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The Notch pathway mediates expansion of a progenitor pool and neuronal differentiation in adult neural progenitor cells after stroke

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Abstract

Molecular mechanisms by which stroke increases neurogenesis have not been fully investigated. Using neural progenitor cells isolated from the subventricular zone (SVZ) of the adult rat subjected to focal cerebral ischemia, we investigated the Notch pathway in regulating proliferation and differentiation of adult neural progenitor cells after stroke. During proliferation of neural progenitor cells, ischemic neural progenitor cells exhibited substantially increased levels of Notch, Notch intracellular domain (NICD), and Hes1, which was associated with a significant increase of proliferating cells. Blockage of the Notch pathway by siRNA against Notch or a γ secretase inhibitor significantly reduced Notch, NICD and Hes1 expression and cell proliferation induced by stroke. During differentiation of neural progenitor cells, Notch and Hes1 expression was downregulated in ischemic neural progenitor cells, which was coincident with a significant increase of neuronal population. Inhibition of the Notch pathway with a γ secretase inhibitor further substantially increased neurons, but did not alter astrocyte population in ischemic neural progenitor cells. These data suggest that the Notch signaling pathway mediates adult SVZ neural progenitor cell proliferation and differentiation after stroke.

Keywords

Notch pathway; neural progenitor cell; proliferation; differentiation; stroke; rat

Introduction

The Notch receptors are transmembrane proteins activated by Delta and Jagged ligands (Jones et al., 1998; Morrison et al., 2000; Gaiano and Fishell, 2002; Hitoshi S, 2002; Guentchev and McKay, 2006; Nagao et al., 2007). On activation, Notch internal cellular domain (NICD) is cleaved by presenilin-1 and the γ -secretase enzyme complex and translocates into the nucleus (Redmond et al., 2000). Within the nucleus, the NICD forms a complex with C-promoter binding factor 1 (CBF1), which activates transcription factors of hairy and enhancer of split (Hes) family (Iso et al., 2003). The Notch signaling pathway plays a pivotal role in maintaining embryonic neural stem cell pool and promotes gliogenesis (Gaiano and Fishell, 2002; Yoon and Gaiano, 2005). Notch signals in collaboration with ciliary neurotrophic factor lead neural

stem cells to generate astrocytes (Nagao et al., 2007). Knockdown of CBF1 promotes conversion of neural stem cells to intermediate progenitor cells that primarily generate neurons (Mizutani et al., 2007). Notch transcripts are expressed in the subventricular zone (SVZ) of adult brain where neural progenitor cells reside (Givogri et al., 2006; Mizutani et al., 2007).

Stroke increases neurogenesis in the SVZ and these newly generated neurons migrate towards the ischemic boundary to replenish damaged neurons (Zhang et al., 2001; Arvidsson et al., 2002; Parent et al., 2002; Jin et al., 2003; Zhang et al., 2004). Cerebral ischemia upregulates Notch 1 and downregulates Hes 5 expression in the SVZ and the dentate gyrus, where neurogenesis occurs (Kawai et al., 2005; Felling et al., 2006). Intraventricular infusion of a Notch ligand, Delta-like 4 (Dll4) along with fibroblast growth factor-2 reduce neural stem cell death resulting in increase of neurogenesis after stroke (Androutsellis-Theotokis et al., 2006). In the present study, using a neurosphere assay that has been extensively employed for investigating the biology of neural progenitor cells (Reynolds and Weiss, 1992; Morshead et al., 1994; Wang et al., 2004; Wang et al., 2005), we investigated the endogenous Notch signaling pathway in mediating proliferation and differentiation of neural progenitor cells derived from the SVZ of adult rats subjected to focal cerebral ischemia.

Experimental procedures

Middle cerebral artery occlusion (MCAo) model

Male Wistar rats (3–6 month old, Charles River, Portage, MI) were employed in this study. The right middle cerebral artery (MCA) was occluded by placement of an embolus at the origin of the MCA, as previously described (Zhang et al., 1997). Briefly, a 16 mm length of catheter containing a single fibrin rich clot was gently advanced within the lumen of the internal carotid artery (ICA). When the catheter reached to the origin of the MCA and the clot was injected into the ICA along with 2–3 μ l of 0.9% saline. In this model, occlusion of the MCA evokes a peak increase of neurogenesis in the SVZ 7 days after stroke (Zhang et al., 2004). Therefore, for in vitro study, rats were sacrificed 7 days after MCAo (n=6). To evaluate the time course of NICD expression, rats were sacrificed 0, 1, 2, 7 and 14 days after stroke (n=3/per time point).

Neurosphere culture

SVZ neural progenitor cells were dissociated from normal (n= 6) and stroke rats (n=6), as previously reported (Reynolds and Weiss, 1992; Morshead et al., 1994; Chiasson BJ, 1999; Wang et al., 2004). The cells were plated at a density of 1×10^4 cells per milliliter in growth medium. Growth medium contains DMEM-F-12 medium (Invitrogen Corporation, Carlsbad, California), 20 ng/ml of epidermal growth factor (EGF, R&D System, Minneapolis, MN) and 20ng/ml basic fibroblast growth factor (bFGF, R&D System, Minneapolis, MN). DMEM-F-12 medium contains L-glutamine (2mM), glucose (0.6%), putrescine (9.6 μ g/ml), insulin (0.025mg/ml), progesterone (6.3ng/ml), apo-transferrin (0.1mg/ml), and sodium selenite (5.2ng/ml). The generated neurospheres (primary sphere) were passaged by mechanical dissociation and reseeded as single cells at a density of 10 cells per microliter in bFGF and EGF-containing media (passage 1 cells). Passage 1–2 cells were processed for various experiments in the present study.

To examine whether SVZ cells have characteristics of neural progenitor cells, immunostaining was performed on the neurospheres with antibodies against nestin and SOX2. Three dimensional images of the neurospheres were acquired using Zeiss confocal microscopy (Zeiss LSM 510 NLO).

To analyze the formation of neurospheres, single cell at a density of 10 cell per microliter were incubated in each well of a 24-well plate (Corning) in growth medium. The number and size of neurospheres were counted at 7 days in vitro (DIV).

To analyze cell proliferation, bromodeoxyuridine (BrdU, 10 μ g/ml), the thymidine analog that is incorporated into the DNA of dividing cells during S-phase, was added into the growth medium 18 hrs prior to termination of incubation. BrdU positive cells were measured.

To analyze phenotypes of neural progenitor cells, neurospheres were mechanically dissociated as single cells. These cells (2.5×10^4 cells/cm²) were plated directly onto lamin-coated glass coverslips in DMEM-F-12 medium containing 2% FBS but without the growth factors for 7 days. This medium was referred as the differentiation medium. Immunocytochemistry was performed with various antibodies to determine phenotypes of neural progenitor cells.

To examine the effect of blockage of the Notch pathway on neural progenitor cell proliferation and differentiation, a γ -secretase inhibitor N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-S-phenylglycine t-Butyl Ester (DAPT; 10 μ M; Calbiochem, EMD Biosciences, San Diego, CA) or an equal volume of DMSO (vehicle) was used. This dose of DAPT has been demonstrated to block the Notch signaling pathway (Hoe and Rebeck, 2005; Olivier et al., 2006; Chigurupati et al., 2007; Fuentealba et al., 2007).

Immunocytochemistry and quantification

Single and double immunofluorescent staining of cultured cells were performed, as previously described (Zhang et al., 2003). The following primary antibodies were used in the present study: mouse anti-BrdU (a marker of proliferation, 1:100, Boehringer Mannheim, Indianapolis, IN), NICD (1:3000, Abcam, Cambridge, MA), mouse anti- β -tubulin III (TuJ-1, a marker of neurons, 1:500, Covance the Development Services Company, MI), rabbit anti-glial fibrillary acidic protein (GFAP, a marker of astrocytes, 1:500, Dako Cytomation California Inc. Carpinteria, CA), rabbit anti-nestin (a marker of neural progenitor cells, 1:100, BD Biosciences, San Jose, CA) and rabbit anti-SOX2 (a marker of neural progenitor cells, 1:50, Santa Cruz Biotechnology, Inc. CA). Cultured cells were fixed in 4% paraformaldehyde for 15–20 min at room temperature. Nonspecific binding sites were blocked with 1% Albumin from bovine serum (BSA) for 60 min at room temperature. The cells were then incubated with the primary antibodies listed above and with CY3-conjugated or FITC-conjugated secondary antibodies. Nuclei were counterstained with 4', 6'-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA). The number of BrdU, NICD, TuJ1, GFAP, nestin and SOX2 positive cells and total DAPI cell number were counted in randomly selected 5 microscopic fields under 20 $\times$ objective and the percentage of immunoreactive cells within the total number of DAPI positive cells were quantified (Wang et al., 2005; Wang et al., 2006).

TUNEL assay was performed to detect apoptotic cells by means of the ApopTagR Peroxidase In Situ Apoptosis Detection Kit (Millipore, Billerica, Massachusetts) following the manufacture's instructions (n=3).

siRNA synthesis and transfection

Notch1 short interfering (si) RNA and scrambled siRNA were purchased from Santa Cruz Biotech. Neural progenitor cells were transfected using the NSC Nucleofector™ Kit (Amax Inc.) following the manufacturer's instructions (n=3). The total amount of siRNA per transfection was kept constant to 0.8 μ g/ml. mRNA levels were measured 48h after transfection (Chen et al., 2008).

Real time-PCR

Total RNA was isolated from neural progenitor cell cultures using the Stratagene Absolutely RNA MicroRNA isolation kit (Stratagene, La Jolla, CA, n=3). Quantitative RT-PCR was performed on an ABI 7000 PCR instrument (Applied Biosystems, Foster City, CA) using 3-stage program parameters provided by the manufacturer, as follows; 2 min at 50°C, 10 min at 95°C, and then 40 cycles of 15 s at 95°C and 1 min at 60°C. Specificity of the produced amplification product was confirmed by examination of dissociation reaction plots. A distinct single peak indicated that a single DNA sequence was amplified during PCR. Each sample was tested in triplicate and samples obtained from three independent experiments were used for analysis of relative gene expression using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The following SYBR Green primers for real-time PCR were designed using Primer Express software (ABI): Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Fwd: ACA ACA TCA TCC CTG CAT CC, Rev: CAC ATT GGG GGT AGG AAC AC); Notch1: (Fwd: TCC TCC TGA GAG TTG TCC TAGC, Rev: GTG GTC TAA GTG ACC ATC AGCA); Hes1: (Fwd: ACA CCG GAC AAA CCA AAG AC, Rev: ATG CCG GGA GCT ATC TTT CT).

Western blot assay

Western blots were performed according to our published methods (Wang et al., 2006). Briefly, lysates from neural progenitor cells and tissue were sonicated for 10 second and centrifuged at 14000g for 20 min (n=3). Protein concentration in the supernatants of cell extract was determined using a BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL). Equal amounts of proteins were loaded on 10% SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred to nitrocellulose membranes and the blots were subsequently probed with the following antibodies: NICD (1:500, Abcam). For detection, horseradish peroxidase (HRP) conjugated secondary antibodies were used (1:2000) followed by enhanced chemiluminescence (ECL) development (Pierce Biotechnology, Inc). Normalization of results was ensured by running parallel Western blots with β -actin antibody. The optical density was quantified using an image processing and analysis program (Scion Image, Ederick, MA).

Statistical Analysis

One-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test was performed. The data were presented as means $\pm$ SE. A value of $p < 0.05$ was taken as significant.

Results

Stroke enhances neural progenitor cell proliferation and promotes neuronal differentiation

To examine whether stroke changes proliferation and differentiation of neural progenitor cells, we employed a neurosphere assay. We first examined proliferation and differentiation in SVZ cells harvested from normal adult rat. When passage 1 cells were plated at a density of 10 cells/ μ l on the non-adhesive culture surface in the growth medium containing bFGF and EGF, these cells formed spheres after 7 days in culture (Fig. 1). The majority of cells in neurospheres were nestin ($99 \pm 3\%$, Fig. 1A and B) and Sox 2 ($75 \pm 3\%$, Fig. 1C and D) immunoreactive, markers of neural progenitor cells. Double immunostaining revealed that $51 \pm 2\%$ of cells in neurospheres were BrdU reactive, an index of proliferating cells, and nestin positive, suggesting that most of cells are proliferating. After passage, these cells were capable of forming secondary neurospheres. When single cells dissociated from neurospheres were reseeded on laminin-coated glass cover-slips (2.5×10^4 cells/ml) in medium without the growth factors for 7 days, these cells differentiated into TuJ1 reactive cells (Fig. 1E), a marker of immature neurons, GFAP positive cells, a marker of astrocytes (Fig. 1F) and O4 positive cells, a marker of oligodendrocytes (Fig. 1G). These data indicate that adult SVZ cells retain

characteristics of neural progenitor cells, self-renew and are multipotent as demonstrated in embryonic neural stem cells (Reynolds and Weiss, 1992; Gritti et al., 1996).

Using this assay, we then examined whether stroke affects neural progenitor cell proliferation and differentiation. SVZ cells were harvested from adult rats subjected to 7 days of stroke or from normal rats. When these cells were cultured in the growth medium for 7 days, significant increases of the number and size of neurospheres were detected in ischemic SVZ cells compared with normal SVZ cells (Fig. 2B, 2J and 2K). Stroke also significantly ($p<0.05$) increased the percentage of BrdU positive cells (Fig 2E and 2L), suggesting that stroke increases neural progenitor cell proliferation.

We next examined the effect of stroke on neural progenitor cell differentiation. Single cells dissociated from a neurosphere were reseeded on laminin-coated glass cover-slips for 7 days in differentiation medium. Phenotypes of these cells were measured by immunostaining. The percentage of TuJ1 (Fig. 2H and 2M) positive cells was significantly higher in ischemic neural progenitor cells than the number in normal neural progenitor cells (Fig. 2G and 2M). In contrast, the GFAP positive cell population was not significantly different between the two groups ($36 \pm 3.3\%$ vs. $29 \pm 3.2\%$ in control, $n=6$). Collectively, these results indicate that stroke augments neural progenitor cell proliferation and enhances neural progenitor cell differentiation into neurons.

The Notch pathway mediates the effect of stroke on neural progenitor cell proliferation and differentiation

The Notch pathway regulates embryonic neural stem cell maintenance and lineage acquisition (Hitoshi S, 2002; Guentchev and McKay, 2006; Nagao et al., 2007). Notch signals are expressed in neural stem and progenitor cells in the SVZ of the adult rodent (Mizutani et al., 2007). To examine whether the Notch pathway is involved in stroke-induced neurogenesis, levels of Notch, NICD, and Hes1 were measured. Real-time RT-PCR analysis of neural progenitor cells cultured in the growth medium showed that stroke substantially increased Notch (Fig. 3A) and Hes1 (Fig. 3B) mRNA levels compared with the levels in normal neural progenitor cells. Double immunofluorescent staining showed that NICD immunoreactivity was localized to nuclei of nestin positive cells (Fig. 3C to 3F), and ischemic neural progenitor cells exhibited more NICD positive nuclei (Fig. 3G and 3I) than normal neural progenitor cells ($p<0.05$, Fig. 3H and 3I). Western blot analysis of SVZ tissue harvested from rats subjected to 1 to 14 days of MCAo revealed that stroke significantly increased NICD levels 2 and 7 days after MCAo (Fig. 3J). In addition, Western blot analysis of the cultured SVZ cells showed a significant increase of NICD protein in ischemic neural progenitor cells (Fig. 3K). These data suggest that stroke activates the Notch pathway.

To examine the effect of the Notch pathway on stroke-increased neural progenitor cell proliferation, Notch was blocked with a siRNA approach. Ischemic neural progenitor cells transfected by siRNA against Notch and scrambled siRNA were cultured in the growth medium for 48h. Real-time RT-PCR analysis showed that siRNA against Notch but not scrambled siRNA significantly reduced of Notch and Hes1 mRNA levels in these cells (Fig. 3A and 3B), suggesting that siRNA against Notch attenuates endogenous Notch in ischemic neural progenitor cells. Furthermore, addition of a γ -secretase inhibitor (DAPT, 10 μ M) into the growth medium significantly decreased NICD and Hes1 levels in neural progenitor cells measured by real-time RT-PCR (Fig. 3B) and Western blots (Fig. 3K), respectively, suggesting that DAPT blocks the Notch signaling pathway.

Blockage of the Notch pathway with DAPT also significantly ($p<0.05$) reduced the number of neurospheres (Fig. 2C and 2J), and the diameter of spheres (Fig. 2C and 2K) and the percentage of BrdU positive cells (Fig. 2F and 2L). In addition, attenuation of endogenous Notch with

siRNA resulted in a significant reduction of the percentage of BrdU positive cells compared with the percentage in non-transfected ischemic neural progenitor cells (Fig. 2L). These data indicate that blockage of the Notch pathway in ischemic neural progenitor cells reduces progenitor cell proliferation.

We then examined the effect of the Notch pathway on neural progenitor cell differentiation. Real-time RT-PCR analysis showed that ischemic neural progenitor cell cultured in the differentiation medium exhibited a significant ($p<0.05$) decrease in mRNA levels of Notch (1.9 ± 0.2) and Hes1 (1.4 ± 0.1) compared with the levels in the growth medium (3.1 ± 0.2 for Notch and 1.9 ± 0.1 for Hes1). Downregulation of Notch and Hes1 expression in ischemic neural progenitor cells was associated with a significant increase of the percentage of TuJ1 (Fig. 2H and 2M). When ischemic neural progenitor cells were cultured in the differentiation medium in the presence of DAPT, the percentage of TuJ1 positive cells were further ($p<0.01$) increased compared with that in neural progenitor cells without DAPT (Fig. 2I and M). In addition, blockage of the Notch pathway with DAPT in normal neural progenitor cells cultured in the differentiation medium augmented the percentage of TuJ1 positive cell ($16.2\pm 1.1\%$ vs $10.5\pm 1.4\%$ in control, $p<0.05$). DAPT did not significantly alter the percentage of GFAP positive cell population in ischemic ($36\pm 3.3\%$ vs. $32\pm 2.4\%$ in DAPT group, $p<0.05$, $n=6$) and normal neural progenitor cells ($29\pm 3.2\%$ vs. $27\pm 1.8\%$ in DAPT group, $p<0.05$, $n=6$). The increase of TuJ1 positive cells could reflect a decrease of cell death. To address the possibility that DAPT may act as a survival factor for neural progenitor cells, we measured TUNEL positive cells and found that the percentage of TUNEL positive cells in the DAPT treated ischemic neural progenitor cells ($0.5\pm 0.01\%$, $n=3$) were not significantly different from the percentage in the ischemic neural progenitor cells ($0.6\pm 0.01\%$, $n=3$), suggesting that blockage of the Notch pathway with DAPT does not cause cell death. Together, these results suggest that inactivation of the Notch signal promotes neuronal differentiation in normal and ischemic adult neural progenitor cells.

Discussion

The present study demonstrates that stroke transiently activates the Notch pathway in SVZ neural progenitor cells of the adult rat. In the proliferation medium, activated Notch signals coincided with substantial increases of neural progenitor cell proliferation. Blockage of the Notch pathway with either siRNA against Notch or the γ secretase inhibitor significantly suppressed ischemia-induced progenitor cell proliferation. Moreover, in the differentiation medium, inactivation of the Notch pathway with the γ secretase inhibitor significantly augmented neuronal population in normal and ischemic neural progenitor cells. These data suggest that the Notch signaling pathway mediates expansion of a neural progenitor pool and neuronal differentiation in adult neural progenitor cells, which may contribute to ischemia-induced neurogenesis.

The Notch signaling pathway regulates neural stem cell proliferation and promotes gliogenesis (Morrison et al., 2000; Nagao et al., 2007). Our in vivo data show that stroke significantly increased NICD expression in SVZ cells as early as 48 h after stroke. Moreover in vitro, neural progenitor cells isolated from the SVZ of the rat subjected to 7 days of stroke exhibited substantial upregulation of Notch and Hes1 genes and increased NICD protein levels localized to nuclei of neural progenitor cells, when the progenitor cells were cultured in the growth medium. On activation, NICD is cleaved by γ -secretase and translocates into the nucleus (Redmond et al., 2000). Within the nucleus, the NICD forms a complex with CBF1, which activates transcription factors of Hes family (Yoon and Gaiano, 2005). Thus, the present study suggests that stroke activates the Notch signaling pathway in adult progenitor cells.

To study the biological effect of endogenous Notch signals on ischemic neural progenitor cells, we employed an in vitro neurosphere assay which has been used for investigating proliferation and differentiation of neural progenitor cells (Reynolds and Weiss, 1992; Morshead et al., 1994; Wang et al., 2004; Wang et al., 2005). Our in vitro data show that during proliferation, non-ischemic neural progenitor cells expressed Notch signals, which is consistent with published studies in neural stem cells derived from the embryonic rodent (Hitoshi S, 2002; Alexson TO, 2006; Androutsellis-Theotokis et al., 2006; Guentchev and McKay, 2006; Mizutani et al., 2007; Nagao et al., 2007). However, activation of Notch signals in ischemic neural progenitor cells was enhanced, which was associated with increases of the progenitor cell proliferation. Blockage of the Notch pathway by attenuation of endogenous Notch with siRNA against Notch or inhibition of γ -secretase with the DAPT suppressed ischemia-increased self-renewal of the progenitor cells. The specificity of DAPT to block the Notch pathway was confirmed by demonstrating that DAPT significantly reduced NICD protein levels and Hes1 expression in neural progenitor cells. These data suggest that the Notch pathway activated by stroke increases expansion of the neural progenitor cell pool. Activation of the Notch pathway by administration of a Notch ligand, Dll4, results in enhancement of neurogenesis in the ischemic brain, which could be mediated by reduction of neural stem cell death (Androutsellis-Theotokis et al., 2006; Oya et al., 2008). The present study suggests that in addition to cell survival, neural progenitor cell proliferation amplified by activation of the Notch pathway may also contribute to neurogenesis in the ischemic brain. Our data are consistent with studies in perinatal hypoxia/ischemia which demonstrate that the Notch pathway activated by leukemia inhibitory factor (LIF) substantially increases SVZ neural progenitor cell proliferation (Covey and Levison, 2007). In addition, hypoxia-inducible factor 1 α (HIF1-1 α) interacts with NICD to maintain neural stem cell in undifferentiated state (Gustafsson et al., 2005). We previously demonstrated elevation of HIF1-1 α mRNA levels in ischemic neural progenitor cell even under normoxic culture conditions (Liu et al., 2007). Taken together, we speculate that the Notch pathway activated by LIF and other factors interacts with HIF1-1 α to promote neural progenitor cell proliferation under ischemic conditions.

In contrast to the progenitor cells in the growth medium, expression of Notch and Hes 1 was substantially downregulated in ischemic neural progenitor cells, when the cells were cultured in differentiation medium. The downregulation of Notch and Hes1 was associated with a significance increase of neurons in ischemic neural progenitor cells. Moreover, blockage of the Notch signaling pathway with the γ -secretase inhibitor further augmented neuronal population, but did not affect cell death measured by a TUNEL assay, suggesting that the increased neuronal population is not resulted from enhancement of cell survival. Hes1 is an essential Notch effector and a negative regulator of neuronal differentiation (Ohtsuka T, 1999). Downregulation of Hes1 in progenitor cells cultured in the differentiation medium could contribute to an increase of neuronal population. However, other Hes family genes, such as Hes 5, could also regulate neuronal differentiation and the Notch signaling pathway regulates biological function of neural progenitor cell independent of Hes family genes (Ohtsuka T, 1999; Kawai et al., 2005; Curry et al., 2006; Felling et al., 2006; Scholzke and Schwaninger, 2007).

The Notch pathway promotes gliogenesis in embryonic neural stem cells (Morrison et al., 2000; Nagao et al., 2007). Interestingly, the present study shows that blockage of the Notch signaling pathway with the γ -secretase inhibitor did not alter astrocyte population. Although exact mechanisms for this result cannot be revealed from the present study, there are some possible explanations. The Notch signaling pathway promotes the generation of astrocytes in collaboration with ciliary neurotrophic factor (CNTF) and growth factors (Motoshi Nagao, 2007). Downregulation of these factors in normal or ischemic neural progenitor cells may attenuate the promotion of gliogenesis by Notch. Interestingly, perinatal hypoxia/ischemia

does not upregulate CNTF expression in SVZ neural progenitor cells (Covey and Levison, 2007).

The Notch pathway mediates multiple aspects of neural progenitor cell biology, including cell cycle progression (Hitoshi S, 2002; Guentchev and McKay, 2006; Nagao et al., 2007). Studies on cell cycle parameters in adult neural progenitor cells indicate that stroke triggers early expansion of the neural progenitor pool via shortening the cell cycle length and retaining daughter cells within the cell cycle, and later lengthening the G₁ phase of the cell cycle leads to daughter cells exiting the cell cycle and differentiating into neurons (Zhang et al., 2006). The temporal profile of NICD upregulated by stroke in the present study is comparable to a temporal profile of expansion of SVZ progenitor cells and generation of neuroblasts after stroke (Zhang et al., 2006). Moreover, in vitro data show that the Notch signaling pathway enhances neural progenitor cell proliferation and neuronal differentiation in ischemic neural progenitor cells. Thus, the present study could provide molecular mechanisms underlying ischemia-induced neurogenesis. The present findings may have the potentially therapeutic effect on neurogenesis during stroke recovery by temporally amplifying and blocking the Notch pathway to expanding a neural progenitor pool and to augmenting neuronal differentiation, respectively. However, there are multiple pathways such as the sonic hedgehog and Wnt pathways in regulating neurogenesis in adult neural progenitor cells. Future studies how the Notch signaling pathway activated by stroke interacts with these pathways are under investigation.

In conclusion, the present study suggests that the Notch signaling pathway enhances expansion of the neural progenitor pool and neuronal differentiation in adult neural progenitor cells after stroke.

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Comprehensive list of abbreviations

SVZ	subventricular zone
NICD	Notch intracellular domain
Hes	Hairy Enhancer of Split
CBF1	C-promoter binding factor 1
MCAo	Middle cerebral artery occlusion
EGF	epidermal growth factor
bFGF	basic fibroblast growth factor
BSA	Albumin from bovine serum
TuJ1	

β -tubulin III

GFAP

glial fibrillary acidic protein

SiRNA

short interfering RNA

RT-PCR

reverse transcription polymerase chain reaction

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Figure 1. SVZ cells have characteristics of neural progenitor cells

SVZ cells cultured in the growth medium formed neurospheres (A to D) and these cells exhibited markers of neural progenitor cells, nestin (A and B, red) and Sox2 (C and D, green). SVZ cells cultured in the differentiation medium (E to G) exhibited phenotypes of neurons (E, TuJ1, red), astrocytes (F, GFAP, green), and oligodendrocytes (G, O4, red). Nuclei were stained with DAPI (blue). Bar =20 μ m in panel A and 100 μ m in panel E.

Figure 2.

Stroke increases neural progenitor cell proliferation and neuronal differentiation when neural progenitor cells in the growth and differentiation medium, respectively. Representative images were obtained for the cells in the growth (A to F) and differentiation medium (G to I). Panels A to C show representative neurospheres derived from normal SVZ cells (A), ischemic SVZ cells (B), and ischemic SVZ cells treated with DAPT (C). Panels D to F show representative BrdU immunoreactive SVZ cells in the normal (D), ischemia (E), and ischemia with DAPT groups (F). Panels G to I show TuJ1-immunoreactive cells in the normal (G), ischemia (H) and ischemia with DAPT (I) groups. Panels J to M are quantitative data of neurosphere number (J) and size (K), the percentage of BrdU positive cells (L), and the percentage of TuJ1 positive cells (M). siNotch represents siRNA against Notch. * $P < 0.05$ and # $P < 0.05$ vs the normal and ischemic groups, respectively. Bar = 100 μm . $n = 6/\text{group}$.

Figure 3.

The effect of stroke on Notch, Hes1 and NICD expression in SVZ neural progenitor cells. SVZ neural progenitor cells were cultured in the growth medium. Real-time RT-PCR analysis shows mRNA levels of Notch (A) and Hes1 (B) in normal and ischemic neural progenitor cells as well as ischemic neural progenitor cells treated with DAPT, siRNA-Notch or scramble siRNA. Double immunofluorescent staining shows that nestin immunoreactive cells (C and F, red) were NICD positive (E and F, green) in the nucleus (D and F, blue). Stroke significantly increased the percentage of NICD positive cells (H and I) compared with the normal group (G and I). Western blot analysis shows protein levels of NICD in SVZ tissue isolated from adult rats during 14 days of stroke (J) and in cultured neural progenitor cells harvested from in

normal, ischemia, ischemia with DAPT groups (K). GAPDH and β -actin were used as internal controls for mRNA and proteins, respectively. * $P < 0.05$ and # $P < 0.05$ vs the normal and ischemic groups, respectively. Bar = 100 μ m. n=3/group.