 signaling regulates neural progenitor fate in the SVZ of young-adult brain. However, whether a similar effect occurs in aged brain is unknown. Therefore, we first determined the expression profile of Notch signaling in the SVZ of aged rat brain. Rats were trained for 3 days prior to dMACO and behavioral tests were performed 72 hr after ischemia. Notch1 inhibitor, activator or vehicle was injected into the lateral ventricle immediately after dMCAO for 3 consecutive days, and rats were then euthanized on 4th day (**Figure 1A**). By Western blot analyses, we found that Notch1 expression in the SVZ of aged brain was significantly decreased compared to young-adult brain (**Figure 1B and C**), which was confirmed by immunocytochemistry (**Figure 1D**).

Confocal images showed that Notch1 protein was predominantly in the DCX-positive cells, but not GFAP-positive cells (**Figure 1E and F**). As shown in **Figure 2A and C**, expression of Jagged1, a Notch1 ligand, was also declined in response to aging. However, Jagged1 was expressed in GFAP-positive cells, but not DCX-positive cells (**Figure 2D and E**).

Next, we asked whether Notch1 signaling activation was also declined in the SVZ with aging. Notch signaling is triggered upon interaction of the receptor with its transmembrane ligand. Upon this ligand-receptor interaction, Notch receptor undergoes to proteolytic cleavage at the membrane by Presenilins. This cleavage releases NICD, which is then translocated to the nucleus where it can interact with a transcriptional activator, resulting in subsequent events. Therefore, NICD is the activated form of Notch1 signaling. As shown in **Figure 3 A and B**, NICD protein in the SVZ of aged brain was significantly reduced compared with young-adult brain. Consistently, immunohistochemistry showed that NICD-positive cells were found in the SVZ of both young-adult and aged brain, but expression level was reduced in aged brain compared with young-adult brain. High-magnification views showed that NICD was located in the nuclei of SVZ cells (insets in **Figure 3C**). In addition, we also found that Hes1, a downstream element of Notch1 signaling, was reduced in the aged brain (data not shown). To determine the phenotype of NICD-expressing cells, triple immunostaining was performed using anti-GFAP, anti-DCX and anti-NICD. Consistent with our findings in young-adult rat (Wang *et al.* 2009b), triple-label immunofluorescence recorded by a two photon confocal microscopy showed that NICD was expressed in the nuclei of DCX-positive cells in the SVZ of aged brain (**Figure 3D and E**).

To further test the significance of Notch1 signaling in the proliferation of SVZ cells in the aged brain, we activated Notch1 signaling by administration of Notch1 extracellular domain antibody (Conboy *et al.* 2003; Wang *et al.* 2009b) and inhibited Notch1 signaling by injection of a soluble Jagged1-Fc complex protein into the lateral ventricle of aged brain and BrdU- and DCX-positive cells in the SVZ were assessed. We found that the numbers of both BrdU- (**Figure 4A**) and DCX-positive (**Figure 4B**) in the SVZ cells were significantly increased after the Notch1 signaling activator injection and decreased after infusion of Notch1 signaling inhibitor, in consistent with the findings in young-adult rat brain (Wang *et al.* 2009b). Double-label immunohistochemistry showed that ~50% of BrdU-positive cells expressed DCX, suggesting that these cells were proliferative neuronal progenitors (**Figure 4C-D**). The confocal images through the series of *z-stacks* indicated that BrdU-positive cells expressed DCX (**Figure 4E**).

Then, we asked whether Notch1 signaling modulated ischemia-induced neurogenesis in aged brain. Focal ischemia was induced in aged rats and Notch1 activator and inhibitor were administrated by intracerebroventricular route. As shown in **Figure 5A-D**, the numbers of both BrdU- and DCX-positive cells in the SVZ were significantly increased 3 days after administration of the Notch1 signaling activator and dramatically reduced 3 days after infusion of the Notch1 signaling inhibitor compared with vehicle-treated group. Similarly, double-label immunohistochemistry also showed that ~50% of BrdU-positive cells in the SVZ expressed DCX after ischemic stroke (**Figure 5E-F**), which were recorded by two photon confocal microscopy

To determine the role of Notch 1 signaling in histological outcome after focal ischemia, we induced focal ischemia, and then administered Notch1 inhibitor, activator or vehicle into the brains for 3 days. As shown in **Figure 6**, infarct volume was significantly decreased in rats treated with Notch1 activator, compared to vehicle- or Notch 1 inhibitor-treated rats after dMCAO. Although the infarct volume was increased in the ischemic rats after treatment of Notch 1 inhibitor, no significant difference was reached.

We also measured neurological deficits in ischemic rats after Notch1 inhibitor, activator or vehicle treatment. We found that motor deficits in the ladder running walking tests (**Figure 7A**), EBST (**Figure 7B**), cylinder test (**Figure 7C**) and limp placing test (**Figure 7D**) were improved in the Notch1 activator-treated rats tested at 3 days after dMCAO, compared to vehicle- or Notch1 inhibitor-treated rats, suggesting involvement of Notch 1 signaling in functional outcome in aged rats after focal ischemia.

DISCUSSION

In the present study, we found that the expression of Notch1 and Notch1 ligand (Jagged1) in the SVZ decreased with aging, in parallel with reduced activation of Notch1 signaling. In addition, we also found that Notch1 and NICD were predominantly expressed in neural precursor cells, but Jagged1 mainly in astrocytes. Enhancing activation of Notch1 signaling promoted neurogenesis, while inhibiting Notch1 signaling decreased neurogenesis, of the SVZ in aged brain. Moreover, ischemia-induced cell proliferation in the SVZ of aged brain was also increased by activating the Notch1 pathway and reduced by inhibiting the Notch1 signaling. Moreover, we also found that the Notch1 activator administration reduced infarct volumes and improved neurological deficits in aged rats after focal cerebral ischemia. Our data suggest that Notch1 signaling plays a critical role in diminished SVZ neurogenesis with aging and modulates the SVZ neurogenesis as well as functional outcome after focal ischemia.

The Notch1 signaling pathway is regarded as one of the main regulators of neural development, of which signaling components have been found to express in neural germinal zone of both embryonic and adult brains (Stump *et al.* 2002; Irvin *et al.* 2004; Givogri *et al.* 2006; Wang *et al.* 2009b). Activation of Notch1 signaling contributes to the maintenance and proliferation of NSCs (Chojnacki *et al.* 2003b; Givogri *et al.* 2006; Ehm *et al.* 2010; Gaiano 2011). Studies have shown that Notch1 expression decreases significantly with aging in the neural germinal zone, including SVZ and SGZ (Givogri *et al.* 2006; Crews *et al.* 2008), which is consistent with our data. Notch1 is a transmembrane protein that is activated by binding the ligands Delta1 or Jagged1. Studies report that the cells expressing the ligands typically need to be adjacent to the Notch1 expressing cell for signaling to occur. Consistent with our previous findings (Wang *et al.* 2009b) and other studies (Givogri *et al.* 2006; Ables *et al.* 2011) in young-adult brain, we also found that Notch1 and NICD were expressed in DCX-positive cells (Type A cells); while Jagged1 was found in neighboring cells of Notch1 expressing cells such as GFAP-positive cells (Type B cells) in the SVZ of aged brain. Such a unique expression pattern may form a certain microenvironments or niches that instruct the fate of NSCs in the SVZ.

With age, the capacity for neurogenesis in adult brain diminishes. We found that the forced Notch1 signaling activation restores the reduced proliferation rate of NSCs in aged brain, which is evidenced by increase in the number of BrdU-positive cells in the SVZ. Conversely, after inhibiting Notch1 signaling, the proliferation level of NSCs in the SVZ of the aged brain is significantly reduced or abolished. In our previous study (Wang *et al.* 2009b), we applied intracerebroventricular administration of Notch1 extracellular domain antibody to activate Notch1 signaling (Conboy *et al.* 2003; Wang *et al.* 2009b) and of a soluble Jagged1-Fc complex protein to inhibit Notch1 signaling (Conboy *et al.* 2003; Wang *et al.* 2009b) and found that manipulating Notch1 signaling could alter the neurogenesis levels in the SVZ of normal young-adult brain. In addition, studies also show that NICD overexpression rescues proliferation and self-renewal of NSCs in the SVZ (Aguirre *et al.* 2010) and inducible knockout Notch1 fails to self-renew and expand of NSCs (Ables *et al.* 2010). These data suggest that Notch1 signaling plays a critical role in the proliferation of NSCs in the SVZ of young adult and aged brain.

Although the basal rate of neurogenesis declines with age (Kuhn *et al.* 1996; Kempermann *et al.* 1998; Cameron & McKay 1999), declined hippocampal neurogenesis in aged mice living in an enriched environment was fivefold higher than in controls, along with significant improvements of learning parameters, exploratory behavior and locomotor activity. Our recent study also showed that FGF-2 and HB-EGF restore neurogenesis in aged brain, and that stroke-induced neurogenesis is present, but reduced in aged brain, suggesting that the aged brain retains the ability to react to functional challenges with a neurogenic response (Kempermann G *et al.* 2002). We and others show that the Notch1 signaling responds to ischemic stroke in young adult brain. After focal ischemia in aged rat, we infused the Notch1 signaling inhibitor into lateral ventricular for 3 days and found that ischemia-induced neurogenesis is significantly reduced after inhibiting Notch1 signaling (Wang *et al.* 2009b). Consistently, transient administration of Notch ligand, Delta-like protein 4 (DLL4) to activate Notch1 increased the numbers of newly generated precursor cells in the SVZ and improves motor skills after ischemic injury (Androutsellis-Theotokis *et al.* 2006). Wang (Wang *et al.* 2009a) *et al* found that expression of Notch, NICD and Hes1 mRNA in neural progenitor cells are significantly increased *in vitro*. siRNA against Notch or a γ -secretase inhibitor significantly reduced Notch, NICD and Hes1 expression and cell proliferation after experimental stroke. As stroke generally occurs in aged populations (Jin 2010), experimental studies employing aged animals may be more clinically relevant than that in young-adult animals. Our findings in aged rat brain are in agreement with and extend these previous studies in young-adult animals. Study shows that hypoxia enhances Notch1 signaling. Hypoxia inducible factor (HIF)-1 binds to NICD and stabilizes it (Gustafsson *et al.* 2005). NICD carries HIF-1 to Notch1-responsive promoters, and the complex enhances transcription, which further support the notion that Notch1 signaling may play a role in stroke-induced neurogenesis. However, a recent study shows that blocking Notch1 signaling by γ -secretase inhibitors exerts a neuroprotective and anti-inflammatory effect in focal cerebral ischemia, suggesting that Notch1 signaling may contribute to ischemic damage (Arumugam *et al.* 2006). In contrast, our study showed that administration of Notch 1 inhibitor did not significantly cause larger infarct volume, which may be due to the fact that the maximized infarct sizes have been reached. However, we found that administration of Notch1 activator not only reduced infarct volumes, but also improved neurological deficits in aged rats after focal cerebral ischemia. Consistently, a more delayed activation of Notch1 signaling through infusion of the Notch1 ligand Dll4 into the lateral ventricle of adult rats had no effect on the infarct size but improved motor skills over a period of 45 days (Androutsellis-Theotokis *et al.* 2006). In addition, a recent study also shows that *Notch 3*^{-/-} mice developed ischemic lesions approximately twice as large as those seen in WT or heterozygous (*Notch 3*^{+/-}) male mice (Arboleda-Velasquez *et al.* 2008). Very recent studies show that activation of Notch signaling is involved in the ischemic tolerance (Yang *et al.* 2012; Zhao *et al.* 2012). The reason for the discrepancy between these studies is unknown, but a possible explanation for these apparently contradicting results may be due to different animal strains and animal models of stroke, and the different Notch 1 signaling inhibitor used in these studies, as γ -secretase inhibitors may not only involve in Notch1 signaling, but also other signaling transduction pathways, which may play a role in neuroprotection in the ischemic brain.

Notch signaling integrates with a number of other pathways, thereby tuning downstream expression to regulate the maintenance of NSCs in both the embryonic brain (Takizawa *et al.* 2003; Shimizu *et al.* 2008) and adult brain (Andreu-Agullo *et al.* 2009). It is likely that Notch signaling controls NSC maintenance, proliferation and survival in the adult brain in cooperation with other signals. One study suggested that modulation of the PI3 kinase pathway and of the Wnt/ β -catenin pathway potentiate Notch-induced promoting effect on adult NSCs (Ehm *et al.* 2010), indicating that the fine-tuning neurogenesis role of Notch1 signaling occurs in cooperation with cross-talking pathways. However, much of this is

speculations and future studies will have to make a clearer understanding of the Notch signaling network. Notch may be a dominant regulator of cell fate, but it does not work alone.

Taken together, our findings in the present study indicate the beneficial effect of Notch1 signaling on neurogenesis in the aged brain under both physiological and ischemic condition. Understanding how Notch1 signaling can modulate neurogenesis has broad implications for the development of clinical treatments aimed at combating injury and neurodegeneration in aged brain.

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Figure 1. Expression of Notch1 in the SVZ of young-adult and aged rat brain

(A) Rats were trained for 3 days prior to dMCAO and motor deficits were assessed 72 hr after ischemia. Notch1 inhibitor, activator or vehicle was injected into the left lateral ventricle immediately after dMCAO for 3 consecutive days. BrdU was injected twice per day for 3 days. Rats were euthanized after behavioral tests for immunocytochemistry and histology analysis. (B) The SVZ was dissected from normal young-adult (Young rats) and aged rat brains and Western blots were performed using anti-Notch1 antibody (top). The blot was reprobed using anti-actin antibody to control for protein loading (bottom). (C) The optical density of the respective band in panel B, which were normalized according the corresponding actin band. Data are means $\pm$ SEM; N = 3 to 4 per group; * p < 0.05, analyzed by Student *t*-test. (D) Notch1 immunoreactivity (brown) in the SVZ of young-adult (top) and aged (bottom) rat brain. Inset: high magnification. (E) Confocal image showed that Notch1 (green) was mainly expressed in DCX-positive cells (red), but not GFAP-positive cells (purple), in the SVZ of aged brain. DAPI (blue) was used to counterstain nuclei. (F) Maximum z-projections (main panels) and selected orthogonal yz (right) and xz (above) sections (with the plane of yz and xz sections indicated by the vertical red and horizontal green lines, respectively) of confocal image stacks. SVZ cells were stained with anti-Notch1 (green), anti-DCX (red) and anti-GFAP antibodies (purple), DAPI (blue) was used to counterstain nuclei.

Figure 2. Expression of Jagged1 in the SVZ of young-adult and aged rat brain

(A) The SVZ was dissected from normal young-adult (Young rats) and aged rat brains and Western blots were performed using anti-Jagged1 antibody (top). The blot was reprobed using anti-actin antibody to control for protein loading (bottom). (B) The optical density of the respective band in panel A, which were normalized according the corresponding actin band. Data are means $\pm$ SEM; N = 3 to 4 per group; * $p < 0.05$, analyzed by Student *t*-test. (C) Jagged1 immunoreactivity (brown) in the SVZ of young-adult (top) and aged (bottom) rat brain. Insets: higher magnification. (D) Triple-label immunofluorescence staining recorded by a two-photon confocal microscopy showed that Jagged1 (green) was mainly expressed in GFAP-positive cells (purple), but not DCX-positive cells (red). DAPI (blue) was used to counterstain nuclei. (E) Confocal image stacks show that SVZ cells were stained with anti-Jagged1 (green), anti-DCX (red) and anti-GFAP antibodies (purple), DAPI (blue) was used to counterstain nuclei.

Figure 3. Expression of NICD in the SVZ of young-adult and aged rat brain

(A) The SVZ were dissected from normal young-adult (Yang rats) and aged rat brains and Western blots were performed using anti-NICD antibody. The blot was reprobed using anti-actin antibody to control for protein loading (bottom). (B) The optical density of the respective band in panel A, which were normalized according the corresponding actin band. Data are means $\pm$ SEM; N = 3 to 4 per group; * $p < 0.05$, analyzed by Student *t*-test. (C) NICD immunoreactivity (brown) in the SVZ of young-adult (top) and aged (bottom) rat brain. Insets: high magnification. (D) Triple-label immunofluorescence staining recorded by a two photon confocal microscopy shows NICD (green) was largely expressed in DCX- (red), but not GFAP-positive cells (purple). DAPI (blue) was used to counterstain nuclei. (E) Confocal image stacks confirm that NICD (green) was expressed in DCX- (red), but not GFAP-positive cells. DAPI (blue) was used to counterstain nuclei.

Figure 4. Modulation of Notch signaling significantly alters cell proliferation in the SVZ of young-adult and aged rat brain

(A) Quantification of BrdU-positive cells in the SVZ of aged brain after administration of Notch1 activator, inhibitor or vehicle. (B) Quantification of DCX-positive cells in the SVZ of aged brain after administration of Notch1 activator, inhibitor or vehicle. (C) Quantification of BrdU/DCX-positive cells in the SVZ of aged brain after administration of Notch1 activator, inhibitor or vehicle. (D) Representative confocal images of BrdU/DCX-immunoreactive cells in the SVZ of aged brain after administration of Notch1 activator. (E) Confocal images through the series of z-stacks confirmed that BrdU-positive cells (red) expressed DCX (green). Data are means $\pm$ SEM; N = 3 to 4 per group.

Figure 5. Modulation of Notch signaling pathway significantly alters cell proliferation in aged rat brain SVZ after dMCAO

(A) Representative images of BrdU-positive cells (red) in the SVZ of aged brain after focal ischemia followed administration of Notch1 activator, inhibitor or vehicle. (B) Quantification of BrdU-positive cells in the SVZ of aged brain after focal ischemia followed administration of Notch1 activator, inhibitor or vehicle. (C) Representative images of DCX-positive cells (green) in the SVZ of aged brain after focal ischemia followed administration of Notch1 activator, inhibitor or vehicle. (D) Quantification of DCX-positive cells in the SVZ of aged brain after focal ischemia followed administration of Notch1 activator, inhibitor or vehicle. (E) Representative images of BrdU/DCX-positive cells (red/green) in the SVZ of aged brain after focal ischemia followed administration of Notch1 activator. Left panel: a confocal 3D image of BrdU/DCX-positive cells in the SVZ; right panel: confocal image z-stacks of BrdU/DCX-positive cells in the SVZ. (F) Quantification of BrdU/DCX-positive cells in the SVZ of aged brain after focal ischemia followed administration of Notch1 activator, inhibitor or vehicle (control). Data are means $\pm$ SEM; N = 3 to 4 per group.

Figure 6. Infarct volume in aged ischemic rats after administration of Notch1 inhibitor or activator

(A) Rats were treated with vehicle, Notch1 inhibitor or activator for 3 consecutive days after dMCAO. The brain sections were stained with crystal violet. (B) Infarct volume was expressed as percentage of hemispheric volume. The significant difference was found between the vehicle- and Notch1 activator-treated dMCAO groups by ANOVA analysis, followed by Newman-Keuls *post hoc* multiple comparison tests.

Figure 7. Neurobehavioral outcome in ischemic aged rats after administration of Notch1 inhibitor or activator

Neurobehavioral function was assessed by the ladder running walking test (forelimb slip steps: left panel; hindlimb slip steps: right panel) (A), EBST (B), cylinder test (C) and limb placing test (D) at 3 days after dMCAO, followed administration of vehicle, Notch1 inhibitor or activator. The significant differences were found between the vehicle- and Notch1 activator-treated dMCAO groups and between the Notch inhibitor- and Notch1 activator-treated dMCAO groups by ANOVA analysis, followed by Newman-Keuls *post hoc* multiple comparison tests.