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Poststroke Subgranular and Rostral Subventricular Zone Proliferation in a Mouse Model of Neonatal Stroke

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Abstract

Stroke in the neonatal brain is an understudied cause of neurologic morbidity. Recently we have characterized a new immature mouse model of stroke utilizing unilateral carotid ligation alone to produce infarcts and acute seizures in postnatal day 12 (P12) CD-1 mice. In this study, the amount of poststroke neural progenitor proliferation was examined in the subgranular (SGZ) of the dentate gyrus and the subventricular zone (SVZ) 7, 14, and 21 days after ischemia (DAI). A single IP injection (50 mg/kg) of bromodeoxyuridine (BrdU) given 2 hr before perfusion fixation labeled newborn cells. Early cell phenotypes were quantified by colabeling with GFAP, nestin, and DCX. Control mice revealed an age-dependent decrease in neural proliferation, with an ~50% drop in BrdU-labeled cell counts at P33 compared with P19 both in the SGZ and in the SVZ. Significant reduction in the amount of neural proliferation in the ipsilateral injured SGZ of ligated mice correlated with both the severity of the stroke-injury and the acute seizure scores. Similar correlations were not detected contralaterally. Contralateral SGZ neural proliferation was initially lowered at 7 DAI but normalized by 21 DAI. In both injured and control brains, ~90% of newborn SGZ cells colabeled with nestin, ~30% colabeled with GFAP, and a few colabeled with DCX. In contrast, poststroke SVZ cell proliferation was enhanced ipsi- more than contralaterally at 7 DAI. In the SVZ, the enhanced neural proliferation normalized to control levels by P33. In conclusion, the neural cell proliferation was differentially altered in the SGZ vs. SVZ after neonatal stroke.

Keywords

neural proliferation; bromodeoxyuridine; neuroblast; hippocampus; subventricular zone

The immature brain is highly susceptible to excitotoxic injury (Johnston, 2005). Perinatal insults such as hypoxia-ischemia and stroke result in excitotoxic injury, followed by sequelae such as cerebral palsy, epilepsy, and impaired cognitive abilities (Koelfen et al., 1995; Delsing et al., 2001; Van Handel et al., 2007). Very little is understood about the mechanisms through which post-stroke plasticity in the developing brain results in the pathogenesis of epilepsy and cognitive impairments. Development of effective interventional and chronic post-stroke treatments would be enhanced by a better

understanding of these mechanisms. Neurogenesis in the immature mammalian brain is highly modulated by excitotoxic insults (Nakatomi et al., 2002; Plane et al., 2004; Scheepens et al., 2003; Ong et al., 2005). It has also been proposed that the capacity for neurogenesis in the neonatal brain is higher than that in the adult brain (Altman and Bayer, 1990; Namba et al., 1995; Vaccarino and Ment, 2004). Harnessing the regenerative abilities of the endogenous neural stem cell pool and its enhanced ability for proliferation in the neonatal brain have become topics of interest in postneonatal stroke management.

The P12 ischemic stroke in CD-1 mice negatively modulated counts of new mature neurons in the SGZ, ipsi- more than contralaterally, when the dividing cells BrdU-labeled at 1 week following the insult were quantified 3 weeks later at P40 (Kadam et al., 2008). SVZ-derived neurogenesis, however, was enhanced, but, unlike the SGZ newborn cells that matured into neurons, SVZ cells were committed to nonneuronal cell types in the neocortex and striatum. These paradoxical results in BrdU-labeled cell counts originating from the two neurogenic niches prompted the question of whether the long-term effect of the neonatal stroke on poststroke cell proliferation was differentially regulated in the SGZ vs. the SVZ as well. The P12 neonatal stroke also resulted in impaired working memory function and simple habituation learning as juveniles (Kadam et al., 2009). Understanding endogenous poststroke neural proliferation over the period of brain maturation will help us better understand the neurogenic response to the injury and lay the foundation for future studies to evaluate the effects of acute interventional and chronic therapies on postneonatal stroke neurogenesis and cognition.

In this study, we investigated endogenous neural proliferation at 3 time points after the insult. The first time point of 1 week after the ischemic insult was chosen so that the stroke injury had time to stabilize after necrotic and apoptotic cell losses. The intervening period also diminished the possible false-positive BrdU labeling of damaged cells undergoing DNA repair. The earlier use of BrdU labeling in this context is suspect and interpretation unclear. The two later time points, 14 and 21 days after the insult, investigated the trends of recovery in SGZ and SVZ endogenous neural proliferation during brain maturation from the neonatal to juvenile mouse. Our approach of examining BrdU labeling 7, 14, and 21 days after ligation is consistent with that taken by other investigators studying neural proliferation after hypoxicischemic injury (Hayashi et al., 2005; Wang et al., 2008). We found that SGZ cell proliferation in control mice showed an age-dependent decrease over 3 weeks that covered an age span from an immature (P19) to a juvenile brain (P33). In the ligated mice, SGZ neural proliferation was consistently lowered in the ipsilateral hippocampus. SVZ neural proliferation in controls reached an age-dependent plateau earlier than the SGZ pool. An increase in SVZ-derived neural proliferation was seen in ligated mice ipsi- more than contralaterally at P19 following the P12 insult, which returned to control levels by P33.

MATERIALS AND METHODS

The experimental paradigm and time points at which histological studies were conducted are given in Figure 1.

Subjects

All research was conducted according to a protocol approved by the Johns Hopkins University School of Medicine Animal Care and Use Committee (IACUC). All litters of CD-1 mice were purchased from Charles River Laboratories Inc. (Wilmington, MA). Newly born litters of pups arrived at age 5 postnatal days (P5) and were allowed to acclimate for 7 days. All the mice were housed in a vivarium maintained at 25°C on a 12:12-hr light:dark cycle, with lights on at 0700 hr. Food and water were available ad libitum. In total, 25 CD-1 mice (13 males and 12 females from eight litters) were used for the three groups of mice in

the study [i.e., the 7 day group (4 ligates and 4 control); 14 day group (4 ligates and 4 control), and 21 day group (5 ligates and 4 control)]. Thus, among the 25 mice, 13 were ligates (8 males, 5 females) and 12 served as sham controls (5 males, 7 females). With the intention of studying the effect of neonatal stroke on endogenous neural proliferation, only ligated mice with a stroke injury (i.e., discernible to the naked eye or under microscopic visualization of cell loss and gliosis in the middle cerebral artery perfusion territory in cresyl violet (CV)-stained sections) were included in the study and stained for analysis. In addition, brains with an obvious stroke injury that after the initial CV staining revealed a complete destruction of the ipsilateral hippocampi and SVZ were not analyzed further.

Surgery

On P12, animals were subjected to permanent unilateral double ligation of the carotid artery. Briefly, animals were anesthetized with isoflurane carried by a 50-50 mixture of O₂ and N₂O. The right common carotid artery was doubly ligated with 6-0 surgisilk and the outer skin closed with 6-0 monofilament nylon. Sham control animals were treated identically except for the carotid ligation.

Acute Seizure Scoring

Seizure activity was scored according to a seizure rating scale as previously reported (Morrison et al., 1996). Every 5 min over the 4 hr following the surgical protocol, the score corresponding to the highest level of seizure activity observed during that period was recorded. Briefly, seizure behavior was scored as follows: 0 = normal behavior; 1 = immobility; 2 = rigid posture; 3 = repetitive scratching, circling, or head bobbing; 4 = forelimb clonus, rearing, and falling; 5 = exhibiting level 4 behaviors repeatedly; and 6 = severe tonic-clonic behavior. After 4 hr, the mice were returned to the dam, and each of their seizure scores was individually summed (i.e., highest behavioral seizure scores assigned to every 5 min block of the 4-hr monitoring period were added) to produce a total acute seizure score.

Bromodeoxyuridine Labeling

Bromodeoxyuridine (BrdU) is a thymidine analog that is incorporated into the DNA during its synthetic phase (S phase) of the cell cycle (Del Rio and Soriano, 1989; Soriano et al., 1991). IP injections of BrdU (50 mg/kg) were given 2 hr before the mice were sacrificed. Three groups of sham control and ligated animals got a single injection of BrdU at 7, 14, or 21 days after the P12 surgeries to label dividing cells. Two hours after BrdU injection, mice were anesthetized with 90 mg/kg chloral hydrate, perfused transcardially with ice-cold 4% paraformaldehyde, postfixed for 12 hr in the same fixative, cryoprotected, and snap frozen.

Histology and Computerized Measurement of Atrophy and SGZ Lengths

Brain atrophy measurements were made as previously described (Kadam et al., 2008). By using MCID 7.0 Elite (InterFocus Imaging Ltd., Cambridge, United Kingdom), hemispheric areas of 20-μm-thick, Nissl-stained coronal sections equally spaced and spanning rostral striatum to caudal hippocampus were measured (n = 10–12 sections per animal). The hippocampi and hemispheres of each analyzed section were outlined separately, and the areas were calculated based on a pixel threshold value that differentiates between brain and background. Hippocampal and hemispheric atrophy was calculated for each section as follows: $[1 - (\text{ipsilateral area} / \text{contralateral area})] \times 100 = \text{percentage ipsilateral hippocampal or hemispheric atrophy}$.

The values from each section were then averaged to calculate the hippocampal and hemispheric brain atrophy for each brain. Lengths of SGZ were measured by tracing open

contours in Neurolucida software (MicroBrightField Inc., Colchester, VT) and acquiring open contour measurements for every dentate gyrus in which total BrdU-labeled cell counts were made.

Double Fluorescent Immunohistochemistry

After blocking for nonspecific reactivity, adjacent series of 20- μ m-thick coronal brain sections were sequentially stained for a cell-lineage marker, followed by BrdU detection and a nuclear stain (Hoechst; Chemicon, Temecula, CA; 33342). Individual series were incubated in the primary antibodies: 1) goat anti-DCX (1:100; Santa Cruz Biotechnology, Santa Cruz, CA; SC-8067), 2) rabbit anti-GFAP (1:10; Immunostar, Hudson, WI), or 3) mouse antinestin (1:150; Chemicon; MAB353). For detection of BrdU incorporation, DNA was first denatured by incubation of slides with HCl (2 mol/liter) at 37°C for 0.5 hr and rinsed for 10 min in 0.1 M boric acid (pH 8.5). After washing in PBS, sections were incubated in mouse anti-BrdU (1:200; Roche, Indianapolis, IN; 1170376). Sections were fixed with 4% paraformaldehyde between markers. The secondary antibodies used were Alexa 594 (1:100; Invitrogen, Carlsbad, CA) for the progenitor cell markers and Alexa 488 (1:400; Invitrogen) for BrdU. Triple labeling was done with nuclear stain (1:2,000; Hoechst) to confirm the number of distinct nuclei colabeled with BrdU in a cluster of proliferating cells under high magnification. Slides were examined with an Olympus (Fluoview) confocal microscope using the FV 1000 confocal system based on an Olympus IX81 inverted microscope stand and wide-field fluorescence optics equipped with filter cubes for fluorophores absorbing in the UV, blue, green, and near-IR ranges.

Quantification of BrdU-Labeled Cells, Expression of Cell-Type Markers, and Colocalization With Newborn Cells

The perfusion, fixation, staining protocols (temperature, incubation length, poststaining storage), and time of data acquisition after coverslipping were kept constant among all three time points (P19, P26, and P33). Methods for quantification of SGZ-derived newborn cells in this model have been described previously (Kadam et al., 2008). To visualize the colocalization of lineage markers with BrdU labeling, in the SGZ, confocal Z stack images were taken with 1- μ m step sizes and 18–20 steps per section. Orthogonal views were used to confirm colocalization. Total counts of BrdU-positive cells were made in the DG, and colocalization with DCX, nestin, and GFAP was confirmed in bilateral hemispheres for three equidistant coronal sections from each series for each brain. Total counts of BrdU-positive cells were made at higher magnification ($\times 20$) using consecutive sections per brain series and average cell densities (i.e., BrdU cells per unit length of SGZ) calculated for each DG bilaterally. Thus, 100% sampling [i.e., equivalent to a sampling grid of counting frames adjacent to one another superimposed over the entire region of interest (ROI)] was done through entire ROI in the maximal number serial coronal sections ($n = 3$) in which the ROI could be clearly defined (i.e., both upper and lower blade of the DG present) in the ipsilateral injured DG (Manaye et al., 2007). The method as described previously by Manaye et al. is a modification of systematic random sampling, in which every cell of interest (i.e., BrdU-positive cell) in the ROI was counted. This approach is equivalent to a grid that has sampling windows adjacent to one another superimposed over the entire ROI. The aim of random sampling is to give every cell of interest an equal chance of being counted, and counting all the cells of interest in the ROI does not bypass this very important rule. In the DG, this was an obvious choice because of the low counts and a well-defined ROI, because the total counts of the cells of interest within the anatomical contours defining the SGZs of the DG were not beyond reasonable counts for a manual counting protocol. Therefore, total cell counts in the entire ROI were made and densities (cells/mm) reported to account for ipsilateral injury-related atrophy in the hippocampi. Some of the animals in the study had massive stroke-related atrophy (ranging from 70% to 100%); the ipsilateral

hippocampi in those sections did not have any or had only a couple BrdU-positive cells. Low BrdU counts were seen only in animals with severe hippocampal atrophy. Additionally, because three series of adjacent sections from the same brains were double-stained with BrdU and a cell type marker and showed consistent counts for BrdU-labeled cells for all mice in the study (sham and ligation-injured), we are confident that the low counts are not as a result of immunolabeling or injection problems. Similarly, in the SVZ, Z stack photomicrographs of immunostained sections were acquired. These stacks were fused, and Image J (U.S. National Institutes of Health; <http://rsb.info.nih.gov/ij/>, 1997–2006) software was used to quantify arbitrarily BrdU-positive cells and cell type marker expression in the SVZ of ligated and control brains. Analysis was done on both ipsi- and contralateral images of the anterorostral trigone of the SVZ at coordinate Bregma 0.9–1 mm for each brain (Paxinos and Franklin, 2001). Single-channel fluorescent images were first converted into binary formats, and intensity thresholds were kept constant for each fluorescent cell type marker. These upper and lower limits of threshold intensity were determined for each stain such that background was satisfactorily eliminated.

Data Analysis

Statistical analyses were performed in SPSS for Windows (SPSS Inc., Chicago, IL). One-way ANOVAs with post hoc tests (Bonferroni's multiple-comparisons test) were carried out to analyze means between the time points (P19, P26, and P33) as group factors. One-way ANOVAs were also used to analyze data comparing shams vs. ligation-injured animals at individual time points with the smaller population. Pairwise comparisons within a group were analyzed by Student's *t*-tests assuming unequal variances. Correlations were reported whenever statistical significance was noted. A probability below 0.05 was considered significant.

RESULTS

Injury Severity, Acute Seizure Scores, and SGZ Neural Proliferation

We have previously described the incidence and pattern of ischemic injury and acute poststroke seizures in this mouse model (Comi et al., 2004; Kadam et al., 2008). We found, similarly to the previous studies, a significant correlation between hippocampal and hemispheric atrophy scores of the ligated mice ($r^2 = 0.6$, $P = 0.03$). In this study, stroke-related atrophy scores for the three groups of ligated mice showed increasing trends for hippocampal (i.e., $61\% \pm 16\%$ at P19, $69.6\% \pm 15\%$ at P26, and $83\% \pm 4\%$ at P33) and hemispheric (i.e., $33.6\% \pm 6.9\%$ at P19, $47.7\% \pm 8.8\%$ at P26, and $43.6\% \pm 5.7\%$ at P33) injury that were not found to be significant. A significant overall positive correlation was found for severity of the brain injury quantified by hippocampal and hemispheric atrophy (see Materials and Methods) at P19, P26, and P33 with the acute seizure scores of the ligated mice (Fig. 2A; $r^2 = 0.6$, $P = 0.04$ and $r^2 = 0.65$, $P = 0.02$ for hippocampal and hemispheric atrophy, respectively). Measurement of mean SGZ lengths of the DGs in which BrdU-positive cells were quantified showed similar lengths in the ipsi- and contralateral DGs of control mice (i.e., $1,953 \pm 103$ and $1,674 \pm 11 \mu\text{m}$ at P19, $1,683 \pm 185$ and $1,619 \pm 113 \mu\text{m}$ at P26, and $1,928 \pm 52$ and $1,724 \pm 48 \mu\text{m}$ at P33, respectively). In the ligation-injured mice, mean SGZ lengths were considerably shorter and more variable ipsilaterally (893 ± 288 at P19, $1,261 \pm 325$ at P26, and $856 \pm 181 \mu\text{m}$ at P33) and were significantly shorter at P19 and P33 ($P = 0.01$ and $P = 0.001$ respectively). The mean contralateral SGZ lengths were shorter than control at P19 and P33, but not significantly ($1,468 \pm 100 \mu\text{m}$ at P19, $P = 0.09$; $1,867 \pm 257 \mu\text{m}$ at P26, $P = 0.4$; and $1,573 \pm 121 \mu\text{m}$ at P33, $P = 0.3$). There was an overall negative correlation between the acute seizure scores and the mean lengths of the SGZ contours measured in micrometers in the hippocampi of ligated mice bilaterally, but they were not significant ($r^2 = -0.41$, $P = 0.18$ and $r^2 = -0.46$, $P = 0.11$ ipsi- and

contralaterally, respectively). Thus the overall acute seizure scores in the model were predictive of the severity of the stroke injury (i.e., as quantified by overall hemispherical and hippocampal atrophy) but not of the shortening of the lengths of ipsilateral SGZs. This finding may be indicative of the relative sparing of the DG compared with the Ammon's horns in the stroke injury-related hippocampal atrophy in the model (see CV-stained sections in Fig. 4).

Temporal profile of SGZ-derived neural proliferation—In control mice, the mean BrdU-positive cell counts in the ipsilateral SGZ when normalized to the unit lengths of their SGZs (i.e., cells per 1 mm of SGZ) were 8.46 ± 1.16 at P19, 7.6 ± 1.7 at P26, and 5.2 ± 0.5 at P33 (Fig. 2B, black bars). These counts were similar to counts/mm in the contralateral SGZ (Fig. 2C, gray bars) at all time points investigated (10.7 ± 0.8 , 7.3 ± 1 , and 5 ± 0.7 , respectively). Therefore, BrdU-labeled cell counts in control CD-1 mice at P33 were ~50% lower than counts noted at P19 in the SGZ. Ipsilateral BrdU-labeled cell counts per unit length of SGZ in ligation-injured mice were found to be lower than control at all three time points [2.5 ± 1.2 at 7 DAI (P19), 4.9 ± 0.4 at 14 DAI (P26), and 2.8 ± 0.9 at 21 DAI (P33); Fig. 2C, black bars] and significantly lower at 7 DAI ($P = 0.013$). At 14 and 21 DAI, BrdU-labeled cell counts per unit length of SGZ were also lower, but not significantly ($P = 0.2$ and $P = 0.07$, respectively). Contralaterally (Fig. 2C, gray bars) SGZ-derived BrdU-labeled cell counts per unit length of SGZ were comparable to those of controls. Over time, the BrdU-labeled cell counts per unit length of the contralateral SGZ in ligation-injured mice also showed an age-dependent reduction (10.5 ± 1.53 at 7 DAI, 6.2 ± 1 at 14 DAI, and 4.4 ± 0.8 at 21 DAI) similar to the trend seen in controls. The age-dependent decline in SGZ cell proliferation/mm of SGZ was significant between P19 and P33 both in sham controls ($P = 0.003$; Fig. 2B) and in contralateral SGZs of ligation-injured mice ($P = 0.008$; Fig. 2C).

Counts of BrdU-labeled cells (total counts and counts normalized to SGZ lengths) correlated to injury severity—A significant correlation was noted between the mean counts (i.e., BrdU-labeled cells per section) of newborn cells in the ipsilateral SGZ and injury severity both in the hippocampus and in the hemispheres of the ligation-injured group of mice (Fig. 2D; $r^2 = -0.74$, $P = 0.004$ and $r^2 = -0.63$, $P = 0.02$, respectively). However, no significant correlations were found between mean BrdU-labeled cell counts (i.e., BrdU-labeled cells per section) in the contralateral SGZ and the severity of the stroke injury (Fig. 2E). Therefore, severity of the stroke injury was predictive of the reduction of proliferation in the ipsilateral neurogenic pool in the SGZ (Fig. 2F) but not in the contralateral neurogenic pool when total cell proliferation counts were compared. When counts of BrdU-labeled cells were normalized to the SGZ lengths of the DGs in which the total counts were made, a negative correlation of cell/mm SGZ to the severity of both their hippocampal and their hemispherical injuries remained but were no longer significant ($r^2 = -0.4$, $P = 0.1$ and $r^2 = -0.3$, $P = 0.3$, respectively). No correlations were found contralaterally.

Colabeling of SGZ newborn cells with cell type markers—Because there was a 2-hr time window between the BrdU injection and the perfusion fixation, we chose the three cell type markers that were most relevant to the BrdU-labeled cell types expected to be labeled by BrdU in that time frame. For all the control brains taken together, the percentage of newborn cells in the SGZ that colabeled with each of the cell type markers showed that ~90% of the newborn cells colabeled with nestin, 20–30% colabeled with GFAP, and very few colabeled with DCX. These percentages were similar to those described for the adult SGZ-derived neurogenesis described by Kempermann et al. (2004).

For control CD-1 mice, we found that at all time points tested most of the newborn cells in the SGZ colabeled with nestin bilaterally (i.e., 88% and 90% at P19, 87% and 87% at P26,

and 89% and 91% at P33 ipsi- and contralaterally, respectively; Fig. 3A, Table I). Percentage cell colabeling with GFAP in control CD-1 mice was 17% and 18% at P19, 24% and 28% at P26, and 22% and 23% at P33 ipsi- and contralaterally, respectively (Fig. 3B, Table I). Very few cells were found to colabel with the immature neuronal marker DCX in control CD-1 mice (i.e., 4% and 4% at P19, 11% and 10% at P26, 10% and 16% at P33 ipsi- and contralaterally, respectively; Fig. 3C, Table I). This distribution of percentage cell types did not change considerably in the ligated mice in the contralateral, uninjured hippocampus. Ipsilaterally, percentage nestin colabeling was not significantly different from control ($63.7\% \pm 22\%$ at 7 DAI, $68.9\% \pm 23\%$ at 14 DAI, and $89.9\% \pm 6\%$ at 21 DAI). In ligation-injured animals, there was a wider variability in nestin-BrdU colabeling that was not seen in controls at 7 and 14 DAI and that returned to control levels by 21 DAI. In injured animals, GFAP colabeling was $29.2\% \pm 11.6\%$ at 7 DAI, $19.9\% \pm 6.8\%$ at 14 DAI, and $62.3\% \pm 11\%$ at 21 DAI ipsilaterally and significantly higher compared with control ($P = 0.03$) at 21 DAI. Also, percentage GFAP colabeling at P33 was significantly higher than at P26 ipsilaterally and at P19 contralaterally ($P = 0.049$ and $P = 0.01$, respectively; number signs in Table I). Percentage DCX colabeling was $1.5\% \pm 0.9\%$ at 7 DAI, $8.6\% \pm 3.2\%$ at 14 DAI, and $12.9\% \pm 6.9\%$ at 21 DAI in the ipsilateral, injured hippocampus. Percentage DCX-positive newborn cell counts at 7 DAI were lower but not significantly different from control, and percentages DCX-positive newborn cells at 14 and 21 DAI were comparable to control. Overall, cell type marker expression in the entire hippocampus was considerably higher for GFAP in the injured side [Fig. 4, compare middle column (ipsilateral injured) with right column (ipsilateral control)] compared with nestin and DCX, which were restricted to the DG (data not shown). This most likely represented GFAP-labeled reactive astrocytes in the hilus and regions of small-cell infiltration representing gliotic scarring seen in the adjacent coronal sections stained with CV. In contrast, nestin and DCX expression remained restricted to the SGZ and pyramidal cell layers of the dentate gyrus.

Injury Severity, Acute Seizure Scores, and SVZ Neural Proliferation

Correlations between arbitrary counts of BrdU-positive cells (see Materials and Methods) in the SVZ were not found to be significant with either acute seizure scores or the severity of the stroke injury in the hemispheres or the hippocampi. At the 2-hr time point, BrdU-positive cells were seen to be restricted to the SVZ and the SVZ anterolateral trigone. At all three time points, coronal sections from both sham and ligated groups of mice showed the occasional BrdU-positive cell in the deep gray matter, but none was seen in the neocortex. These few cells seen in the deep gray matter may represent a scarce population of local progenitors.

Temporal profile of SVZ-derived neural proliferation

The amount of SVZ-derived neural proliferation showed an age-dependent decline in control CD-1 mice (see Fig. 7A; see also Supp. Info. Fig. 1, right column) that was comparable ipsi- and contralaterally [i.e., arbitrary Image J counts done on SVZ trigone were 153 ± 19 and 163 ± 23 at P19, 82 ± 11 and 71 ± 4 at P26, and 69 ± 7 and 75 ± 14 at P33 ipsi- and contralaterally, respectively]. SVZ BrdU-labeled cell counts at P33 were ~50% of counts at P19, reaching a plateau between P26 and P33 (ipsilateral group factor significance $P = 0.01$ and 0.005 , respectively; Supp. Info. Fig. 1, right column; contralateral group factor significance $P = 0.008$ and $P = 0.011$, respectively). Compared with controls, arbitrary counts in the ligated mice (see Fig. 7B; see also Supp. Info. Fig. 1, left and middle columns) showed an enhanced amount of SVZ neural proliferation ipsi- more than contralaterally that was highest at 7 DAI (ipsilateral group factor significance $P = 0.01$ between P19 and P33) and returned to control levels by 21 DAI [i.e., 333 ± 80 and 233 ± 93 at 7 DAI (P19), 139 ± 69 and 118 ± 36 at 14 DAI (P26), and 46 ± 7 and 58 ± 17 at 21 DAI (P33), respectively].

Cell-type marker expression in the SVZ

GFAP marker expression profiles remained stable in bilateral SVZ of control CD-1 mice (see Fig. 7C); no age-dependent changes were evident ipsi- or contralaterally over the time points investigated (i.e., 767 ± 78 and 732 ± 60 at P19, 695 ± 148 and 877 ± 227 at P26, and 906 ± 174 and 733 ± 100 at P33, respectively). Compared with controls, in ligation-injured mice (see Fig. 7D), there was a marked increase in total GFAP expression ipsi- more than contralaterally [i.e., $1,519 \pm 91$ (200%) and 878 ± 194 at 7 DAI (P19), $1,096 \pm 124$ and 955 ± 74 at 14 DAI (P26), and $1,166 \pm 223$ and 866 ± 114 at 21 DAI (P33), respectively] in and around the SVZ (i.e., area analyzed with Image J was the entire micrograph depicted in Fig. 5 for all sections). This increase was significantly higher at P19 in the ipsilateral SVZ compared with control ($P = 0.001$).

In contrast, nestin expression was restricted to the SVZ (Fig. 6A) and showed a significant age-dependent decline (Fig. 7E; ipsilateral group factor significance $P = 0.01$ between P19 and P33) in the control mice (i.e., 500 ± 47 and 436 ± 56 at P19, 348 ± 49 and 412 ± 69 at P26, and 279 ± 20 and 298 ± 42 at P33 ipsi- and contralaterally, respectively). Compared to that in ligated mice (Figs. 6A, 7F), nestin expression rose and remained elevated ipsilaterally (significant at P33 compared with controls, $P = 0.004$), whereas contralaterally nestin expression exhibited a temporary elevation at 7 DAI (contralateral group factor significance $P = 0.04$ and $P = 0.01$ compared with P26 and P33, respectively) and declined over time [i.e., 807 ± 156 (160%) and 620 ± 71 at 7 DAI (P19), 521 ± 88 (150%) and 397 ± 58 at 14 DAI (P26), and 580 ± 60 (200%) and 358 ± 25 at 21 DAI (P33) ipsi- and contralaterally, respectively].

The immature neuronal marker DCX expression (Fig. 6B), as with nestin expression, was restricted to the SVZ and also showed an age-dependent decline (Fig. 7G) both ipsi- and contralaterally in control CD-1 mice (i.e., 455 ± 55 and 333 ± 50 at P19, 139 ± 14 and 77 ± 21 at P26, and 111 ± 35 and 103 ± 21 at P33, respectively), which reached a plateau between P26 and P33. Ligated mice (Fig. 7H) showed a marked increase in DCX expression ipsilaterally [$1,339 \pm 433$ (300%) at 7 DAI (P19), 360 ± 128 (260%) at 14 DAI (P26), and 240 ± 122 (200%) at 21 DAI (P33)]. Contralateral DCX expression was not dissimilar from control (419 ± 79 at 7 DAI, 270 ± 92 at 14 DAI, and 123 ± 12 at 21 DAI). Both sides showed an age- and time-dependent decrease (Fig. 7G; group factor significance $P = 0.005$, P19 vs. P26 and P33 bilaterally; Fig. 7H, group factor significance P19 vs. P33 $P = 0.03$ and $P = 0.02$ ipsiand contralaterally, respectively) in DCX expression.

Colocalization of BrdU-positive cells with cell type markers in the SVZ could not be accurately quantified because of the large number of BrdU-positive cells crowded within the trigone of the anterior horn of the lateral ventricle. However, similarly to the colocalization profiles detected in the SGZ, most of the newborn cells were observed to colocalize with nestin at all three time points. A subset of the newborn cells colocalized with GFAP at all time points investigated. These GFAP-BrdU cells often were subependymal in location and may represent the pool of type B cells. No to very few newborn cells were seen to colabel with DCX at the 2-hr time point.

The method of image J analysis used in this study to quantify arbitrarily BrdU-positive cells and cell type marker expression in the SVZ resulted in low variability within the sham control groups at all three time points. These results justified confidence for the arbitrary quantifications acquired using the same parameters within sections from the experimental brains with variable severity of injury and larger variability in Image J arbitrary values. Previous studies using Image J for automated quantification of fluorescently tagged cell somas using similar protocols revealed nearly identical neuron counts and trends of anatomical changes similar to those scored manually (Liu et al., 2005). Cell counts in the

SVZ were an issue in our study, because the large number and clumping of the dividing cells in the region made them difficult to count accurately, which is why we went to the Image J method.

Influence of Animal Gender on Poststroke Injury and Neural Proliferation

The mice in the ligation-injured group did not show significant sex differences (male $n = 8$, female $n = 5$) in their mean hippocampal ($78\% \pm 7.5\%$ and $62.8\% \pm 13\%$, respectively) percentage atrophy scores. The difference in hemispherical percentage atrophy scores ($48\% \pm 5\%$ and $31.8\% \pm 4.2\%$ males and females, respectively) were marginally significant ($P = 0.048$). Acute seizure scores in ligated mice were not significant by gender. Endogenous neural proliferation counts in the SGZ and SVZ were not significantly different by gender, nor were the effects on poststroke neural proliferation significantly different by gender at any of the three time points investigated.

DISCUSSION

The aim of the present study was to characterize the poststroke neural proliferation in this new mouse model of neonatal ischemic strokes by studying the trends as a function of time. The areas of interest were neurogenic niches in the ipsilateral injured and contralateral uninjured SGZ and in the ipsi- and contralateral SVZ. This study has four findings: 1) In line with the age-dependent decrease of neurogenesis detected in rats (Kuhn et al., 1996; Seki and Arai, 1995), control CD-1 mice showed age-dependent reduction in BrdU-labeled cells both in the SGZ and in the SVZ, which at P33 was ~50% of the counts at P19. 2) In ligated mice, SGZ cell proliferation was persistently negatively modulated at all three time points investigated, suggesting long-term impairment of the process in the injured hippocampi. In contrast, contralateral uninjured hippocampi showed proliferation rates comparable to control. 3) In ligated mice, SVZ cell proliferation was higher than control at 7 DAI ipsilaterally but returned to control levels by 21 DAI. 4) Percentage cell type distribution profiles in the SGZ did not change drastically between ligated and control mice for nestin- or DCX-positive newborn cells. However, a significantly higher percentage of GFAP-positive BrdU-labeled cell counts was noted ipsilaterally at 21 DAI. In summary, as noted by others investigators, SVZ cell proliferation was enhanced in response to the neonatal insult (Arvidsson et al., 2002; Goings et al., 2004; Plane et al., 2004; Hayashi et al., 2005), whereas SGZ neural proliferation was lowered ipsi- but not contralaterally.

Prior studies with this model of unilateral ischemia unexpectedly found that, in addition to ipsilateral SGZ neurogenesis, contralateral neurogenesis in the SGZ was also negatively modulated for cells born 1 week after ischemia that survived maturation and integration for 3 weeks following BrdU labeling. These findings could have resulted from either decreased SGZ neural proliferation or decreased survival of the newborn neurons. From the present study, we can conclude that the previously reported long-term decrease in SGZ neurogenesis results, at least in large part, from a poststroke decrease in SGZ neural proliferation ipsilaterally. We also wanted to know whether this decrease in SGZ neural proliferation was a transient or sustained phenomenon at temporally distant time points as the neonatal brain matured. The study reported here reveals that the comparable proliferation rates seen contralaterally may indicate poor maturation and survival over time. Ipsilaterally, SGZ poststroke neural proliferation remained low at all three time points; this suggests that therapeutic measures introduced after the stroke that are also neurally proliferative could boost ipsilateral SGZ neurogenesis and help prevent loss of new neurons during maturation contralaterally.

With the current study, the SVZ neural proliferation at 7 DAI was clearly higher than control, ipsi- more than contralaterally, indicating that SVZ neural proliferation was

enhanced for a specific period after the insult in the model. We can conclude based on these results that the poststroke increased long-term SVZ-derived neurogenesis previously reported results in large part from a poststroke increase in neural proliferation. This transient poststroke enhancement in neural proliferation, together with findings from our chronic survival studies showing migration of SVZ-derived BrdU-labeled cells into the injured cortex and striatum (Kadam et al., 2008), is encouraging. Future studies aimed at modulating the cell fate commitment or survival of the newborn cells could take advantage of this window, which likely starts soon after the stroke but returns to control levels by P26. In addition, the age-dependent decline of neuroblast proliferation rates seen in controls and mirrored in the contralateral uninjured hemispheres of ligated mice further defines the advantageous window for intervention present in the developing brain with a higher capacity for neuroregeneration compared with a juvenile brain.

BrdU is available for ~30 min (Packard et al., 1973) after an injection, so only a proportion of dividing cells is labeled by the single-injection protocol used in this study. Also, the duration of the S phase for proliferative cells in the dentate gyrus of the mouse has been estimated to be ~8 hr (Nowakowski et al., 1989). Therefore, by perfusing the mouse brains at 2 hr following the BrdU injection, we labeled the pool of dividing progenitor cells that were in the DNA synthetic phase of the cell cycle for ~1.5–2 hr prior to fixation. Because this pool of BrdU-labeled cells would not have had adequate time to show mature cell commitment markers, early progenitor cell markers (nestin, GFAP, and DCX) that label the type 1, 2a, 2b, and 3 cells (Kempermann et al., 2004) were examined. These cell types represent the first four of six total developmental milestones of hippocampal neurogenesis, with type 1 cells (GFAP/nestin⁺) representing the stem cell pool (Gage et al., 1995). Type 2a (GFAP⁻/nestin⁺), type 2b (nestin⁺/DCX⁺), and type 3 (nestin⁻/DCX⁺) cells represent the next three consecutive stages of transiently amplifying putative progenitor cells that also represent increasing neuronal differentiation. The type 3 cells denote the end of the cell cycle, which is followed by the postmitotic state, in which cell maturation and survival dictate cell fate. Type 1 cells have been shown to divide into intermediate GFAP⁺ progenitors (i.e., type D cells described by Seri et al., 2001), and investigation of these subtypes in future studies may provide valuable insight into post-stroke susceptibility and modulation of rates of proliferation of these subsets that survive the ischemic insult.

In this study, ~90% of the BrdU-labeled cells in the SGZ of control mice were nestin positive, which likely represented the entire population of GFAP⁺ progenitors [i.e., radial glial (type 1) and intermediate type D cells] and the putative progenitors with undetermined (type 2a) or determined (type 2b) lineages [Gage et al., 1995; for details see review by Kronenberg et al., 2003 (Figs. 1, 3)]. The 20–30% BrdU-labeled cells in the SGZ that colabeled with GFAP likely represent the type 1 progenitors and their intermediates and therefore were a subset of the nestin-positive pool (Filippov et al., 2003). This indicates that the remaining 60–70% of the nestin-positive newborn cells were either type 2a or type 2b cells. The DCX-positive newborn cells amounted to 5–10% of the BrdU-labeled cell population. Insofar as both type 2b and type 3 cells show DCX expression, a portion of this DCX-positive pool represented a subset of the nestin-positive pool (type 2b). The remainder represented the pool of type 3 DCX-positive but nestin-negative newborn cells. Approximately 10% of the BrdU-labeled cell population did not colabel with nestin; some of these cells might have been the type 3 DCX-positive-only cells. Insofar as all three cell types are putative progenitors, the larger pool of putative type 2a cells may represent the higher capacity for proliferation reported for this cell type compared with the type 1 (i.e., putative neural stem cell), type 2b, and type 3 cells (Kronenberg et al., 2003). Type 1 cells have been described (Kronenberg et al., 2003; Kempermann et al., 2004) to have low proliferative activity (Seri et al., 2001) and to have pyramidal somas with a dominant apical process traversing the granule cell layer, with processes in the inner molecular layer.

Therefore, by staining with nestin, GFAP, and DCX, the new cell populations labeled <2 hr before fixation in our study showed early cell type proliferation profiles in immature CD-1 mice similar to those found in prior studies (Gage et al., 1995; Filippov et al., 2003; Kronenberg et al., 2003) that have characterized the early stages of SGZ neurogenesis.

The ligated mice used in this study did not show significant differences from controls, for the profile described above, except for significant increases of percentage GFAP-positive newborn cells at P33. This may suggest reversal of more committed progenitors (types 2a and 2b) back to more immature forms (i.e., radial glial cell types or cell type 1) over time to compensate for the consistently lowered neural proliferation in the ipsi-lateral SGZ or symmetric divisions of type 1 cells to replenish the neural stem cell pool. However, the mean percentage nestin-positive newborn cells was about 60–70% at 7 and 14 DAI ipsilaterally; this may also indicate suppression of the type 2a proliferation in the injured hippocampi. Percentage nestin-positive newborn cells returned to match control by 21 DAI; this may indicate recovery of the proliferation capacity back to control levels by 3 weeks. Seaberg and van der Kooy (2002) have previously used culture methods to show that the stem cells derived from the lateral ventricle and other ventricular subependymal regions directly adjacent to the hippocampus exhibit long-term self-renewal and multi-potentiality and can produce separate neuronal and glial progenitors compared with the limited self-renewal capacity of SGZ-derived cell proliferation. This suggests that neuron-specific progenitors and not multipotential stem cells are the source of newly generated DG neurons throughout adulthood. This may explain the paradoxical response of the SVZ cell proliferation compared with that of the SGZ and the absence of an altered cell type commitment profile following stroke in the SGZ.

An important feature of this neonatal mouse model is the occurrence of acute seizures following the stroke; this feature models the frequent presentation of neonatal stroke with acute seizures. A significant finding of this study was the predictive value of the acute seizure scores for the severity of the stroke injury in the hippocampus (i.e., overall atrophy). The negative but not predictive correlation of acute seizure scores to the lengths of SGZs in the injured brains emphasizes the nonuniform stroke injury within subpopulations of primary neurons in the DG. Severe seizures during early childhood have been associated with adverse effects on postnatal granule cell neurogenesis (McCabe et al., 2001; Mathern et al., 2002). Therefore, in addition to the ischemic stroke, the acute seizures may affect early brain development. The negative modulation of the neural maturation after long survival delays in our previous study (Kadam et al., 2008) may among other possibilities be a reflection of the adverse effect of seizures on cell proliferation, survival, and maturation. Additionally, the reduced neurogenesis may play a pivotal role in epileptogenesis following perinatal brain injury. The data show that long-term proliferation rates were affected by the ligation injury. Future studies to investigate the effect of successful acute seizure suppression following stroke on postnatal neurogenesis are planned. Interventions that help both to reduce the ischemic injury and to block the acute seizures in this model may provide valuable insight into neonatal stroke management.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
Diagram of the experimental protocol.

Fig. 2.

Stroke injury, seizure scores, and SGZ neural cell proliferation. **A:** Significant positive correlations were noted between the acute seizure scores in ligated animals and the severity of their stroke injuries. **B,C:** Mean counts of BrdU-positive cells per unit length of SGZ (cells/mm) in the ipsilateral (black bars) and contralateral (gray bars) DGs in control and stroke-injured mice with significant negative modulation in ipsilaterally injured hippocampi at P19. **D:** Significant negative correlation of severity of injury to the reduction of ipsilateral SGZ-derived neural proliferation was seen ipsilaterally but not contralaterally (**E**) in stroke-injured mice. **F:** Representative DGs at P26 with BrdU-labeled cells in the SGZ counterstained with Hoechst. Black bars, hippocampal atrophy; gray bars, hemispheric atrophy (applies to A,D,E); * $P = 0.013$ at P19. Scale bar = 250 μm .

Fig. 3.

Representative colabeling of BrdU-positive cells with cell type markers in the SGZ of the dentate gyrus at P26. **A–C** are confocal stacked images with orthogonal views to ascertain colocalization of the SGZ along the X and Y axis, showing representative colabeling of BrdU-positive nuclei with nestin (**A**), GFAP (**B**), and DCX (**C**). Panels at right are single-channel views of the double labeling with the red channel converted to magenta for better viewing by color-blind readers and BrdU labeling (green). Scale bar = 25 μ m.

Fig. 4. GFAP expression in ipsilateral injured hippocampi compared with control. Left panel of CV-stained coronal sections shows stroke-related injury in representative samples from all three time points, demonstrating the relative sparing of the DG compared with the Ammon's horns (especially CA3 and CA1). Middle panel has consecutive sections from the same brains showing GFAP-labeled sections that demonstrate the profile of GFAP expression in reactive astrocytes as well as astrocytic infiltration of the dentate hilus compared with controls (right panel) at all three time points ($\times 10$ magnification). Scale bar = 250 μm .

Fig. 5.

A: CV-stained coronal sections showing the SVZ underlying the rostral horn of the lateral ventricle, which is a known niche of neural progenitors. **B:** Corresponding adjacent coronal sections showing the same region with BrdU-labeled proliferating cells (green) and GFAP expression levels (red) in the SVZ using immunohistochemistry ($\times 10$ magnification). Up-regulation of GFAP expression in and around SVZs of injured brains at all time points compared with controls represents reactive astrocytes forming the gliotic scars in and around injured areas. Scale bar = 250 μm .

Fig. 6. Nestin and DCX expression levels in the SVZ from representative coronal sections. Both markers are seen to be restricted to the SVZ and SVZ trigone. This reveals the expansion of the SVZ trigone compared with controls at 7 DAI, which decreases over time in the injured brains. Nestin (**A**) expression was seen to go up at 7 DAI and to remain elevated compared with the contralateral SVZ, where it showed a trend of returning to control levels. DCX (**B**) showed increased expression at 7 DAI but showed trends of returning to control levels bilaterally by 14 DAI. Scale bar = 250 μ m.

Fig. 7.

A–H: Temporal profile of SVZ neural proliferation and cell type marker expression. Image J analysis of expression profiles of BrdU and cell type marker expression at 7, 14, and 21 DAI quantified the trends seen at the three poststroke time points, examples of which are represented by the micrographs in Figures 3–5. Black number sign, significantly different between groups ipsilaterally; gray number sign, significantly different between groups contralaterally. *Significantly different ispi- vs. contralateral within group.

TABLE I

Mean Percent of Total BrdU Positive Cells that Co-labeled with each Cell-type Marker in the SGZ

	Control	Ligates-contralateral	Ligates-ipsilateral
P19(7DAI)			
BrdU-nestin	88.2 ± 3	87.6 ± 4.2	63.7 ± 22
BrdU-GFAP	17.5 ± 5	16 ± 2.2	29.2 ± 11.6
BrdU-DCX	3.8 ± 1.6	8.6 ± 1.5 (<i>P</i> =0.05)	1.5 ± 0.8
P26 (14DAI)			
BrdU-nestin	87.2 ± 6.4	90.3 ± 3.4	68.9 ± 23.2
BrdU-GFAP	23.8 ± 3.25	24.4 ± 3	19.9 ± 6.8
BrdU-DCX	10.8 ± 4.3	10.1 ± 1.9	8.6 ± 3.1
P33(21DAI)			
BrdU-nestin	89.5 ± 2.7	82.9 ± 5.5	90 ± 6.1
BrdU-GFAP	22.5 ± 7.7	32.9 ± 4	62.3 ± 11.5 (<i>P</i> <0.03)
BrdU-DCX	10 ± 4.3	14.1 ± 3	13 ± 6.9