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Ascl1 lineage cells contribute to ischemia-induced neurogenesis and oligodendrogenesis

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Neural and oligodendrocyte progenitor cells in the adult brain express Ascl1 (also known as Mash1), a basic helix-loop-helix transcription factor. We examined the progeny and fate of this progenitor population in adult male *Ascl1-CreER^{TM};R26R-stop-yellow* fluorescent protein mice subjected to right middle cerebral occlusion over 60 days after stroke using inducible Cre recombination to label Ascl1-expressing cells at poststroke days 2 to 6 *in vivo*. Seven days after stroke, a substantial increase in Ascl1 lineage cells was detected in the ipsilateral subventricular zone (SVZ), striatum, and corpus callosum. These cells exhibited proliferating progenitor cell phenotypes (Sox2$^+$, BrdU$^+$, and Ki67$^+$). Although Ascl1 lineage cells in the ipsilateral SVZ gradually decreased during 14 to 60 days after stroke, Ascl1 lineage cells in the ischemic striatum revealed a remarkable increase during this period. Thirty and sixty days after stroke, Ascl1 lineage cells in the ischemic striatum gave rise to GABAergic neurons and mature oligodendrocytes. In contrast, none of the Ascl1 lineage cells in the contralateral striatum exhibited neuronal and oligodendrocyte phenotypes. Moreover, Ascl1 lineage cells in the corpus callosum were only fated to become mature oligodendrocytes. Our data suggest that Ascl1 lineage cells contribute to stroke-induced neurogenesis and oligodendrogenesis in the adult ischemic brain.
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Introduction

The adult rodent subventricular zone (SVZ) of the lateral ventricle is composed of migratory neuroblasts, actively proliferating progenitor cells, and quiescent neural stem cells (Lois and Alvarez-Buylla, 1994; Doetsch *et al*, 1997). Neuroblasts in the SVZ travel the rostral migratory stream to the olfactory bulb where they differentiate into granule and periglomerular neurons throughout adult life (Morshead *et al*, 1994). Subventricular zone neural stem cells generate oligodendrocyte progenitor cells and oligodendrocytes that distribute to the corpus callosum (Menn *et al*, 2006). Cerebral ischemia promotes proliferation of actively proliferating SVZ cells and recruits SVZ neuroblasts to the ischemic boundary regions (Jin *et al*, 2001; Zhang *et al*, 2001; Arvidsson *et al*, 2002; Parent *et al*, 2002). In contrast, the contribution of neural progenitor cells to oligodendrogenesis in the ischemic brain is understudied, although stroke triggers oligodendrogenesis (Gregersen *et al*, 2001; Dewar *et al*, 2003; Zhang *et al*, 2009). Moreover, molecular mechanisms underlying stroke-induced neurogenesis and oligodendrogenesis have not been extensively investigated.

Ascl1 (also known as Mash1) is a basic helix-loop-helix transcription factor, which is transiently expressed in neural progenitor cells (Guillemot *et al*, 1993; Battiste *et al*, 2007). During development, Ascl1 directs neurogenesis and oligodendrogenesis (Parras *et al*, 2007). *In vivo* lineage tracing of Ascl1-expressing cells in the SVZ of the adult mouse reveals that actively proliferating SVZ cells express Ascl1 and that Ascl1 lineage cells are fated to become neurons in the olfactory bulb and oligodendrocytes in the corpus callosum, but not astrocytes (Kessaris *et al*, 2006; Battiste *et al*, 2007; Kim *et al*, 2007). New neurons and oligodendrocytes have promising potential to facilitate ischemic brain repair (Lindvall *et al*, 2004; Zhang and Chopp,

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2009). As Ascl1 is a transcription factor with essential regulatory functions in directing both neurogenesis and oligodendrogenesis (Battiste *et al*, 2007; Parras *et al*, 2007), we investigated *in vivo* the generation of Ascl1 lineage progenitor cells and their ultimate fate in ischemic brain by means of the inducible *Ascl1-CreER^{TM}* mouse.

Materials and methods

All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Henry Ford Hospital.

Bigenic *Ascl1-CreER^{TM}*; *R26R-Stop-Yellow* Fluorescent Protein Mice

A pair of *Ascl1-CreER^{TM}* mice was kindly provided by Dr Johnson (University of Texas Southwestern Medical Center) (Kim *et al*, 2007). The *Ascl1-CreER^{TM}* mouse is a bacterial artificial chromosome transgenic mouse in which sequences encoding a Cre recombinase fused with a modified estrogen receptor replace the Ascl1 coding sequence (Battiste *et al*, 2007; Kim *et al*, 2007). A pair of *R26R-stop-yellow fluorescent protein* (*YFP)* mice, a Cre recombinase reporter strain (Srinivas *et al*, 2001), was purchased from Jackson Laboratory (Bar Harbor, ME, USA). Bigenic *Ascl1-CreER^{TM}*; *R26R-stop-YFP* mice were generated by breeding the *Ascl1-CreER^{TM}* mouse with the YFP reporter mouse. Mice were genotyped by PCR using genomic DNA and primers previously published for *Ascl1-CreER^{TM}* (Battiste *et al*, 2007; Kim *et al*, 2007) and R26R reporter (Srinivas *et al*, 2001) mice.

Animal Model of Stroke and Tamoxifen Treatment

Adult male *Ascl1-CreER^{TM}*; *R26R-stop-YFP* mice aged 2 to 3 months with the genotype *Ascl1-CreER^{TM}/+*;*R26R-stop-YFP/+* were used in this study. The right middle cerebral artery (MCA) was permanently occluded by inserting a 6-0 nylon filament as described previously (Zhang *et al*, 1998). These mice received intraperitoneal injection of tamoxifen (300 mg/kg, Sigma Chemical, St Louis, MO, USA) in sunflower seed oil daily for 5 consecutive days starting 48 hours after stroke. The 48 hours time point was selected because we previously showed a significant increase in proliferating SVZ cells 2 days after stroke (Zhang *et al*, 2004). The dose of tamoxifen was selected on the basis of published studies (Kim *et al*, 2007). These animals were killed 7, 14, 30, and 60 days after stroke (Figure 1A). Nonischemic mice with the genotype *Ascl1-CreER^{TM}/+*;*R26R-stop-YFP/+* or the genotype *Ascl1-CreER^{TM}/−*;*R26R-stop-YFP/−* mice received the same tamoxifen treatment and were killed after 5 days of injection and used as controls.

Bromodeoxyuridine Labeling

Bromodeoxyuridine (BrdU), the thymidine analog that is incorporated into the DNA of dividing cells during the S phase, was used for mitotic labeling (Sigma Chemical). Ischemic mice were intraperitoneally injected with BrdU (100 mg/kg) daily for 5 consecutive days starting 48 hours after stroke.

Brain Tissue Preparation and Immunohistochemistry

Animals were transcardially perfused with heparinized saline, followed by 4% paraformaldehyde. The brains were removed from the skull, fixed further in 4% formaldehyde for 4 hours at 4°C, and then transferred into 30% sucrose in phosphate-buffered saline for 24 hours. The brains were embedded and frozen in optimal cutting temperature compound. A series of 30-μm-thick brain coronal sections were cut in a cryostat from the bregma (1.18 mm to −0.82 mm) for the mouse (Franklin and Paxinos, 1997).

Every fifth section was used for immunohistochemistry, as described previously (Zhang *et al*, 2001). The following antibodies were used in this study: sheep anti-BrdU (1:100, Abcam, Cambridge, MA, USA), mouse anti-nestin (1:100, BD Bioscience, Franklin, NJ, USA), rabbit anti-Ki67 (1:300, Thermo, Fremont, CA, USA), goat anti-doublecortin (DCX, 1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), got anti-Sox2 (1:500, Santa Cruz Biotechnology), mouse anti-NeuN (1:500, Chemicon/Millipore, Billerica, MA, USA), chicken anti-green fluorescent protein (1:500, Aves Labs, Tigard, OR, USA), rabbit anti-DARPP32 (1:200, Cell Signaling Technology, Danvers, MA, USA), mouse anti-calbindin (1:800, Swant/Fisher Scientific, Waltham, MA, USA), rabbit anti-GAD-47 (1:500, Sigma Chemical), rabbit-anti-GFAP (glial fibrillary acidic protein) (1:10,000, Dako, Carpinteria, CA, USA), rabbit anti-NG2 (1:800, Chemicon/Millipore), and mouse anti-2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase, 1:200, Chemicon/Millipore). Cell nuclei were stained with DAPI (4′, 6′-diamidino-2-phenylindole).

Double immunofluorescent images were acquired using a Zeiss (Zeiss, Thornwood, NY, USA) LSM 510 Meta-NLO system with Coherent Chameleon Ti:Sa laser. Three-color images were scanned using 488 nm argon, 543 HeNe, and Chameleon (750 nm for DAPI) lasers.

Quantification of Yellow Fluorescent Protein$^+$ Cells

Stereological unbiased estimates of the total numbers of YFP$^+$ cells within the regions of interest were obtained using a microcomputer imaging device stereology software (3D Fractionator, InterFocus Imaging, Cambridge, England), which drives the Ludl X-Y-Z-motorized stage of the actual microscope stage (Olympus BX61, Olympus, Center Valley, PA, USA) focus position. The YFP+ cells were identified with antibodies against green fluorescent protein. In brief, using the automated optical fractionator method, we drew the corpus callosum, striatal, and SVZ areas on coronal sections at a ×4 objective. A higher power (a ×60 objective, NA 1.4) was then selected, and the system used random systematic sampling to sample 30% of the defined region. When the system moved to the first location within the region of interest, a counting frame was placed over the selected area. We then counted the number

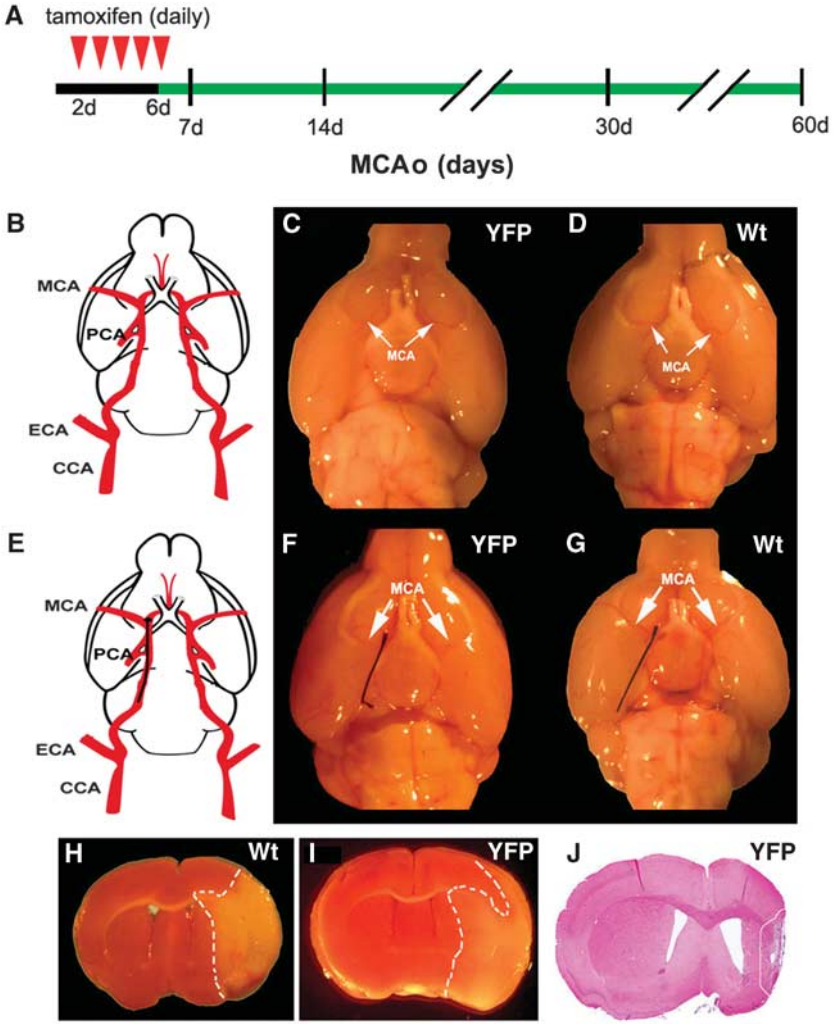


Figure 1 Experimental protocol and ischemic lesion. (**A**) A diagram of experimental design for analysis of the progeny and fate of Ascl1-expressing cells. Tamoxifen was injected (intraperitoneally) daily for 5 consecutive days starting 2 days after the onset of stroke (red arrowheads) and *Ascl1-CreER*^{TM};*R26R-stop-YFP* mice were killed 7, 14, 30, and 60 days after stroke. (**B**) A schematic drawing of mouse carotid and cerebral arteries. (**C** and **D**) Gross structure of the MCA (arrows) in *Ascl1-CreER*^{TM};*R26R-stop-YFP* (panel **C**, YFP) and wild-type mice (panel **D**, Wt). (**E**) Schematic drawing of the mouse right internal carotid artery with a filament (black). (**F** and **G**) A nylon filament within the right intracranial segment of the internal carotid artery of *Ascl1-CreER*^{TM};*R26R-stop-YFP* (panel **F**, YFP) and wild-type mice (panel **G**, Wt). (**H** and **I**) Brain coronal sections show ischemic lesion (outlined by white dash line) in the territory supplied by the right MCA assayed by triphenyltetrazolium chloride staining 48 hours after MCAo in wild-type (panel **H**, Wt) and *Ascl1-CreER*^{TM};*R26R-stop-YFP* (panel **I**, YFP) mice. (**J**) A coronal section stained by H&E of an infarction 1 month after MCAo in *Ascl1-CreER*^{TM};*R26R-stop-YFP* mouse (YFP). CCA, common carotid artery; ECA, external carotid artery; H&E, hematoxylin and eosin; MCA, middle cerebral artery; PCA, posterior cerebral artery; MCAo, middle cerebral artery occlusion; YFP, yellow fluorescent protein.

of immunostained cells by focusing up and down and marking targets within the counting frame. Data are presented as an estimate of the total number of green fluorescent protein + cells in defined regions.

Statistical Analysis

Data were evaluated for normality. Data transformation would be considered if data were abnormal. The average of the two measurements was taken for each mouse at each location at each time point. One-way analysis of variance was used to study the time effect on each region of interest. The analysis began with testing for the overall time effect, followed by pairwise group comparisons. All data are presented as mean ± s.e. Statistical significance was set at $P < 0.05$.

Results

Occlusion of the Middle Cerebral Artery Results in Ischemic Lesion

To examine whether *Ascl1-CreER*^{TM};*R26R-stop-YFP* mice have any gross cerebral vascular abnormality,

we compared gross cerebral vessels in *Ascl1-CreER^{TM};R26R-stop-YFP* mice aged 2 to 3 months with age-matched wild-type mice. The gross structure of the circle of Willis and the MCA in *Ascl1-CreER^{TM};R26R-stop-YFP* mice (Figures 1B and 1C) were comparable with the structure in wild-type mice (Figures 1B and 1D). Occlusion of the right MCA (MCAo) with a nylon filament in these mice resulted in an ischemic lesion in the territory supplied by the MCA assayed by triphenyltetrazolium chloride staining (Figures 1E to 1I). These data indicate that MCAo in *Ascl1-CreER^{TM};R26R-stop-YFP* mice generates an ischemic lesion.

Stroke did not Alter the Ascl1-Expressing Cell Profile in the Contralateral Hemisphere

Before tracking the progeny of Ascl1-expressing cells in the ischemic brain, we verified tamoxifen-inducible Cre-mediated recombination targeted at Ascl1-expressing cells in the young adult mice without MCAo. *Ascl1-CreER^{TM};R26R-stop-YFP* mice aged 2 to 3 months were administered tamoxifen daily for 5 five consecutive days and the brains were harvested 1 day after the last injection. The YFP$^+$ cells were detected in the SVZ of the lateral ventricles, the corpus callosum, and striatum (Figure 2). Using unbiased stereology analysis, we estimated YFP$^+$ cells. We found that the number of YFP$^+$ cells in the SVZ, corpus callosum, and striatum was $2{,}032 \pm 79$, $3{,}351 \pm 133$, and 614 ± 83, respectively (Table 1). In contrast, in the absence of tamoxifen, *Ascl1-CreER^{TM};R26R-stop-YFP* mice did not exhibit any YFP$^+$ cells in their brains (data not shown). These data indicate successful recombination in Ascl1-expressing cells.

Double immunostaining revealed that many (86%) of the YFP$^+$ cells in the SVZ were SOX2$^+$ (Table 1, Figures 2A to 2C), a marker of neural progenitor cells, and $\sim$63% of YFP$^+$ cells were DCX$^+$ (62.7 ± 3.7, Figures 2E to 2G), a marker of neuroblasts. The YFP$^+$ cells were also BrdU$^+$ after 24 hours exposure (Table 1, Figures 2D and 2H). These data indicate that Ascl1-expressing cells comprise transient amplifying cells and neuroblasts in the SVZ of the lateral ventricles, which is consistent with the published studies (Kim *et al*, 2007, 2008).

In the corpus callosum, many YFP$^+$ cells appeared as doublets or clusters and were Ki67$^+$ (Figures 2I to 2L, Table 1), suggesting that these YFP$^+$ cells are actively proliferating *in situ*. Approximately 77% of YFP$^+$ cells were SOX2$^+$ and some YFP$^+$ cells were NG2$^+$ (Table 1). The YFP$^+$/DCX$^+$ or YFP$^+$/GFAP$^+$ cells were not detected in the corpus callosum (data not shown). These data are consistent with published studies that the adult corpus callosum contains many Ascl1-expressing oligodendrocyte progenitor cells (Kim *et al*, 2007, 2008). In contrast to the SVZ and corpus callosum, <1% of YFP$^+$ cells in the striatum were proliferating (Table 1).

To examine whether stroke affects Ascl1-expressing cells in the contralateral hemisphere, *Ascl1-CreER^{TM};R26R-stop-YFP* mice were subjected to MCAo and tamoxifen was administered daily for 5 five days starting 2 days after MCAo. These mice were killed 1 day after tamoxifen treatment (7 days after MCAo). We estimated YFP$^+$ cells in the contralateral hemisphere (nonischemic hemisphere) 7 days after stroke and found that the number of YFP$^+$ cells in the SVZ, corpus callosum, and striatum of the contralateral hemisphere was $2{,}261 \pm 99$, $3{,}346 \pm 122$, and 764 ± 98, respectively, which is comparable with the numbers in the corresponding regions of nonischemic mouse (Table 1). In the contralateral SVZ, YFP$^+$ cells exhibited phenotypes of transient amplifying cells and neuroblasts (Tables 1 and 2), whereas in the contralateral corpus callosum, YFP$^+$ cells exhibited phenotypes of oligodendrocyte progenitor cells 7 days after stroke (Table 1). The YFP$^+$ cells were not actively proliferating in the contralateral striatum (Table 1). Collectively, these data indicate that stroke does not change the number and phenotypes of Ascl1-expressing cells in the contralateral hemisphere, although recombination was induced 1 day after stroke. Therefore, we used an Ascl1-expressing cell profile in the contralateral hemisphere as a reference for the following experiments.

Stroke Increases Ascl1 Lineage Cells in the Ipsilateral Subventricular Zone

To follow the Ascl1-expressing cells in the ischemic brain, *Ascl1-CreER^{TM};R26R-stop-YFP* mice were subjected to MCAo and tamoxifen was administered daily for 5 five days starting 2 days after MCAo. These mice were killed 7, 14, 30, and 60 days after MCAo (Figure 1A). Stereology analysis revealed that 7 days after stroke, the number of YFP$^+$ cells in the ipsilateral SVZ increased by 70% ($3{,}825 \pm 166$, $P<0.05$) compared with that in the contralateral SVZ (Figure 2M). Among these YFP$^+$ cells, 30, 71, and 87% were BrdU$^+$, Ki67$^+$, and Sox2$^+$ (Table 1), respectively, whereas 65% were DCX$^+$ ($65.4\% \pm 3.4\%$, Figure 3A). These data suggest that after stroke, Ascl1-expressing cells remain as transient amplifying cells and neuroblasts in the SVZ. Fourteen days after stroke, the number of YFP$^+$ cells in the ischemic SVZ was reduced by 49% compared with the number 7 days after stroke (Figure 2M), which was still significantly ($P<0.05$) higher than the number in the contralateral SVZ where YFP$^+$ cells decreased by 60% (Figure 2M). Many YFP$^+$ cells extended from the ipsilateral dorsal and ventral SVZ toward the ischemic striatum and these cells exhibited bipolar morphology with a long leading process or a cell body with several small processes (Figure 3B), suggesting that YFP$^+$ cells in the ipsilateral SVZ migrate to the ischemic striatum. The number of YFP$^+$ cells in the contralateral and ipsilateral SVZs continuously decreased 30 and

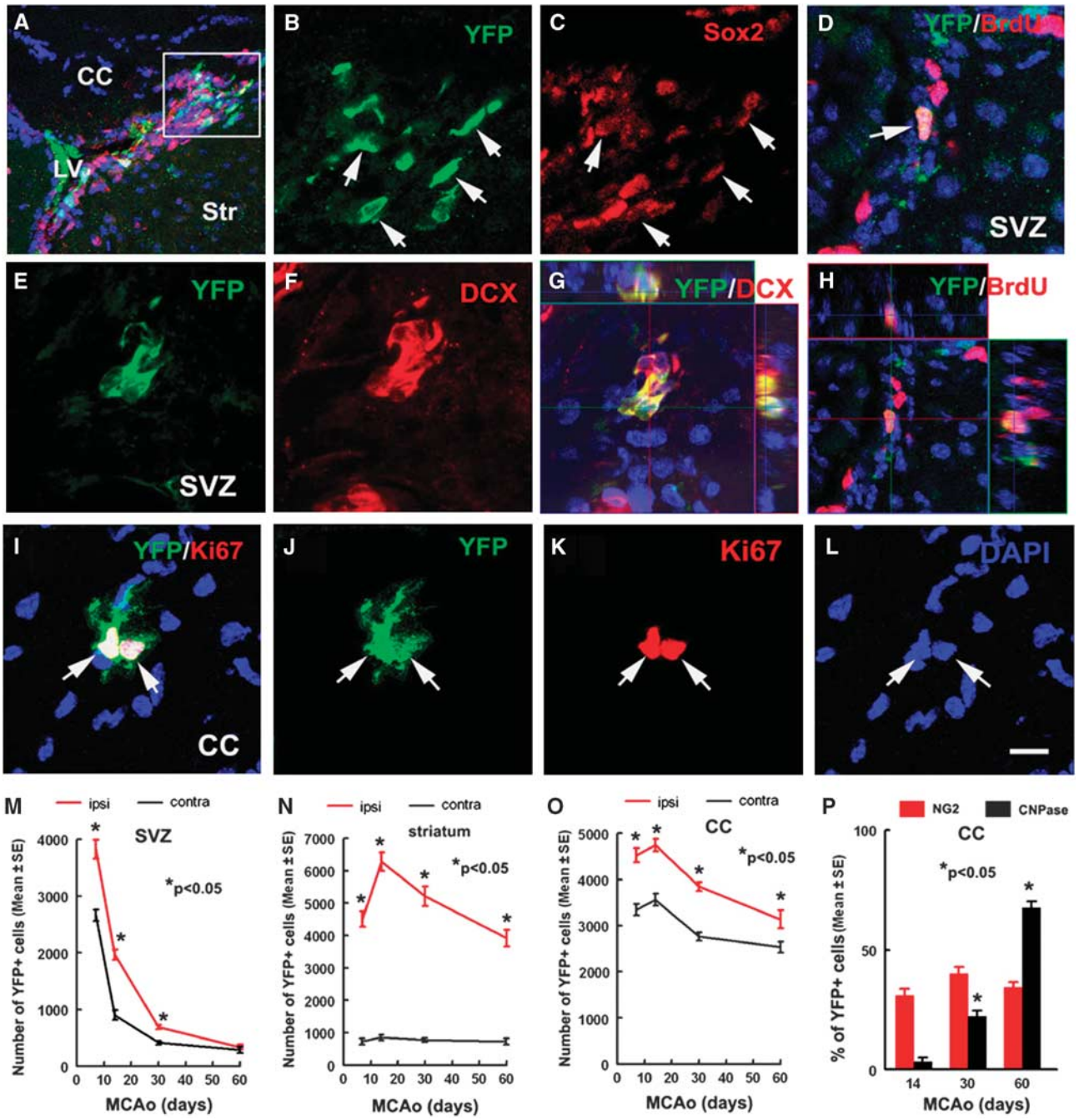


Figure 2 YFP$^+$ cells are proliferating progenitor cells or neuroblasts in the SVZ and corpus callosum of nonischemic *Ascl1-CreER^{TM};R26R-stop-YFP* mice. Double immunostaining shows that YFP$^+$ cells (**A**, **B**, arrows) were Sox2$^+$ (panels **A**, **C**, arrows), YFP$^+$ cells (**E**, **G**, arrows) were DCX$^+$ (panels **F**, **G**, arrows), or YFP$^+$ cells (**D**, **H**, arrow) were BrdU$^+$ (panels **D**, **H**, arrow). (Panels **G** and **H**) Orthogonal views. A pair of YFP$^+$ cells (**I**, **J**, and **L**, arrows) was Ki67$^+$ (panels **I** and **K**, arrows) in the corpus callosum. (Panels **M**–**P**) Line graphs show that the estimated number of YFP$^+$ cells in the SVZ (panel **M**), striatum (panel **N**), and corpus callosum (panel **O**) 7, 14, 30, and 60 days after stroke. (Panel **P**) Percentage of NG2$^+$ and CNPase$^+$ cells in the ipsilateral corpus callosum 14, 30, and 60 days after stroke. $n = 6$ mice for 7, 30, and 60 days and $n = 5$ mice for 14 days. $^*P < 0.05$ versus contralateral for panels **A**–**C**, $^*P < 0.05$ versus 14 days for panel **D**. Bars = 10 μm. Panels **B** and **C** were from a box area in panel **A**. BrdU, bromodeoxyuridine; CC, corpus callosum; DCX, doublecortin; MCAo, middle cerebral artery occlusion; SVZ, subventricular zone; YFP, yellow fluorescent protein.

60 days after stroke (Figure 2M). Interestingly, ~9 and 12% of YFP$^+$ cells remained in the ipsilateral and contralateral SVZ, respectively, 60 days after stroke compared with the number 7 days after stroke (Figure 2M). The YFP$^+$ cells that remained in the SVZ were GFAP negative (data not shown).

Table 1 Percentage of double immunoreactive YFP$^+$ cells in nonischemic and ischemic brains

	SVZ			Striatum			CC		
	Normal	*Contra*	*Ipsi*	*Normal*	*Contra*	*Ipsi*	*Normal*	*Contra*	*Ipsi*
Number of YFP$^+$ cells	2,032 ± 78	2,261 ± 99	3,825 ± 166*#	614 ± 83	764 ± 98	4,514 ± 237*#	3,351 ± 133	3,346 ± 122	4,527 ± 150*#
% (Cells counted)									
BrdU/YFP	24.1 ± 2.2 (874)	23.2 ± 1.8 (964)	29.7 ± 2.1*# (1,016)	0.38 ± 0.1 (412)	0.37 ± 0.2 (437)	20.01 ± 1.4# (892)	21.6 ± 1.7 (1,012)	22.8 ± 1.2 (972)	26.3 ± 1.2# (948)
KI67/YFP	58.5 ± 3.1 (865)	60.2 ± 4.4 (1,022)	71.1 ± 2.2*# (1,236)	0.33 ± 0.2 (478)	0.38 ± 0.1 (502)	33.47 ± 1.9*# (967)	35.3 ± 2.4 (986)	36.4 ± 2.4 (832)	41.4 ± 2.9 (884)
SOX2/YFP	83.1 ± 2.8 (1,078)	86.6 ± 2.7 (1,112)	87.3 ± 2.4 (1,621)	0.58 ± 0.2 (503)	0.53 ± 0.2 (476)	47.94 ± 3.8*# (864)	76.6 ± 2.6 (1,168)	78.6 ± 3.5 (874)	82.1 ± 2.0 (983)

CC, corpus callosum; contra, contralateral; ipsi, ipsilateral; SVZ, subventricular zone; YFP, yellow fluorescent protein.
Data are presented as mean ± s.e. Normal indicates data obtained from nonischemic rats killed 1 day after 5 five consecutive day injections of tamoxifen.
* and # are $P < 0.05$ versus contralateral and normal regions, respectively. There were no significant differences between normal and contralateral regions.

Stroke Increases Ascl1 Lineage Cells that Become Neurons and Oligodendrocyte Progenitor Cells in the Ischemic Striatum

In the ischemic striatum, 7 days after stroke, the number of YFP$^+$ cells increased by 4.6-fold ($P < 0.01$) from 764 ± 98 in the contralateral striatum to 4,514 ± 237 in the ipsilateral striatum (Figure 2N). Approximately 20, 34, and 48% YFP$^+$ cells were BrdU$^+$ (20% ± 1.46%), Ki67$^+$ (33.5% ± 1.9%), and Sox2$^+$ (47.9% ± 3.8%), respectively, indicating that the majority of Ascl1-expressing cells in the ischemic striatum are actively proliferating progenitor cells. Less than 10% of YFP$^+$ cells in the ischemic striatum were DCX$^+$ (Table 2), and none of the YFP$^+$ cells were colabeled with a pan-neuronal marker NeuN$^+$ at this time point. In contrast to the SVZ where YFP$^+$ cells decreased, 14 days after stroke, the number of YFP$^+$ cells in the ischemic striatum further increased ($P < 0.05$) compared with 7 days after stroke (Figures 2N and 3B). The DCX$^+$ cell population also substantially increased to 21% of YFP$^+$ ($P < 0.05$, Table 2), suggesting that Ascl1 lineage neuroblasts in the SVZ migrate to the ischemic striatum during this period. Some of the YFP$^+$ cells formed doublets and exhibited mitosis (Figures 3D to 3F), indicating that these cells are actively proliferating in the ischemic striatum. Previous studies have indicated that ~80% or more of stroke-induced new neurons die in the ischemic striatum during 2 to 6 weeks after stroke (Arvidsson *et al*, 2002). We found that the number of YFP$^+$ cells in the ischemic striatum did not decrease during 30 to 60 days compared with the number 7 days after stroke (Figures 2N and 3G). Within the ischemic striatum, YFP$^+$ cells distributed to the ischemic boundary (Figure 3G). The YFP$^+$/NeuN$^+$ cells were observed in the striatal ischemic boundary starting 14 days after stroke (8%, Table 2, Figures 4A to 4D) and the number of YFP$^+$/NeuN$^+$ cells increased by 30 (24%, Table 2, Figures 4E to 4H) and 60 (26%, Table 2, Figures 4I to 4L) days after stroke. The YFP$^+$/NeuN$^+$ cells exhibited multiple long processes (Figures 4E to 4L). Approximately 20% of YFP$^+$ cells exhibited the phenotype of calretinin (Table 2, Figures 4M to 4P), a calcium-binding protein that has been used as a marker of GABAergic interneurons (Gabbott and Bacon, 1996). Few YFP$^+$ cells were DARPP32$^+$ (Figures 4Q to 4T), a marker of medium-sized spiny neurons (Ivkovic and Ehrlich, 1999). However, calbindin and glutamic decarboxylase 67 (GAD-67) immunoreactivity was not detected in YFP$^+$ cells (data not shown). Studies in the adult rodent brain show that a restricted dorsomedial region of the striatum adjacent to the SVZ generates new neurons (Dayer *et al*, 2005). However, we did not detect YFP$^+$/NeuN$^+$ cells in the contralateral striatum at the time points examined in this study. Our results suggest that Ascl1 lineage cells are destined to become neurons in the ischemic striatum.

Table 2 Percentage of YFP$^+$ cells coexpressing neuronal and oligodendrocyte phenotypes in the ischemic striatum

Days (sample size)	*Percentages (counted cells)*				
	DCX/YFP	*NN/YFP*	*Calretinin/YFP*	*NG2/YFP*	*CNPase/YFP*
7 ($n=6$)	9.5 ± 1.0 (1,021)	0	0	21.9 ± 1.9 (774)	0
14 ($n=5$)	21.2 ± 1.8*(1,154)	8.4 ± 1.7 (1,012)	0	30.9.4 ± 2.8*(1,024)	3.4 ± 1.7 (984)
30 ($n=6$)	12.7 ± 1.2*(1,062)	23.5 ± 2.5$^{\#}$ (1,226)	17.7 ± 2.3 (1,124)	43.5 ± 2.7*(1,134)	22.4 ± 2.2$^{\#}$ (972)
60 ($n=6$)	NC	26.3 ± 2.9$^{\#}$ (1,092)	20.7 ± 3.3 (1,118)	32.9 ± 2.1*(1,232)	36.7 ± 3.2$^{\#}$ (1,022)

CNPase, 2′,3′-cyclic nucleotide 3′-phosphodiesterase; DCX, doublecortin; NC, not counted; NN, NeuN; YFP, yellow fluorescent protein.
Data are presented as mean ± s.e.
* and $^{\#}$ $P < 0.05$ versus 7 and 14 day groups, respectively.

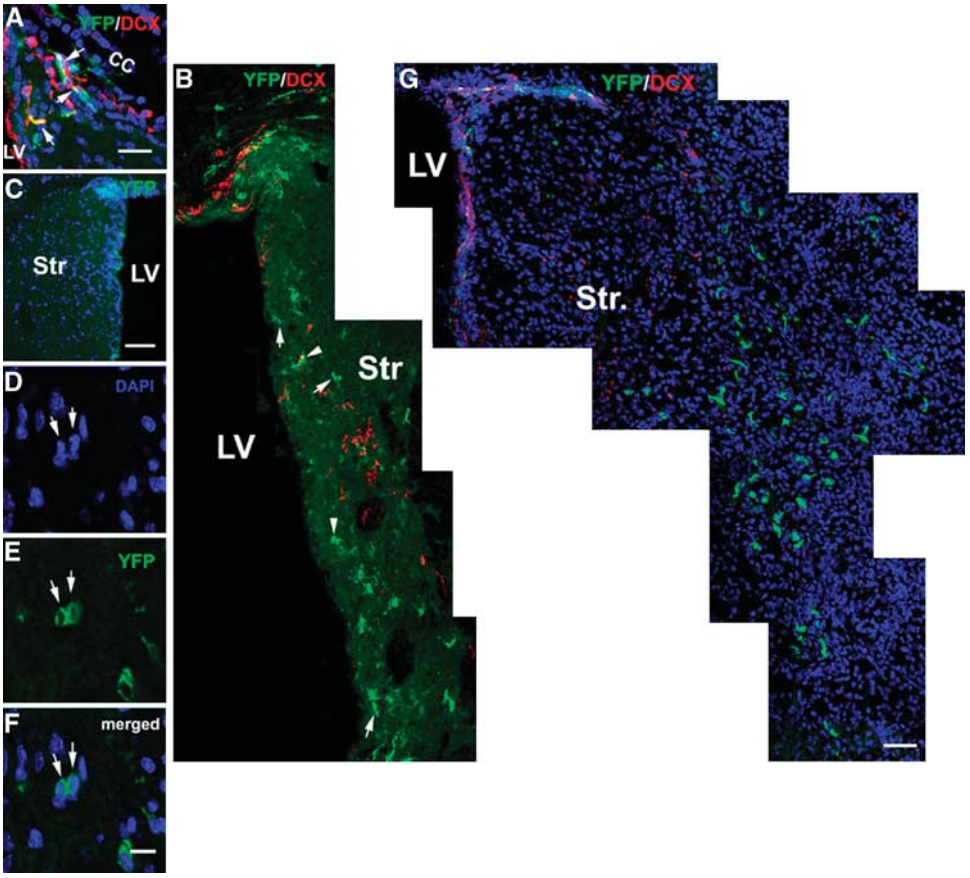


Figure 3 Distribution of YFP$^+$ cells in the ischemic striatum. Double immunostaining shows YFP$^+$/DCX$^+$ cells (**A**, arrows) in the ipsilateral SVZ 7 days after stroke. Many YFP$^+$ cells with bipolar (**B**, arrows) and multiple branches (panel **B**, arrowheads) were distributed in the ipsilateral dorsal and ventral SVZ, whereas YFP$^+$ cells were localized within the contralateral SVZ (**C**) 14 days after stroke. (**D**, **E**) A YFP$^+$ cell in telophase (arrows) during mitosis in the ischemic striatum. (**G**) Thirty days after stroke, YFP$^+$ cells were distributed to the ischemic boundary region. It must be noted that there were some YFP$^+$ cells that remained in the SVZ. Bars = 20 μm in panels **A** and **G**, 100 μm in panel **C**, and 10 μm in panel **F**. CC, corpus callosum; DCX, doublecortin; LV, lateral ventricle; Str, striatum; SVZ, subventricular zone; YFP, yellow fluorescent protein.

In the ischemic striatum, some of the YFP$^+$ cells exhibited morphologic characteristics of oligodendrocyte progenitor cells and their cell bodies and processes were immunoreactive for the chondroitin sulfate proteoglycan NG2 (Table 2, Figure 5A). Some YFP$^+$ cells exhibited faint NG2 immunostaining (Figures 5B to 5E) 30 days after stroke, and orthogonal reconstruction of confocal images revealed that the cell bodies of YFP$^+$ cells with faint or strong NG2 immuostaining were within the imaged tissue (Figure 5E), indicating that cells with weak NG2 immunosignals are not owing to tissue cutting. This observation suggests that these YFP$^+$ oligodendrocyte progenitor cells may differentiate into mature oligodendrocytes. By immunostaining mature oligodendrocytes with antibodies against CNPase and myelin basic

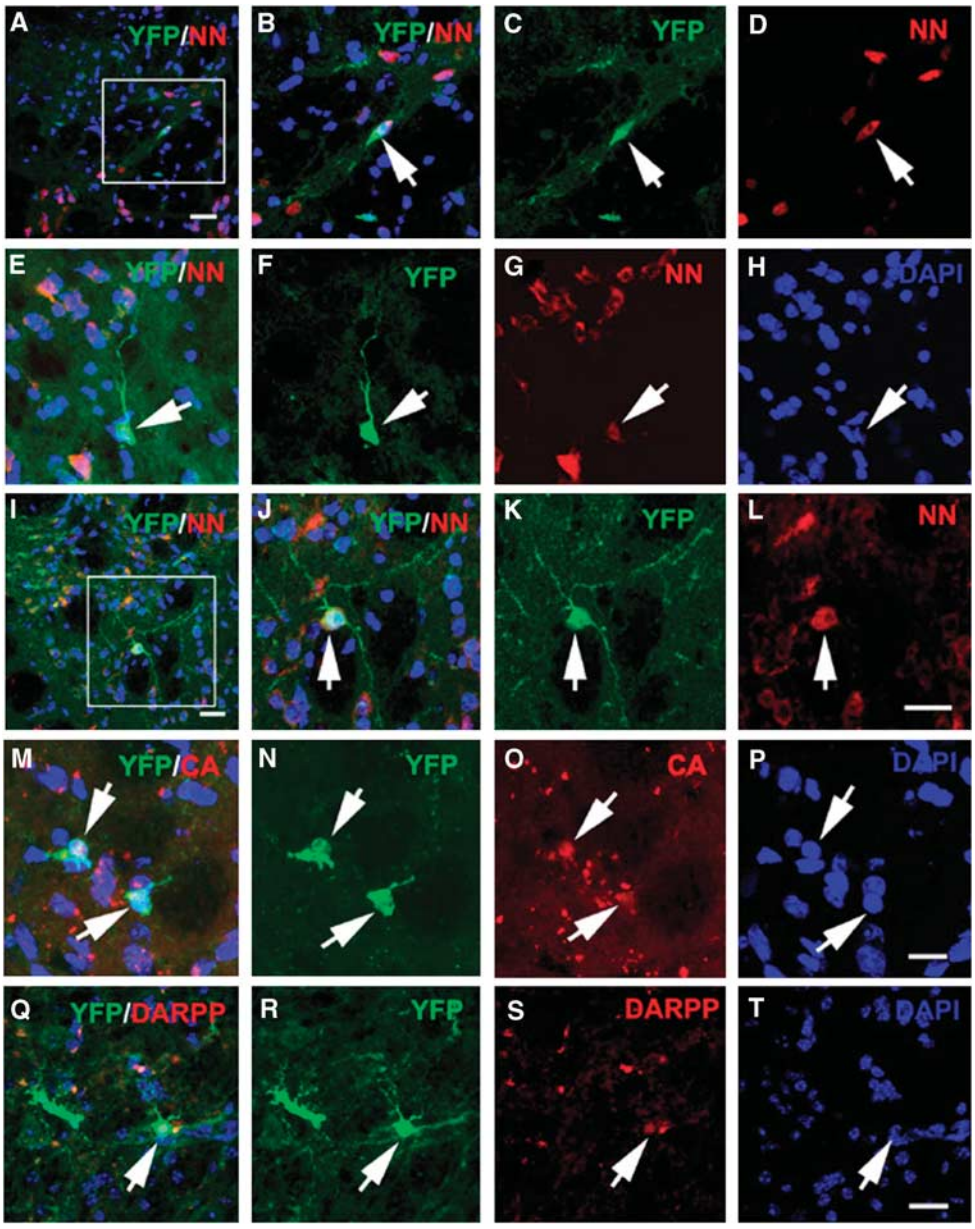


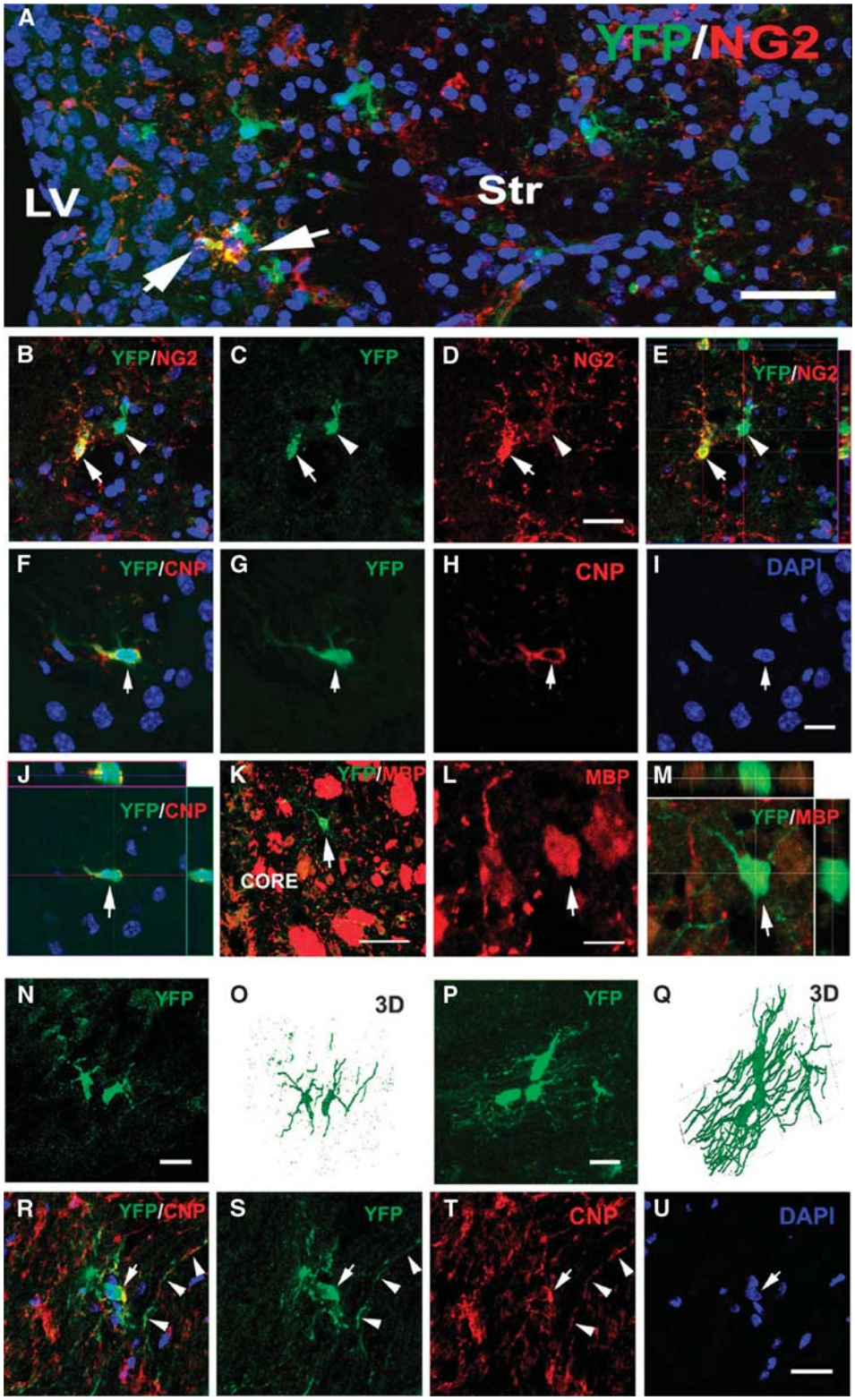
Figure 4 Neuronal phenotypes of YFP$^+$ cells in the ischemic striatum. Double immuostaining shows YFP$^+$/NN$^+$ cells in the ischemic striatum 14 (**A**–**D**, arrow), 30 (**E**–**H**, arrow), and 60 (**I**–**L**, arrow) days after stroke. Some YFP$^+$ cells (**M** and **N**, arrows) were calretinin$^+$ (**O**, arrows) and some (panel **Q** and **R**, arrow) were DARPP 32$^+$ (**Q** and panel **S**, arrow). (Panel **H**, panel **P**, and **T**) are DAPI nuclei. Panels **B**–**D** were from a box area in panel **A**. Panels **J**–**L**) from a box area in panel **I**. . Bars = 20 μm in panels **A**, **I**, **L**, and **T**; 10 μm in **P**. CB, calbindin; DARPP, DARPP 32; NN, NeuN; YFP, yellow fluorescent protein.

protein (MBP), we found an increase in YFP$^+$/CNPase$^+$ and YFP$^+$/MBP$^+$ cells in the ischemic striatum during 30 to 60 days of stroke (Table 2, Figures 5F to 5M). These data indicate that in addition to neurons, Ascl1 lineage cells are fated to become oligodendrocyte progenitor cells and mature oligodendrocytes.

Stroke Increases Ascl1 Lineage Cells that Become Oligodendrocytes in the Ischemic Corpus Callosum

Stroke significantly ($P<0.05$) increased the number of YFP$^+$ cells in the ischemic corpus callosum compared with the number in the contralateral corpus callosum during 7 to 60 days after ischemia (Figure 2O). Immunostaining revealed that 7 days after stroke, 26 and 41% of YFP$^+$ cells were BrdU$^+$ and Ki67$^+$, respectively, in the ipsilateral corpus callosum (Table 1) and that many YFP$^+$ cells were SOX2$^+$ (82%, Table 1), indicating that Ascl1 cells in the corpus callosum are actively proliferating progenitor cells. Three-dimensional confocal images revealed that 7 and 14 days after stroke, YFP$^+$ cells had multiple radial processes and thin bushy branches (Figures 5N and 5O), which resemble oligodendrocyte progenitor cells (Marshall *et al*, 2005;

Ness *et al*, 2005). However, 30 and 60 days after stroke, many YFP$^+$ cells exhibited multiple parallel fine process sheaths aligned with white matter axonal fiber tracts (Figures 5P and 5Q) reminiscent of myelinating oligodendrocytes (Ness *et al*, 2005; Menn *et al*, 2006). During this period, we observed the cell bodies and proximal processes of YFP$^+$ cells were CNPase$^+$ (Figures 5R to 5U), indicating that they were synthesizing myelin. The number of YFP$^+$/CNPase$^+$ cells substantially increased 30 and 60 days after stroke (Figure 2P). We did not detect any YFP$^+$ cells in the corpus callosum that were NeuN$^+$



or GFAP$^+$ (data not shown). These results suggest that Ascl1-expressing cells in the corpus callosum differentiate into oligodendrocyte progenitor cells and mature oligodendrocytes.

Discussion

This study provides *in vivo* fate-mapping evidence that stroke substantially increased Ascl1 lineage cells in the ipsilateral SVZ, striatum, and corpus callosum and that the increased Ascl1 lineage cells were fated to become neurons and oligodendrocytes in the ischemic striatum and oligodendrocytes only in the corpus callosum. These data indicate that Ascl1 lineage cells contribute to stroke-induced neurogenesis and oligodendrogenesis in the adult ischemic brain.

Ascl1 Lineage Cells Become Neurons in the Ischemic Striatum

Ascl1 is a pro-neural basic helix-loop-helix transcription factor that is transiently expressed in cells that are within the cell cycle (Guillemot *et al*, 1993; Battiste *et al*, 2007). Cells expressing Ascl1 in the *Ascl1-CreER^{TM}* mouse can be labeled by tamoxifen-inducible Cre-mediated recombination (Kim *et al*, 2007, 2008). Cre recombination is detectable within 6 hours after tamoxifen treatment, and it persists for $\sim$24 hours (Hayashi and McMahon, 2002). In this study, we labeled cells expressing Ascl1 by tamoxifen-inducible Cre-mediated recombination for 5 days starting 2 days after stroke, which permitted us to follow the progeny and fate of Ascl1 progenitor cells arising within the first week of the onset of stroke. Our data show that stroke substantially augmented Ascl1 lineage cells in the ischemic striatum and the increased Ascl1 cells persisted at least for 60 days after stroke. A continuous increase in the number of YFP+ cells caused by additional Cre-mediated recombination is unlikely, because Cre recombination ceases 24 hours after tamoxifen injection (Hayashi and McMahon, 2002). Thus, migration of Ascl1 lineage cells in the SVZ toward the ischemic striatum and *in situ* proliferation of Ascl1 lineage cells likely contribute to an increase in YFP$^+$ cells in the ischemic striatum. Previous studies have shown that stroke recruits SVZ neuroblasts to the ischemic striatum (Jin *et al*, 2001; Zhang *et al*, 2001; Arvidsson *et al*, 2002; Parent *et al*, 2002). Our lineage tracking experiments show that 7 days after stroke, there was already a 4.6-fold increase in Ascl1-expressing cells and that only 10% of Ascl1 lineage cells were neuroblasts (DCX positive). These findings suggest that in addition to recruitment of SVZ Ascl1-expressing cells, considerable *in situ* dividing Ascl1-expressing cells likely contribute to the augmentation of Ascl1 lineage cells in the ischemic striatum. During development, Ascl1 progenitor cells contribute to GABAergic interneurons in the striatum (Kim *et al*, 2007, 2008). However, in the adult brain, Ascl1 progenitor cells give rise to neurons only in the olfactory bulb and the dentate gyrus of the subgranular zone (Kim *et al*, 2007, 2008). Consistently, this study did not detect any Ascl1 lineage neurons in the contralateral striatum where Ascl1 cells were present. In marked contrast to observations in the contralateral striatum, Ascl1 cells in the ischemic striatum were fated to become mature neurons (NeuN positive) and some of the Ascl1 lineage neurons exhibited the GABAergic interneuron phenotype (calbindin positive). We previously showed that stroke triggers embryonic molecule expression in SVZ neural progenitor cells (Liu *et al*, 2007). Collectively, these data suggest that stroke may evoke embryonic molecular signals to redirect Ascl1 lineage cells into neurons in the striatum. A recent study has shown that adult Ascl1-expressing hippocampal neural progenitor cells exhibit substantial plasticity by changing their fate from neurons to oligodendrocytes (Jessberger *et al*, 2008). Our results suggest that adult Ascl1-expressing cells in the SVZ and striatum exhibit plasticity under ischemic conditions. The present data also suggest that there is a window for Ascl1 lineage cells to differentiate into neurons, because 30 days after stroke Ascl1 lineage neurons no longer increase. Further studies aimed to identify molecules that trigger differentiation of Ascl1 lineage cells into neurons that may amplify neurogenesis after stroke are warranted.

Ascl1 Lineage Cells Become Oligodendrocyte Progenitor Cells and Oligodendrocytes in the Corpus Callosum and Striatum

Oligodendrocytes are the myelin-forming glial cells in the adult brain (Levison and Goldman, 1993;

Figure 5 Oligodendrocyte phenotypes of YFP$^+$ cells in the ischemic corpus callosum and striatum. Double immunostaining shows that YFP$^+$ cells in the ischemic striatum were NG2$^+$ (**A**, arrows) 14 days after stroke. YFP$^+$ cells (**B**, **C**, **E**,) with strong (panel **B** and **D**, arrow) or weak (panels **B** and **D**, arrowhead) were in the ischemic striatum 30 days after stroke. Panel **E** is an orthogonal view. (**F**–**I**) A YFP$^+$ (panel **F**, panel **G**, panel **I**, and **K**, arrow) and CNPase$^+$ (panels **F** and **H**, arrow) or MBP$^+$ (**L**, arrow) cells in the ischemic striatum 30 days after stroke. (**J** and **M**) Orthogonal views. (**N**–**Q**) YFP$^+$ cells (panels **N** and **P**) and confocal Z-stack 3D reconstructions of the YFP$^+$ cells (panels **O** and **Q**) in the ipsilateral corpus callosum 14 (panels **N** and **O**) and 30 (panels **P** and **Q**) days after stroke. Double immunostaining shows that an YFP$^+$ cell (**R**, **S**, and **U**, arrow) in the ipsilateral corpus callosum was CNPase$^+$ in its cell body (**T**, arrow) and proximal processes (panel **T**, arrowheads). Bar = 40 μm in panel **A**, 20 μm in panels **D**, **I**, **N**, **P**, and **U**, 50 μm in panel **K**, and 10 μm in panel **L**. CNPase, 2′,3′-cyclic nucleotide 3′-phosphodiesterase; core, ischemic core; LV, lateral ventricle; Str, striatum; YFP, yellow fluorescent protein; 3D, three dimensional.

Menn *et al*, 2006). Mature oligodendrocytes do not proliferate and new oligodendrocytes are derived from nonmyelinating oligodendrocyte progenitor cells (Carroll *et al*, 1990). The corpus callosum of the adult rodent contains heterogeneous oligodendrocyte progenitor cells (Gonzalez-Perez *et al*, 2009). Most $NG2^+$ cells in the adult brain originate from Ascl1 lineage cells (Parras *et al*, 2007). Ascl1 could be the earliest oligodendrocyte marker in the corpus callosum based on its expression before Olig2 and Sox10 expression, two early markers of oligodendrocyte progenitor cells (Kim *et al*, 2007). Approximately 50% of oligodendrocyte progenitor cells in the corpus callosum are within the cell cycle (Rivers *et al*, 2008). This study confirms that Ascl1 lineage oligodendrocyte progenitor cells are actively proliferating and that these oligodendrocyte progenitor cells differentiate into myelinating oligodendrocytes. Oligodendrocytes are highly susceptible to ischemic insults and the loss of myelin leads to axonal damage after stroke (Pantoni *et al*, 1996). The contribution of progenitor cells to oligodendrogenesis in the ischemic brain has not been extensively investigated (Dewar *et al*, 2003). Oligodendrocyte progenitor cells exist in the corpus callosum, the striatum, and the SVZ (Levison and Goldman, 1993; Menn *et al*, 2006). Our lineage tracking experiments show that a considerable population of Ascl1 lineage cells contributes to $NG2^+$ oligodendrocyte progenitor cells and mature oligodendrocytes in the ischemic corpus callosum and striatum, indicating that stroke augments oligodendrogenesis by Ascl1 lineage cells. We observed that in addition to the striatal Ascl1 lineage $NG2^+$ cells, Ascl1-expressing cells extended from the SVZ to the ischemic striatum were $NG2^+$, suggesting that stroke-induced oligodendrocytes in the striatum differentiate not only from the SVZ but also from parenchymal Ascl1 lineage $NG2^+$ cells. These data indicate that both SVZ- and striatum-derived Ascl1 lineage cells are important for remyelination in the ischemic brain. However, oligodendrocytes in the corpus callosum likely differentiate from local Ascl1 lineage $NG2^+$ cells. This study also revealed Ascl1 lineage oligodendrocytes in the ischemic striatum and corpus callosum, which is consistent with previous findings that Ascl1 lineage oligodendrocytes are heterogeneous and there are different sub-populations of oligodendrocytes in the brain (Parras *et al*, 2007). During stroke recovery, expansion of the white matter in the ischemic damaged areas has been observed (Li *et al*, 2009). It is possible that the newly formed Ascl1 lineage oligodendrocytes with myelin sheath observed in this study may function to remyelinate damaged and sprouting axons in the ischemic brain. Remyelination reinstates saltatory conduction and resolves functional deficits of axons (Franklin and Ffrench-Constant, 2008). Thus, amplification of Ascl1 lineage oligodendrogenesis could potentially promote axonal repair, leading to improvement in neurologic function.

The Ascl1 lineage cells gave rise to oligodendrocyte progenitor cells and to mature oligodendrocytes in the corpus callosum even after stroke, which is consistent with previous observations showing that the Ascl1 progeny do not differentiate into neurons in the corpus callosum (Kim *et al*, 2007, 2008). This is in contrast with Ascl1 lineage cells in the ischemic striatum that gave rise to neurons and oligodendrocytes, suggesting that differences in the local environment may be responsible. The fate determination of Ascl1 lineage cells is context dependent and additional molecular components are required for Ascl1 lineage cells to differentiate into neurons (Kim *et al*, 2007, 2008).

This study establishes the fate of Ascl1 lineage cells in the ischemic brain, which is consistent with essential regulatory functions of Ascl1 in directing both neurogenesis and oligodendrogenesis (Kim *et al*, 2007, 2008). Given the fact that recombination is only detected in $\sim$10% of Ascl1-expressing cells in *Ascl1-CreER^{TM}* mice, this study indicates that Ascl1 lineage cells make a substantial contribution to neurogenesis and oligodendrogenesis in the ischemic brain. Amplification of Ascl1 expression has the potential to facilitate endogenous brain repair during stroke recovery.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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