Nestin-CreER Mice Reveal DNA Synthesis by Nonapoptotic Neurons following Cerebral Ischemia–Hypoxia

The standard method of detecting neurogenesis uses bromodeoxyuridine (BrdU) to label DNA synthesis followed by double labeling with neuronal markers. However, DNA synthesis may occur in events unrelated to neurogenesis including aneuploidy and abortive cell cycle reentry. Hence, it is important to confirm neurogenesis with methods other than BrdU incorporation. To this end, we have generated transgenic nestin-CreERT2 mice that express tamoxifen-inducible Cre recombinase under the control of a nestin enhancer. When crossed with a ubiquitous Enhanced Green Fluorescent Protein (EGFP)-Cre-reporter line, the bitransgenic animals can reveal the nestin-positive progenitors and their progeny with EGFP after tamoxifen induction. This system has many applications including visualization of embryonic neural progenitors, detection of postnatally transformed radial glial cells, and labeling adult neural progenitors in the subventricular zone (SVZ). To examine the contribution of SVZ progenitors to cell replacement after stroke, tamoxifen-induced mice were challenged with focal ischemia or combined ischemia–hypoxia followed by BrdU injection. This analysis revealed only very few EGFP-positive cells outside the SVZ after focal ischemia but robust DNA synthesis by hippocampal neurons without immediate cell death following ischemia–hypoxia. These results suggest that the nestin-CreERT2 system is a useful tool for detecting embryonic and adult neurogenesis. They also confirm the existence of nonproliferative DNA synthesis by old neurons after experimental brain injury.

Keywords: BrdU, ischemia–hypoxia, lineage tracing, MCAO, neural stem cells, tamoxifen

Introduction

Adult neurogenesis is often studied using bromodeoxyuridine (BrdU) or other thymidine analogues to label DNA synthesis in nascent neurons during the S-phase of the cell cycle. Although BrdU immunocytochemistry is a powerful method, it has shortcomings that may complicate the study of adult neurogenesis after brain injury (Nowakowski and Hayes 2000; Rakic 2002; Kempermann 2005). For example, BrdU incorporation merely indicates DNA synthesis, which may occur in old neurons after cell cycle reentry or in events leading to aneuploidy (Rehen et al. 2001; Herrup et al. 2004). Furthermore, the existence of viable polyploid neurons in normal brains suggests that nonproliferative DNA synthesis may not lead to immediate cell death (Kingsbury et al. 2005). We have recently shown that the combination of cerebral ischemia and hypoxia stimulates DNA synthesis in damaged neurons (Kuan et al. 2004). Hence, in studies of disease and injury, it is particularly important to verify de novo neurogenesis using methods other than BrdU incorporation.

Due to these limitations of BrdU immunocytochemistry, several genetic methods have recently been developed for detecting neurogenesis. An established method is to infect neural progenitors with replication-deficient retrovirus carrying a lineage marker such as the green fluorescence protein (GFP) (Noctor et al. 2001; van Praag et al. 2002). However, this method requires precise injection of the virus into embryos or animals, which can become technically demanding or label only a small number of progenitors. Another useful tool is the transgenic nestin-GFP mice, which express GFP under the control of the nestin promoter or enhancer sequence (Yamaguchi et al. 2000; Kawaguchi et al. 2001; Mignone et al. 2004). Although nestin-GFP mice allow visualization of neural progenitors, they have limited use for lineage tracing because the descendants no longer express GFP after the progenitor-specific nestin gene is downregulated. Recently, a transgenic tetracycline-inducible Cre system was introduced to label nestin-positive progenitors and their progeny (Yu et al. 2005). However, this system is complicated by the requirement of 3 separate transgenes (TTA, TetOp-Cre, and the Cre-reporter gene) to detect the descendants of nestin-positive progenitors.

Recently, the tamoxifen-inducible Cre/loxP recombination system has shown promise as a new strategy to label specific progenitors and their descendants. In this system, transgenic mice are generated using a cell type–specific promoter to express Cre recombinase fused with a tamoxifen-specific binding domain of mutated estrogen receptor (Cre-ERT1 or Cre-ERT2) (Imai et al. 2001; Hayashi and McMahon 2002). When crossed with a ubiquitous Cre-reporter line, the resulting bitransgenic animals allow for temporally controlled lineage tracing after tamoxifen induction. This system has been used to detect adult neural progenitors that respond to Sonic hedgehog signaling in Gli1-CreERT2 mice (Ahn and Joyner 2005), as well as the progeny of neural progenitors using the nestin promoter and enhancer sequences in nestin-CreERT2 mice (Carlen et al. 2006; Imayoshi et al. 2006). We have independently generated nestin-CreERT(T1) mice. Here we report that the nestin-CreERT system can be used in different ways for studying neurogenesis. We have also used this method in conjunction with BrdU immunocytochemistry to demonstrate the existence of DNA synthesis by nonapoptotic neurons in the hippocampus following ischemia–hypoxia brain injury.

Materials and Methods

Generation of Transgenic Mice

A 1.5-kb polymerase chain reaction (PCR) product containing the nestin second intron enhancer/hsp68 minimal promoter (a gift of Dr S.
Goldman) was subcloned into the pcDNA3 vector using the BgIII/HindIII sites, thus replacing the cytomegalovirus promoter. A 1.6-kb PCR product encoding Cre recombinase fused to the mutant estrogen receptor (a gift of Dr A. McMahon) was cloned into the nestin/pcDNA3 construct using the NotI/ApaI sites. The nestin/hsp68-CreER construct was released from its backbone using HindIII and then injected into the pronuclei of fertilized C57BL/6 eggs by the in-house transgenic mouse facility. Founders were detected by both PCR and Southern blot analysis using a 500-bp probe within the region encoding Cre recombinase.

Tamoxifen Treatment and Histology
To induce Cre activity in bitransgenic (nestin-CreER and CAG-CAT-EGFP) mice, pregnant dams were induced with a single dose of tamoxifen (Sigma, St Louis, MO) dissolved in corn oil (5 mg per 40 g body weight) on the indicated embryonic days, and adult animals were injected once daily for 4 days with 4 mg per 30 g body weight of tamoxifen dissolved in corn oil. Adult animals were sacrificed by overdose of Avertin, followed by transcardiac perfusion of 4% paraformaldehyde. Brains were postfixed overnight in 4% paraformaldehyde, cryoprotected using 30% sucrose, embedded in Tissue-Tek Optimal Cutting Temperature Compound (Sakura Finetek USA, Torrence, CA), and flash frozen using 2-methyl-butane chilled with dry ice. Frozen brains were sectioned using a cryostat at either 16 μm thickness for mounting on slides or 50 μm for free-floating staining. Standard histological techniques were employed using the following antibodies: EGFP (Molecular Probes, 1:1500), nestin (Chemicon, Temecula, CA, 1:1000), Glutamate Aspartate Transporter (GLAST) (Chemicon, Temecula, CA, 1:4000), glial fibrillary acidic protein (GFAP) (Dako, Glostrup, Denmark, 1:1000), tyrosine hydroxylase (Chemicon, Temecula, CA, 1:1000), doublecortin (DCX) (Chemicon, Temecula, CA, 1:4000). NeuN (Chemicon, Temecula, CA, 1:1000). Secondary/tertiary antibodies used included biotinylated anti-mouse, anti-rabbit, and anti-guinea pig (Vector Labs, Burlingame, CA 1:400); AlexaFluor488-conjugated anti-rabbit (Molecular Probes, Eugene, OR 1:500); and AlexaFluor594-conjugated streptavidin (Molecular Probes, Eugene, OR 1:1000). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) stain was performed as previously described (Kuan et al. 2004). For double labeling with BrdU, the second epitope was stained for, as usual, to completion. This was followed by acid treatment (2 N HCl and 0.5% Triton-X100 diluted in phosphate-buffered saline [PBS]) for at least 1 h to denature DNA/expose BrdU epitope, washed with PBS, and stained by standard protocol thereafter using rat anti-BrdU (Serotech 1:100) followed by biotinylated anti-rat (Vector Labs, Burlingame, CA 1:100), and AlexaFluor594-conjugated streptavidin. All tissues prepared for fluorescence microscopy were counterstained with 4’,6-diamidino-2-phenylindole (Molecular Probes, Eugene, OR), and mounted using Vectashield (Vector Labs, Burlingame, CA) to preserve fluorescence. Samples were visualized using either an Olympus epifluorescent microscope (BX-51) or a Zeiss confocal microscope (LSM 510).

Organotypic Slice Cultures
Slice cultures were performed as previously described with minor modifications (Gal et al. 2006). Briefly, EGFP-positive embryos were removed from the uterus on E12.5 by a C-section and quickly immersed in ice-cold artificial cerebrospinal fluid (126.0 mM NaCl, 3.0 mM KCl, 1.3 mM MgSO4, 2.5 mM CaCl2, 1.2 mM Na2HPO4, 26 mM NaHCO3, and 20 mM dextrose). Brains were supported with 3% low melting agar and cut into 250 μm thickness for a vibraslicer (WPI, Sarasota, FL). Subsequently, slices were gently separated and moved to collagen-coated culture inserts (Millipore, Bedford, MA) over 1 ml Neurobasal media (GIBCO, Grand Island, NY; supplemented with 5% B27 and 1% N2 supplements, 1% Glutamak, 10% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and 0.5% penicillin/streptomycin) and incubated at 37 °C and 5% CO2 atmosphere.

Multiphoton Live Imaging
Multiphoton Live Imaging was performed on a LSM 510 Meta confocal scanning microscope (Carl Zeiss Inc., Thornwood, NY) coupled to a Mira 900F pumped with 8.0 W Verdi laser (Coherent Inc., Santa Clara, CA) tuned to 890 nm to excite EGFP. After a 2-h recovery period in the incubator, slices were moved to a RC25 recording chamber (Warner Instruments, Hamden, CT), maintained at 36.5 °C, and constantly perfused with culture media (same as above). We focused our data collection on dividing cells in the ventricular zone (VZ) and subventricular zones (SVZs) and migrating cells in the intermediate zone. 2 stacks (20 × 2 μm) were collected every 10 min from within the slice through a 63× water-immersed objective. Time series were imported into the Axiovision software (Carl Zeiss) for 3-dimensional deconvolution and 4-dimensional reconstruction.

Animal Model of Stroke
The cerebral ischemia-hypoxia model was performed as described (Adhami et al. 2006). The middle cerebral artery occlusion (MCAO) model was performed as described (Longa et al. 1989).

Results
Generation of Nestin-CreER Mice
We generated 3 lines of nestin-CreER mice using the rat nestin second intron enhancer fused to a minimal hsp68 promoter (E/nestin-hsp68 promoter, see Kawaguchi et al. 2001) to express a tamoxifen-inducible Cre recombinase. The nestin-CreER mice were then crossed to a line of Cre-dependent EGFP-reporter mice (Nakamura et al. 2006) to derive bitransgenic nestin-CreER/EGFP-Cre-reporter mice (hereafter referred to as bitransgenic mice). In bitransgenic mice, the E/nestin-hsp68 promoter directs the expression of CreER protein in nestin-positive progenitors (step 1 in Fig. 1A). In the absence of tamoxifen, the CreER protein remains in an inactive state outside the nucleus. After tamoxifen induction, the active metabolite 4-hydroxytamoxifen allows CreER to enter the nucleus (step 2) and excise the CAT gene allowing EGFP expression driven by the ubiquitous CMV-β-actin promoter (step 3). Thus, in contrast to nestin-EGFP mice, the nestin-CreER system converts nestin expression in precursors into permanent lineage tracing of their descendant cells after tamoxifen induction (Fig. 1B).

Imaging Early Neural Progenitor Proliferation
In bitransgenic embryos dosed with tamoxifen at E16.5 (4 mg to the mother) and sacrificed on E18.5, many EGFP-expressing cells were detected in the telencephalic VZ and SVZ (Fig. 1C). Some EGFP-positive cells in the VZ have long nestin-positive processes, indicating that they belong to the radial glia subpopulation of neural progenitors (radial glial cells [RGC], arrows in Fig. 1C,D) (Noctor et al. 2001). However, there are many more non-RGC EGFP-expressing cells in the VZ and SVZ (asterisk in Fig. 1C,D). To test whether these EGFP-expressing cells are also progenitors, we employed real-time confocal imaging of brain slices to look for cell division. In this experiment, bitransgenic embryos dosed with tamoxifen (2 mg) on E10.5 and E11.5 were sacrificed on E12.5, and their brains were harvested for telencephalic slice cultures. This real-time analysis showed cell division and interkinetic nuclear oscillation of EGFP-positive progenitors in the VZ (data not shown). In addition, proliferation of EGFP-positive cells in the SVZ of E12.5 cortex can be visualized with a high signal-to-noise ratio (Fig. 1E,F; see Supplementary Video). Together, these results suggest the nestin-CreER system labels embryonic neural progenitors.
Detecting Adult Neurogenesis by Progenitors in the SVZ

The nestin-CreER system can be used to detect adult neurogenesis by initiating tamoxifen induction after birth. To test this application, we injected 4 doses of tamoxifen (4 mg) to 5-week-old bitransgenic mice and sacrificed them 10 days later to detect EGFP-expressing cells. We found many EGFP-positive cells in the SVZ near the lateral ventricles (Fig. 3A), some of which also express GFAP (Fig. 3B, see also Supplementary Fig. 2). This result suggests the nestin-Cre-ER system can label the astrocyte-like SVZ precursors (B cells) and their descendants in the adult brain (Doetsch et al. 1999). Consistent with this notion, we detected many EGFP-positive cells expressing the migratory neuroblast marker DCX in the rostral migratory stream (RMS) of tamoxifen-dosed bitransgenic mice (Fig. 3D-F). In addition, many EGFP-expressing cells were found exiting the RMS to migrate toward the glomerular regions in the olfactory bulb (Fig. 3f,k). The EGFP-positive cells in the periglomerular region express interneuron markers including tyrosine hydroxylase (Fig. 3f-l).

We found that the expression of EGFP requires tamoxifen induction in both male and female bitransgenic mice (Fig. 3l, n > 10), indicating the stringency of the inducible CreER(T1) activity. However, there are limitations of the nestin-CreER system. In all 3 lines of nestin-CreER mice, the dentate gyrus subgranular zone (SGZ) progenitors were not labeled, in contrast to the consistent labeling of SVZ progenitors in every tamoxifen-induced bitransgenic animal. Some EGFP-positive cells were occasionally found in the normally nonneurogenic regions after tamoxifen induction, including the SVZ of the third ventricle, the hippocampal pyramidal cell layer, and the cingulate cortex (Supplementary Fig. 3). The cause of this "false-negative" and "false-positive" labeling may be due to the E/nestin-hsp68 promoter in the transgenic mice (see Discussion).

Minimal SVZ-Derived Neurogenesis and Ectopic Migration following Focal Ischemia

Previous studies have indicated that cell replacement after stroke primarily comes from the SVZ progenitors, rather than the dentate gyrus SGZ progenitors (Arvidsson et al. 2002). Thus, although the nestin-CreER system does not label SGZ progenitors, it can still be used for detecting the SVZ-derived neurogenesis and potential cell replacement following stroke. For this study, bitransgenic mice were given 4 doses of tamoxifen over 4 days and then rested for 14 days to allow the clearance of tamoxifen prior to the stroke challenge. After stroke, the tamoxifen-dosed mice were injected with 4 doses of BrdU (100 mg/kg) over 2 days before they were sacrificed either 2 or 3 days poststroke.
7 days after injury (Fig. 4A). Because previous studies suggested that combined cerebral ischemia--hypoxia is a stronger stimulus of aberrant DNA synthesis than focal ischemia alone (Katchanov et al. 2001; Kuan et al. 2004), we challenged the mice with either MCAO (Fig. 4) or unilateral carotid occlusion followed by hypoxia (ischemia--hypoxia, Fig. 5). In control experiments, no EGFP-expressing cells were found in the MCAO-challenged bitransgenic mice that did not receive tamoxifen induction (Fig. 4B; n = 5), indicating that MCAO does not cause leaky EGFP expression in the nestin-CreER system. In tamoxifen-dosed and MCAO-challenged bitransgenic mice, the vast majority of EGFP-expressing cells were located in the SVZ after 2- or 7-day survival (Fig. 4C; n = 5). On the contralateral side of the brain, the signals of BrdU labeling were restricted in the SVZ and RMS (Fig. 4D). In contrast, on the lesion side, there were many BrdU-incorporated cells in the striatum but not in the hippocampus (Fig. 4E,F). Surprisingly, only very few BrdU-labeled cells in the damaged striatum also expressed EGFP (Fig. 4G--I). Moreover, we did not find EGFP-positive cells expressing the migratory neuroblast marker DCX (Fig. 4J,K), whereas we can readily detect migratory neurons expressing DCX in the RMS (Fig. 4L). Based on the observed distribution pattern of EGFP-positive cells, we concluded that there was very little contribution of cell replacement by the SVZ progenitors following focal cerebral ischemia.

Aberrant DNA Synthesis after Cerebral Ischemia--Hypoxia

We previously reported that severe cerebral ischemia--hypoxia produces many TUNEL and BrdU double-labeled cells in the pyramidal layer of the hippocampus of adult rodents, suggesting that apoptotic neurons undergo aberrant DNA synthesis (Kuan et al. 2004). We have since modified the ischemia--hypoxia model allowing titration of the severity of ischemic--hypoxic insult (Adhami et al. 2006). This modified model of cerebral ischemia--hypoxia allows us to test whether aberrant DNA synthesis always leads to cell death.

We found that, following a mild ischemic--hypoxic insult when the brain damage is mostly restricted to the striatum, there was still robust BrdU labeling in the hippocampus, and to a lesser degree in the striatum and cortex, in over two-thirds of the tamoxifen-dosed bitransgenic mice at 7 days after stroke (Fig. 5A, n > 10). In these animals, the dentate granule neurons and the hippocampal neurons showed a high level of BrdU immunoreactivity (Fig. 5B). The BrdU immunoreactivity was specific because, in control staining, there was no immunosignal in adjacent sections when the primary anti-BrdU antibody was not added (Fig. 5C). These BrdU-positive hippocampal neurons showed strong signals of NeuN labeling (Fig. 5D,E), but importantly, they were not labeled by the TUNEL staining (Fig. 5G). There were some EGFP-expressing cells in the hippocampus, but they were distributed outside the pyramidal cell layer and close to the posterior periventricle (arrows in Fig. 5F,H). Together, these results suggest that the intense BrdU incorporation in the hippocampus after cerebral ischemia--hypoxia is due to aberrant DNA synthesis, which can occur in non-apoptotic neurons.

A recent study suggested that cyclin-dependent kinase (CDK) 5 controls the cell cycle arrest of postmitotic neurons (Cicero and Herrup 2005). Consistent with this notion, we have found
the protein level of CDK5 selectively reduced in the striatum and hippocampus on the lesion side, whereas the amounts of CDK2 and cyclin D1 were not obviously changed (Fig. 5). Together, these results suggest that cerebral ischemia-hypoxia may disturb the cell cycle arrest state of postmitotic neurons leading to aberrant DNA synthesis.

Discussion

Nestin-CreER Mice as a Tool for Detecting Neurogenesis

There is a growing trend of developing new methods for detecting neurogenesis to complement BrdU immunocytochemistry (van Praag et al. 2002; Ahn and Joyner 2005; Spalding et al. 2005; Yu et al. 2005; Imayoshi et al. 2006). Here we report the generation of nestin-CreER mice, which detects nestin-positive progenitors and their descendant cells after tamoxifen induction. Based on the literature and our results, this seems a reasonable approximation for detecting neurogenesis. Despite some uncertainty regarding the sensitivity and specificity of nestin expression in neural progenitors, for practical purposes, the promoter and enhancer sequences of the nestin gene is a useful tool for directing transgene expression in neuro-epithelial precursors that can give rise to both neurons and glia (Lendahl et al. 1990; Zimmerman et al. 1994, Kempermann 2005). The nestin promoter or enhancer sequence has been used to drive expression of the EGFP-reporter gene, allowing both the visualization of precursor cells in vivo and isolation of progenitors from adult brains (Yamaguchi et al. 2000; Kawaguchi et al. 2001; Sawamoto et al. 2001; Mignone et al. 2004). Hence, we have used the previously characterized E/nestin-hsp68 promoter (Kawaguchi et al. 2001) to generate nestin-CreER mice and examined its applications.

Our results suggest that the nestin-CreER system has several merits for neurogenesis-related research. First, when crossed to an EGFP-reporter line, this system reveals cell morphology via the cytoplasmic expression of EGFP, thus allowing for real-time imaging of progenitor proliferation and assisting in cell-type identification of their progeny. Second, the nestin-CreER system has the flexibility of temporal control of lineage tracing, which can be used to label some specific populations of cells. For example, we have shown that late-embryonic injection of tamoxifen selectively labels the astrocytically transformed RGC in the postnatal brain. This opens the possibility of prospective labeling of RGC and examining their postnatal developmental fate under experimental conditions. Third, the nestin-CreER system can be used to introduce Cre/loxP-based gene mutation in neural progenitors and to track the consequences in their descendant cells via a second Cre-dependent EGFP-reporter

Figure 3. The nestin-CreER system labels adult neurogenesis from SVZ to olfactory bulbs. (A-C) Bitransgenic animals dosed with tamoxifen as adults present EGFP-labeled, GFAP-positive progenitors in the SVZ. (D-F) EGFP-positive cells in the RMS express DCX, a marker of migratory neurons. (G-I) EGFP-positive cells differentiate into periglomerular interneurons, some of which express tyrosine hydroxylase. (J, M) In these animals, EGFP-positive cells can be found from the SVZ to the RMS and appear to migrate through the mitral cell layer (M) and the external plexiform layer (EPL) onto the glomeruli (GL) in the olfactory bulb. (L) No EGFP-expressing cells were found in bitransgenic animals not receiving tamoxifen. Scale bar: 40 μm in (A-I), 400 μm in (J-L).
Gene. This could be a particular useful application for identifying gene mutations in neural stem cells that cause brain tumors (Fomchenko and Holland 2005).

However, the present study also revealed several limitations of the nestin-CreER system. The most notable deficiency is that it does not label the adult dentate gyrus SGZ progenitors (false negative). This defect is consistent with E-nestin-GFP mice where no GFP was reported in the dentate gyrus of adult brains (Kawaguchi et al. 2001). We suggest that additional promoter sequences of the nestin gene may be needed to direct transgene expression in the SGZ population of adult neural progenitors (e.g., see Imayoshi et al. 2006). Also, we detected cells in brain regions that were not previously known to contribute to adult neurogenesis, and presumably, represent false-positive labeling. This may also be owing to the lack of inhibitory elements in the E-nestin construct. Alternatively, these false-positive cells may indicate a low grade or overlooked expression of nestin by nonprecursor cells in the adult brain. This should be taken into consideration when interpreting cells labeled by the nestin-CreER system: although the labeled cells descend from E-nestin-positive precursors, it remains an approximation of neurogenesis that has to be confirmed by additional methods.

Neurogenesis Versus Aberrant DNA Synthesis following Stroke

Previous studies suggested that focal cerebral ischemia stimulates the proliferation of SVZ precursors and redirects the migration of their descendants into the stroke-damage striatum (Arvidsson et al. 2002; Jin et al. 2003; Teramoto et al. 2003; Zhang et al. 2004). However, these studies primarily used BrdU incorporation combined with cell markers to detect new neurons, which could potentially include old neurons that have resumed DNA synthesis after stroke. In the present study, we have taken advantage of the ability of the nestin-CreER system to label SVZ-derived new neurons in examining this issue. Our experimental protocol includes a 2-week interval between tamoxifen induction and stroke challenge, which allows for the clearance of tamoxifen from the animal body and avoids labeling stroke-induced nestin-positive cells in the brain parenchyma that are presumably nonneurogenic (Kronenberg et al. 2005).

Surprisingly, we found only very few EGFP-positive cells derived from prelabeled SVZ progenitors, in contrast to the large number of BrdU-incorporated cells in the ischemic striatum (Fig. 4). Neither did we detect any EGFP-positive cell that expresses DCX, a marker for migrating neuroblasts.
Although we cannot exclude the possibility that neuronal replacement can occur after a longer survival period, our results are in line with previous estimations that less than 1% of BrdU-positive cells express the neuronal marker NeuN by 5 weeks after focal ischemia (Teramoto et al. 2003), and only 0.2% of deceased striatal neurons are replaced by new neurons by 6 weeks (Arvidsson et al. 2002). Thus, our results underscore the limited capacity of spontaneous neuroregeneration after stroke.

Future studies are warranted using the nestin-CreER system to test whether external growth factors increase the regeneration potential of endogenous precursors.

The obvious contrast between the large number of BrdU-incorporated cells and the small number of SVZ-derived, EGFP-positive cells in stroke-challenged nestin-CreER mice raises a critical question concerning the source of these BrdU-labeled cells. Some of them may be macrophages or activated microglia. Another plausible cause of BrdU incorporation is cell cycle reentry by old neurons following brain injury (Rakic 2002; Herrup et al. 2004; Kempermann 2005). Although the concept of DNA synthesis by stressed old neurons is widely accepted, it is generally believed that this is a prelude to cell death (Liu and Greene 2001). Our previous study showing a large number of BrdU and TUNEL double-labeled hippocampal neurons following a severe ischemic-hypoxic insult also gave support to the notion that aberrant cell cycle reentry leads to immediate cell death (Kuan et al. 2004). However, the recent discovery of a large number of polyploid neurons being functionally active in healthy brains (Kingsbury et al. 2005) prompted us to reexamine whether a milder insult of cerebral ischemia-hypoxia can trigger aberrant DNA synthesis without induction of cell death.

To test this hypothesis, we have modified the cerebral ischemia-hypoxia model of stroke allowing titration of the severity of the insult (Adhami et al. 2006). Using the nestin-CreER system, here we show a large number of hippocampal neurons display a BrdU-positive, but TUNEL-negative staining profile at 7 days after a mild cerebral ischemic-hypoxic insult (Fig. 5). The lack of EGFP expression by BrdU-incorporated hippocampal neurons suggests that they are not derived from SVZ progenitors but rather due to aberrant DNA synthesis by old neurons. Thus, one cannot assume that aberrant DNA synthesis by damaged neurons will always quickly lead to cell death and therefore not confound the interpretation of neurogenesis by BrdU incorporation.

There is no simple solution to the conundrum of distinguishing de novo neurogenesis from aberrant DNA synthesis following experimental models of brain injury. However, better understanding of the mechanisms that maintain the quiescent (G0) state of postmitotic neurons and identifying the factors, such as ischemia-hypoxia, that could disrupt this cell cycle regulation may provide insights into a future solution.

Supplementary Material

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

Notes

We thank Drs A. McMahon and S. Goldman for providing plasmids, Ms S. Falcone and the Children’s Hospital Medical Center transgenic core for pronuclei injection, and Ms A. Schloemer for preparation of the manuscript. KAB is recipient of a National Institutes of Health (NIH)
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