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Neonatal Hypoxic/Ischemic Brain Injury Induces Production of Calretinin-Expressing Interneurons in the Striatum

ZHENGANG YANG^{1,*}, YAN YOU¹, and STEVEN W. LEVISON²

¹ Institutes of Brain Science and State Key Laboratory of Medical Neurobiology, Fudan University, Shanghai, People's Republic of China 200032

² Department of Neurology and Neurosciences, UMDNJ—New Jersey Medical School, Newark, New Jersey 07103

Abstract

Ischemia-induced striatal neurogenesis from progenitors in the adjacent subventricular zone (SVZ) in young and adult rodents has been reported. However, it has not been established whether the precursors that reside in the SVZ retain the capacity to produce the full range of striatal neurons that has been destroyed. By using a neonatal rat model of hypoxic/ischemic brain damage, we show here that virtually all of the newly produced striatal neurons are calretinin (CR)-immunoreactive (+), but not DARPP-32⁺, calbindin-D-28K⁺, parvalbumin⁺, somatostatin⁺, or choline acetyltransferase⁺. Retroviral fate-mapping studies confirm that these newly born CR⁺⁺neurons are indeed descendants of the SVZ. Our studies indicate that, although the postnatal SVZ has the capacity to produce a range of neurons, only a subset of this repertoire is manifested in the brain after injury.

Indexing terms

neurogenesis; neural stem cells; stroke; subventricular zone; calretinin; medium spiny neurons

Neurogenesis normally occurs in two regions of the adult rodent brain, the hippocampus and the olfactory bulb (OB; Alvarez-Buylla and Lim, 2004; Ming and Song, 2005; Zhao et al., 2008). Recent studies suggest that the mature human brain also generates new neurons in these two regions (Curtis et al., 2007; Eriksson et al., 1998). The persistence of neurogenesis throughout life raises the possibility that the resident neural stem cell can mount a regenerative response to replace neurons lost after stroke or other insults. Indeed, ischemia-induced production of new striatal neurons from neural stem/progenitor cells from the adjacent subventricular zone (SVZ) in adult rodents has been reported by several groups (Arvidsson et al., 2002; Jin et al., 2003; Parent et al., 2002; Teramoto et al., 2003; Thored et al., 2006; Yamashita et al., 2006). With doublecortin (Dcx), an immature neuronal marker, BrdU/Dcx double positivity has been observed streaming from the immature mouse and rat SVZ into the adjacent damaged striatum at 1–2 weeks following neonatal hypoxic/ischemic (H/I) brain injury (Felling et al., 2006; Ong et al., 2005; Plane et al., 2004; Yang and Levison, 2006, 2007), mirroring what occurs in the adult brain in models of stroke. At 4–5 weeks of recovery some BrdU-labeled Dcx-positive cells express NeuN (a mature neuron marker).

*Correspondence to: Zhengang Yang, PhD, Institutes of Brain Science and State Key Laboratory of Medical Neurobiology, Fudan University, 138 Yi Xue Yuan Road, Shanghai, People's Republic of China 200032. yangz@fudan.edu.cn.

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More than 95% of the neurons in the striatum are γ -aminobutyric acid (GABA)-ergic medium-sized spiny projection neurons, and under 5% of the neurons are local interneurons. DARPP-32 is present in virtually all of the striatal medium-sized spiny projection neurons (Ouimet et al., 1998). Calbindin (CB), a calcium-binding protein, also is a selective marker for projection neurons in the striatum (Liu and Graybiel, 1992). Unlike striatal projection neurons, striatal interneurons are a heterogeneous group of four largely nonoverlapping populations that have been classified as parvalbumin (PV)⁺, calretinin (CR)⁺, somatostatin (SOM)⁺, and choline acetyltransferase (ChAT)⁺ neurons (Kawaguchi et al., 1992; Marin et al., 2000). It has been reported that, subsequent to ischemic injury in the adult, the newly generated neurons are predominantly medium spiny projection neurons, as might be expected (Arvidsson et al., 2002; Parent et al., 2002). To date, the phenotype of the striatal neurons produced after neonatal hypoxia/ischemia has not been established. Therefore, in the present study, we used a widely accepted rat model of neonatal H/I (Rice et al., 1981; Vannucci and Vannucci, 1997) to assess whether the full range of striatal neurons, or only a subset, is regenerated after cerebral injury. Surprisingly, the types of neurons produced in the neonatal brain after injury are quite different from those produced in the adult after a similar injury.

MATERIALS AND METHODS

Neonatal hypoxia/ischemia

Timed pregnant Wistar rats (Charles River, Wilmington, DE) were maintained in the Laboratory Animal Care accredited facility for at least 3 days. After normal delivery, the litter size was adjusted to 12 pups per litter. Cerebral H/I was produced in 6-day-old rats (day of birth being P0) by a permanent unilateral common carotid artery ligation, followed by systemic hypoxia (Vannucci rat model of neonatal H/I; Rice et al., 1981; Vannucci and Vannucci, 1997). Briefly, pups were lightly anesthetized with isoflurane (4% induction, 2% maintenance). Once they were fully anesthetized, a midline neck incision was made and the right common carotid artery was isolated by blunt dissection and then ligated with 3-0 silk. The incision was then sutured, and animals were returned to the dam for 1.5 hours. The pups were then exposed to 80 minutes of humidified 8% O₂/92% N₂ in jars submerged in a 37°C water bath. Control animals were separated from the dam for the same amount of time as experimental animals but were otherwise not manipulated. Approximately 60% of the animals sustained infarcts that involved both the ipsilateral striatum and the neocortex, and these animals were used in this study. All experiments on animals were carried out in accordance with institutional guidelines, and the authors further attest that all efforts were made to minimize the number of animals used and their suffering.

Bromodeoxyuridine injections

Intraperitoneal injections of the S-phase marker bromodeoxyuridine (BrdU; 50 mg/kg body weight; Sigma, St. Louis, MO) were given twice daily for 3 days after 2 days recovery from H/I (P9–P11). Animals were perfused 2 weeks (n = 3), 5 weeks (n = 4), 2 months (n = 3), and 5 months (n = 3) after H/I. As a positive control, intraperitoneal injections of BrdU (50 mg/kg body weight) were given to a pregnant rat once daily for 4 days from embryonic days 14 to 17 (peak neurogenesis in the striatum; Rymar et al., 2004), and the pups were perfused at P42.

Retrovirus injections

Replication-incompetent retroviruses encoding the marker gene human placental alkaline phosphatase (AP) were harvested from the psi2 DAP cell line (ATCC CRL-1949), concentrated, titered, and tested for helper virus as described previously (Levison and Goldman, 1993). The titer was 1×10^7 colony-forming units/ml. P5 rat pups were

anesthetized, and 2 μ l of retrovirus with 8 μ g/ml of polybrene was injected stereotactically into lateral ventricles at A: 1.2, L: $\pm$ 1.3, D: 2.5 mm. Rats were killed 5 weeks ($n = 5$) after H/I (days 36 after the DAP virus injection). Forty-micrometer sections at 240- μ m intervals were collected and processed for either AP histochemistry or AP immunofluorescence in combination with neuronal markers (Wichterle et al., 2001).

Immunohistochemistry

Animals were anesthetized with a mixture of ketamine (75 mg/kg) and xylazine (5 mg/kg) prior to intracardiac perfusion with 4% paraformaldehyde. Brains were post-fixed with 4% paraformaldehyde overnight and then cryoprotected for at least 24 hours in 30% sucrose in 0.1 M phosphate buffer (pH 7.4). The brain samples were frozen in embedding medium (O.C.T.; Sakura Finetek, Torrance, CA) on a dry ice/ethanol slush.

Immunofluorescence staining was performed on 40- μ m free-floating sections. Sections for BrdU staining were pretreated with 2 N HCl for 1 hour at room temperature to denature DNA. Sections were blocked for 1 hour in Tris-buffered saline (TBS; pH 7.4) with 10% donkey serum, 1% bovine serum albumin (BSA), and 0.5% Triton X-100. Primary antibodies were incubated for 24 hours at 4°C. The antibodies used in this study are described in Table 1. All antibodies are available commercially. The expected cellular morphology and distribution of staining for each antibody that was obtained for each primary antibody employed were consistent with earlier studies.

Secondary antibodies were incubated for 2 hours at room temperature (all from Jackson ImmunoResearch, West Grove, PA; 1:200). All secondary antibody combinations were carefully examined to ensure that there was no cross-talk between fluorescent dyes or cross-reactivity between secondary antibodies, especially for anti-rat and anti-mouse secondary antibodies. No signal above background was obtained when the primary antibodies were replaced with preimmune sera. The sections were then washed, counter-stained with 4',6'-diamidino-2-phenylindole (DAPI; Sigma; 1 μ g/ml) for 5–10 minutes, and coverslipped with Gel/Mount (Biomed, Foster City, CA).

Antibody characterization

Rabbit polyclonal antiserum to human placental AP was raised by repeated immunization of rabbits with highly purified human placental AP. This antibody recognizes human placental AP, a membrane-bound enzyme normally synthesized by syncytiotrophoblasts of the placenta (manufacturer's technical information; Wichterle et al., 2001).

Rat monoclonal anti-BrdU, clone BU1/75 ICR1, reacts with BrdU in single-stranded DNA, BrdU attached to protein carrier, or free BrdU (manufacturer's technical information). No staining was observed in cases in which animals were not infused with BrdU.

Mouse monoclonal anti-CB, clone CB-955, stains a 28-kD band in immunoblots of the rat brain and does not react with other members of the EF-hand family, such as calbindin-D-9K, CR, PV, S-100a, S-100b, S100A2, and S100A6 (manufacturer's technical information). Preabsorption of this antibody with calbindin D-27 kDa protein purified from chick and rat brains, or from rat kidney, completely abolished calbindin immunostaining in the brain (Pasteels et al., 1987; Pinaud et al., 2007). The staining that we observed with this antibody was identical to that described previously (Liu and Graybiel, 1992).

Mouse monoclonal anti-CR recognizes a 31-kDa band on Western blots of the brain, spinal cord, and retina of the rat and human (manufacturer's technical information). The staining that we observed in the striatum was identical to that described previously (Dayer et al., 2005; Rymar et al., 2004).

Rabbit polyclonal anti-CR recognizes both the calcium-bound and calcium-unbound conformations of CR in immunoblot of the rat brain (by manufacturer). The staining that we obtained was identical to that obtained with the mouse anti-CR.

Mouse monoclonal anti-ChAT, clone 1E6, detects a single band of 68 kDa on Western blots of rat and chick brain (manufacturer's technical information). The staining that we obtained with this antibody was identical to that described previously (Thal et al., 1992).

Rabbit polyclonal anti-DARPP-32 detects a single band at 32 kDa on Western blots of rat brain or retina (manufacturer's technical information; Partida et al., 2004). The staining that we obtained with this antibody was identical to that described previously (Ouimet et al., 1998).

Goat polyclonal anti-DARPP-32 detects two bands at 32 kDa and 64 kDa on Western blot of mouse brain (manufacturer's technical information). We obtained identical staining patterns using the Cell Signaling (Beverly, MA) antibody and the Santa Cruz (Santa Cruz, CA) antibody.

Goat polyclonal anti-Dcx detects a single band at 40 kDa on Western blot of adult rat OB (Brown et al., 2003; Suzuki et al., 2007). We found that it stains cells with the same morphology and cell distribution in the damage striatum as described previously (Arvidsson et al., 2002).

Rabbit polyclonal anti-GFAP detects a single band at 50–53 kDa on Western blot of the adult mouse cortex (Toma et al., 2001). It stains cells with the proper morphologies and distribution as expected for astrocytes (Yang et al., 2007).

Rabbit polyclonal anti-GST- π detects a single band at 26 kDa on Western blots of the human, mouse, and rat brain. It reacts with GST- π and does not react with other isozymes on Western blots (manufacturer's technical information). The staining we obtained with this antibody was identical to that described previously (Hsieh et al., 2004; Tansey and Cammer, 1991).

Rabbit polyclonal anti-Iba1 detects a single band of about 17 kDa on Western blot of the rat brain (Ito et al., 1998). This antiserum reacts with Iba1 of human, mouse, and rat (manufacturer's technical information). The staining that we obtained with the Iba1 antibody was identical to that described previously (Ito et al., 1998).

Mouse monoclonal anti-NeuN recognizes two or three bands in the 46–48-kDa range and another band at approximately 66 kDa on Western blots (manufacturer's technical information). The staining that we obtained with the NeuN antibody was identical to that described previously (Mullen et al., 1992).

Mouse monoclonal anti-PV recognizes a 12-kDa protein in immunoblots of brain and muscle. This antibody is directed against an epitope in the first calcium-binding site and specifically stains the calcium-bound form of parvalbumin (manufacturer's technical information). The staining that we obtained with the anti-PV antibody was identical to that described previously (Schlosser et al., 1999).

Rabbit polyclonal anti-Pbx1/2/3 detects three bands at 44 kDa (Pbx 2), 50 kDa (Pbx 3), and 55 kDa (Pbx 1) on Western blot. It reacts with Pbx 1, Pbx 2, and Pbx 3 of human, mouse, and rat origin on Western blot (manufacturer's technical information). The staining that we obtained with the anti-Pbx antibody was identical to that described previously (Arvidsson et al., 2002).

Mouse monoclonal anti-Rip stains oligodendrocytes from early in their differentiation into adulthood (Friedman et al., 1989; Song et al., 2002). The antigen that this monoclonal antibody recognizes has not been well defined but may be CNPase (Jim Salzer, unpublished observations). The staining that we obtained with this antibody was identical to that originally described (Friedman et al., 1989).

Rabbit polyclonal anti-SOM reacts with SOM of human, mouse, and rat origin and recognizes a 17-kDa band on Western blot (manufacturer's technical information). The staining that we obtained was identical to that previously described (Rushlow et al., 1995).

Mouse monoclonal anti-TH recognizes an epitope on the outside of the regulatory N-terminus and recognizes a protein of approximately 59–61 kDa on Western blot (manufacturer's technical information). The staining that we obtained was identical to that previously described (Clarke et al., 1988).

Microscopy

Fluorescently immunolabeled sections were analyzed on a Zeiss LSM410 confocal laser scanning microscope using the following filter sets with the indicated wavelengths (in nm) for the excitation laser line and emission filters: Cy2, 488/(510–540); rhodamine, 568/(590–610). Confocal Z sectioning was performed at 0.5- μ m intervals with a $\times 63$ (NA = 1.40) or a $\times 40$ (NA = 1.30) oil-immersion objective. Images were acquired and three-dimensional images reconstructed in the Zeiss LSM software and cropped, adjusted, and optimized in Adobe Photoshop 9.0 (Adobe Systems Inc., San Jose, CA). Photoshop was also used to convert red-green fluorescence images to magenta-green images and to switch some images from color mode to black-and-white mode. Images of enzyme histochemistry labeled sections and some fluorescently immunolabeled sections were acquired using an Olympus BX 41 microscope.

Cell quantification and statistical analysis

Because the caudolateral striatum was severely damaged (striatal tissue loss) in the animal model, rostral striatal regions were analyzed. Forty-micrometer brain coronal sections were collected from the anterior tip of the corpus callosum. The number of CR⁺ cells in the striatum 5 months after H/I was counted using a Zeiss LSM410 confocal laser scanning microscope. Briefly, six sections at 240- μ m intervals were quantified per brain. For each section, six fields in the contralateral striatum and one to four fields in the intact area or damaged area of the ipsilateral striatum were analyzed (see Fig. 7, Supp. Info. Fig. 3). We defined the penumbra as that region that surrounded the ischemic core (which was devoid of cells) and that contained visibly affected tissue. Confocal Z sectioning was performed at 0.5- μ m intervals using Plan-Apochromat $\times 63$ oil-immersion objective (NA = 1.40) in each field (200 μ m $\times$ 200 μ m; see Fig. 7F,G). This method allowed us to count accurately the number of CR⁺ cells in the damaged area of the ipsilateral striatum (Yang and Levison, 2007). The density of CR⁺ cells in the striatum was obtained by dividing the total volume analyzed by the total number of cells counted ($n = 3$). For example, the total volume analyzed in contralateral striatum per brain was 6 fields $\times$ 6 sections $\times$ 40- μ m section thickness $\times$ 200 μ m $\times$ 200 μ m = 0.0576 mm³. The density of CR⁺ cells in the contralateral striatum was obtained by dividing 0.0576 by the total number of cells counted.

Data are presented as means $\pm$ SEM. Comparisons of densities of CR⁺ cells in the striatum were performed by one-way ANOVA followed by Fisher post hoc LSD (least significant difference) test. Comparisons were interpreted as significant at $P < 0.05$.

RESULTS

H/I brain injury induces production of CR⁺ interneurons in the striatum

In the present study, we used a widely accepted rat model of perinatal H/I that produces unilateral brain damage as a consequence of an acute reduction of blood flow and oxygenation to mimic the disruption in the delivery of nutrients and oxygen to the brain that is a primary cause of neurologic injury during the perinatal period. At 2 weeks or 5 weeks following neonatal H/I there is a compensatory production of new neurons in the ipsilateral striatum compared with the contralateral striatum (Supp. Info. Fig. 1; Yang and Levison, 2007). Because the range of neuronal subtypes produced after neonatal H/I has not been established, here we phenotyped these newly produced striatal neurons using distinct neuronal markers combined with BrdU or retrovirus. Surprisingly, no BrdU⁺/DARPP-32⁺ or BrdU⁺/CB⁺ cells were found (Fig. 1A,B), despite the fact that many BrdU⁺/NeuN⁺ cells were observed in the ipsilateral striatum 5 weeks after H/I (Fig. 2A–C, Supp. Info. Figs. 1F, 2A,B). More surprisingly, we also failed to find BrdU⁺ cells labeled for PV, SOM, or ChAT (Fig. 1C–E). By contrast, large numbers of BrdU⁺/CR⁺ and BrdU⁺/CR⁺/NeuN⁺ cells could be found in the damaged striatum 5 weeks after H/I (Fig. 1F–H).

The failure to label neurons other than CR⁺ cells was not due to technical problems; injecting BrdU into rats on embryonic days 14–17 (at the peak neurogenesis in the striatum) combined with immunostaining on P42 rats revealed that BrdU⁺/DARPP-32⁺ and BrdU⁺/PV⁺ cells were plentiful in the striatum (Fig. 2F,G, and Supp. Info. Fig. 2D,E). Many of these BrdU⁺/NeuN⁺ neurons could be classified as projection neurons, insofar as they were medium sized (10–15 μ m in diameter) and the NeuN protein was distributed both in their nuclei and in their perinuclear cytoplasm (Fig. 2D,E, Supp. Info. Fig. 2C). By contrast, the NeuN protein in the newborn striatal neurons after neonatal H/I was restricted to the nucleus (Fig. 2A–C, Supp. Info. Fig. 2A,B), a phenotype typical of interneurons (Dayer et al., 2005; Mullen et al., 1992).

BrdU may be taken up by cells undergoing DNA repair, so we validated our BrdU labeling studies with replication-incompetent retroviruses encoding the marker gene human placental AP. DAP retroviruses were injected into the lateral ventricles 1 day before inducing H/I, which we have shown labels dividing cells in the SVZ (Yang and Levison, 2007). Confirming the BrdU study results, the AP⁺ cells with neuronal morphologies were CR⁺ in the damaged striatum 5 weeks after H/I (Fig. 1I–M). These AP-labeled CR⁺ interneurons were nonhomogeneously distributed in the damaged striatum, with more AP⁺/CR⁺ cells in the ischemic penumbra and a few in the intact area. Consistent with BrdU studies, the cell bodies of AP⁺/CR⁺ cells were mainly oval or fusiform, and their diameters never exceeded 10 μ m (range 4–8 μ m; Fig. 1I–M; Yang and Levison, 2007). In studies in progress in which patch-clamp recordings were obtained from two green fluorescent protein (GFP) retrovirally infected cells with neuronal morphologies at 5 weeks of recovery, the cells possessed resting membrane potentials near –60 mV, and they had high input resistances and long time constants, as would be expected from a neuron (A. Corbett, unpublished observations). However, no AP-labeled DARPP-32⁺, CB⁺, PV⁺, SOM⁺, and ChAT⁺ cells were found. In the contralateral striatum, the occasional AP/CR⁺ cell was observed, but the number of these double-positive cells was extremely low. Together, these results indicate that, after neonatal H/I brain injury, neural progenitors in the SVZ that have recently divided migrate into the damaged striatum, where they differentiate into CR⁺ interneurons.

Newborn immature neurons express Pbx and CR in the striatum after H/I

Because more than 40% of newborn neurons in the striatum differentiated into DARPP-32⁺ neurons after adult stroke (Arvidsson et al., 2002), the scarcity of DARPP-32 neurons

generated after neonatal H/I insult was highly unexpected. Previous studies have used expression of Pbx to mark developing striatal medium-sized spiny projection neurons (Arvidsson et al., 2002). Consistent with the established expression of Pbx in medium spiny neurons, DARPP-32⁺ and CB⁺ striatal neurons expressed Pbx protein (Fig. 3A,B). However, some of the PV⁺ and CR⁺ cells also expressed Pbx (Fig. 3C,D). These data suggest that Pbx could not be a specific marker of the striatal medium-sized spiny projection neuron, because Pbx is also expressed in interneurons (Redmond et al., 1996; Toresson et al., 2000). We also found many BrdU⁺/Pbx⁺ cells (Fig. 3E), and more than 90% of the Dcx⁺ cells expressed Pbx (Fig. 3F–H) in the ipsilateral striatum after 2 weeks and 5 weeks of recovery from H/I. Pbx immunoreactivity was stronger in these newborn neurons compared with the other striatal neurons (Fig. 3E–H).

Dcx is a microtubule-associated protein expressed by neuronally committed precursors and immature neurons and has been used in numerous studies to mark newly generated neurons. Therefore, we performed Dcx double staining with different striatal neuronal markers to identify the phenotype of the new neurons generated during recovery from H/I. Again, more than 60% of the single Dcx⁺ cells in the damaged striatum also expressed CR (Fig. 3I–L); however, none was DARPP-32⁺, CB⁺, PV⁺, SOM⁺, or ChAT⁺. These results further support the conclusion that only CR⁺ interneurons are produced in the striatum after neonatal H/I.

Long-term effect of H/I on the pattern of neuronal distribution in the striatum

Animals were killed 5 months following the insult to evaluate the extent of cell replacement as well as the distribution of these new neurons in the striatum after a long period of recovery from H/I. Even at this late time point, many Dcx⁺ cells with morphologies of migrating neuroblasts could be observed interspersed between the affected SVZ and striatum (Fig. 4B,E). In contrast, Dcx⁺ cells were extremely rare in the contralateral striatum (Fig. 4A,C). Furthermore, clusters of small-diameter NeuN⁺ and Dcx⁺/NeuN⁺ cells could be observed in the ischemic penumbra (Fig. 4D,E). These results are consistent with the view that the production of striatal interneurons after H/I is sustained (Yang and Levison, 2007). Although well developed, heavily labeled PV⁺ cells were clearly recognized in the contralateral striatum, PV⁺ cells were rare in the ischemic penumbra (Fig. 4F–J). Similarly, DARPP-32⁺ cells were also rare in the penumbra of the ipsilateral striatum (Fig. 5A,B,E,F). Given that progenitors in the postnatal SVZ also give rise to a small number of dopaminergic periglomerular cells (identified by the presence of TH, the rate-limiting enzyme in dopamine synthesis) of the OB throughout life (Batista-Brito et al., 2008a; Betarbet et al., 1996; Merkle et al., 2007; Yang, 2008), we also performed TH immunostaining. However, no TH⁺ cells were found either in the contralateral or in the ipsilateral striatum 5 months after H/I (Fig. 5C,D,G,H). Our failure to stain for TH⁺ neurons was not due to technical limitations, insofar as TH⁺ terminals that originated from dopaminergic neurons in the midbrain and established synaptic connections with striatal medium-sized spiny neurons were abundant in the contralateral striatum and in the intact area of the ipsilateral striatum (Fig. 5C,D,G,H).

Neurogenesis induced by ischemia in the lesioned striatum is sustained for several months (Figs. 4B, 6; Thored et al., 2006; Yang and Levison, 2007). If only CR⁺ interneurons were generated, continued neurogenesis should induce more CR⁺ interneurons in the ipsilateral striatum after long-term recovery from H/I. As anticipated, we observed a large number of CR⁺ cells in the ischemic penumbra of the ipsilateral striatum 5 months after H/I (Fig. 7B,D, Supp. Info. Fig. 3B,E,F). Surprisingly, many more CR⁺ cells were also found in the intact area of the ipsilateral striatum compared with the contralateral striatum (Fig. 7A–D, Supp. Info. Fig. 3). In the ischemic penumbra, these CR⁺ cells were either clustered or scattered, and some of them were still labeled by BrdU (Fig. 7B,D, Supp. Info. Figs. 1H, 3B,E,F). Quantitative analysis revealed that the density of CR⁺ cells in both the intact and the

damaged areas of the ipsilateral striatum was significantly higher than in the contralateral striatum ($P \leq 0.05$; one-way ANOVA with Fisher LSD post hoc test; $n = 3$; Fig. 7E).

Long-term effect of H/I on the pattern of glial distribution in the striatum

Although the striatum sustained significant damage from neonatal H/I, nuclear DAPI staining revealed a higher cell density in the ischemic penumbra of the ipsilateral striatum 5 months after H/I (Fig. 8A–D). Immunostaining for GFAP and for Iba-1 (for astrocytes and microglia, respectively) established that there was extensive gliosis in the neonatal brain after injury (Fig. 8E–H). By contrast, staining for GST- π and RIP (for oligodendrocytes) revealed fewer oligodendrocytes in the ipsilateral striatum compared with the contralateral striatum (Fig. 8I,J). These results are consistent with the vulnerability of immature oligodendrocytes and poor regeneration of oligodendrocytes in the white matter and neocortex after neonatal H/I (Back et al., 2002; Ness et al., 2001).

DISCUSSION

Although the brain was once regarded as an organ that was incapable of repairing itself, this view is no longer tenable, and there is substantial interest in evaluating both the extent to which cell replacement normally occurs and the extent to which this naturally occurring regenerative response can be expanded. Here we show that, during recovery from a moderate neonatal H/I injury, there is sustained migration of newborn neuroblasts from the SVZ into the damaged area, where they differentiate into CR⁺ interneurons. The conclusion is based on several lines of supporting evidence: 1) BrdU labeling studies show coregistration with CR, whereas BrdU⁺ cells failed to express markers of other neurons that are either residents of the striatum or descendants of the SVZ; 2) retroviral fate mapping studies show that CR⁺ interneurons are found and that no retrovirally labeled cells with neuronal morphologies expressed other neuronal subtype makers; 3) newborn young neurons (Dcx⁺ cells) express only CR, rather than the other markers of striatal neurons; 4) small-diameter neurons and CR⁺ interneurons accumulate in the ipsilateral striatum at long intervals of recovery from H/I; and 5) virtually no DARPP-32⁺, PV⁺ cells and few TH⁺ terminals are found in the ischemic penumbra at long intervals of recovery from H/I.

Neuroepithelial precursors in the medial (MGE), lateral (LGE), and caudal ganglionic eminence are the principal sources of interneurons migrating tangentially to the different regions of the developing rodent brain. Studies suggest that precursors in the LGE but not the MGE also give rise to the medium spiny projection neurons in the striatum (Marin et al., 2000; Stenman et al., 2003; Wichterle et al., 2001). It has been hypothesized that most of the stem cells in the adult SVZ are descendants of the embryonic dorsal LGE based on similarities in transcription factor profiles (Marin and Rubenstein, 2001; Waclaw et al., 2006; Young et al., 2007).

Under normal circumstances, the adult SVZ does not generate new striatal medium spiny neurons (Merkle and Alvarez-Buylla, 2006; Stenman et al., 2003). The slowly dividing SVZ stem cells generate rapidly dividing transit-amplifying progenitors that in turn generate neuroblasts that migrate forward along the rostral migratory stream (RMS) into the OB, where they give rise to several subtypes of olfactory interneurons throughout life (Kohwi et al., 2007; Merkle et al., 2007; Yang, 2008; Young et al., 2007). In the intact brain, neuroblasts in the SVZ do not all join the RMS, and some of them instead migrate into the striatum and differentiate into CR⁺ interneurons (Dayer et al., 2005). However, compared with neuroblasts that migrate into the OB, the number of neuroblasts entering the adult striatum is very low (Dayer et al., 2005; Nacher et al., 2001; Yang et al., 2004). Ischemic brain injury, such as that induced by middle cerebral artery occlusion or global ischemia, induces an infarct in the striatum that results in the death of large numbers of striatal

neurons, including both projecting medium sized spiny and distinct interneurons. Previous studies have shown an increase in neuroblasts with migratory profiles interspersed between the SVZ and the striatum in both young and adult animals (Arvidsson et al., 2002; Felling et al., 2006; Jin et al., 2003; Ong et al., 2005; Parent et al., 2002; Plane et al., 2004; Teramoto et al., 2003; Yamashita et al., 2006; Yang and Levison, 2006, 2007). In adult brains after stroke or excitotoxic injury, it has been reported that these neuroblasts can give rise not only to striatal projection neurons and striatal interneurons but also to CA1 hippocampal pyramidal neurons (Arvidsson et al., 2002; Collin et al., 2005; Nakatomi et al., 2002; Parent et al., 2002; Teramoto et al., 2003). Magavi et al. have suggested that SVZ progenitors may also give rise to neocortical pyramidal neurons after synchronous apoptotic degeneration of corticothalamic neurons (Chen et al., 2004; Magavi et al., 2000).

With the widely accepted Vannucci model of neonatal H/I (Rice et al., 1981; Vannucci and Vannucci, 1997), our studies show quite different results. Although robust production of new striatal neurons from SVZ progenitors occurs after neonatal H/I, these newborn neurons are restricted to only one subtype, CR⁺ interneurons. Over time, there is a twofold increase in the density of CR⁺ cells in the ipsilateral striatum. Although our results differ from those obtained in the adult, they are consistent with what might be predicted when one considers the temporal and spatial properties of the developing telencephalic eminences and postnatal SVZ (Batista-Brito et al., 2008b; Butt et al., 2005; Flames et al., 2007; Fogarty et al., 2007; Luskin, 1993; Marin et al., 2000; Merkle et al., 2007; Xu et al., 2004; Yang, 2008; Young et al., 2007). During the neonatal period and continuing into adulthood, SVZ cells continually give rise to granular cells and CR⁺, TH⁺, CB⁺ periglomerular interneurons that migrate to the OB (Kohwi et al., 2007; Merkle et al., 2007; Yang, 2008; Young et al., 2007). However, among all of the newborn interneurons in the adult OB, the vast majority are CR⁺ (Batista-Brito et al., 2008a; Yang, 2008). Our results show that only CR⁺ interneurons but not other subtypes of interneurons are generated in the cortex and striatum after neonatal H/I (Yang et al., 2007). These data are reminiscent of studies showing that olfactory bulbectomy does not stop the production of neuroblasts in the SVZ, nor their migration, but these neuroblasts are more likely to differentiate into CR⁺ neurons and to integrate into neighboring brain parenchyma (Jankovski et al., 1998; Kirschenbaum et al., 1999). This raises the possibility that neuroblasts reaching the damaged striatum are already committed to a CR⁺ interneuron fate or that virtually only CR⁺ committed progenitors have the ability to migrate into the damaged striatum after neonatal H/I. This interpretation is further supported by recent findings that neural stem cells in the SVZ are restricted in the types neurons that they can generate and are not susceptible to being respecified in vivo or in vitro (Merkle et al., 2007). It is also worth noting that recent studies have shown that progenitors from both the pallium and septum (Kohwi et al., 2007; Ventura and Goldman, 2007; Young et al., 2007), but not from the LGE (De Marchis et al., 2007; Kohwi et al., 2007), can give rise to CR⁺ interneurons in the OB. Thus, newly born CR⁺ cells in the damaged striatum after H/I may not be generated from LGE-derived neural stem/progenitor cells.

Neonatal H/I is a disruption of blood and oxygen delivery to the brain and represents a primary cause of neurologic injury in the newborn. Neonatal H/I occurs in one or two of 1,000 live term births, and more than 50% of surviving preterm infants sustain permanent brain damage from this type of injury (Vannucci and Hagberg, 2004; Volpe, 1997). Several studies indicate that the neurogenesis that occurs after ischemic lesions can enhance behavioral performance and brain self-repair (Jin et al., 2004; Leker et al., 2007; Nakatomi et al., 2002; Thored et al., 2006). Because CR⁺ interneurons represent only 0.5% of all striatal neurons in the adult rat brain (Rymar et al., 2004), our data collectively suggest that the regenerative capacity of the neural stem/progenitors in the SVZ after neonatal H/I brain injuries is very limited and that epigenetic reprogramming may be necessary. For instance, it has been shown that noggin and brain-derived neurotrophic factor can collaborate to

increase medium-sized spiny neuron addition to the striatum from SVZ progenitors in adult normal and R6/2 huntingtin mutant mouse brains (Chmielnicki et al., 2004; Cho et al., 2007). These studies suggest a means to increase medium spiny neuron production from neonatal SVZ cells. Alternatively, given the encouraging results of recent studies showing that somatic cells can be genetically reprogrammed to a pluripotent state by using forced transcription factor expression (Takahashi and Yamanaka, 2006), gene therapies may be more effective in expanding the therapeutic potential of the dividing neural stem cells in the SVZ to promote functional recovery after brain injuries.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Calretinin (CR)⁺ interneurons are generated in the striatum after H/I. **A–E:** BrdU was injected on days 3–5 of recovery from H/I (P9–P11), and BrdU immunofluorescence was combined with immunostaining for neuronal markers at 5 weeks (P41) after H/I. No BrdU⁺/DARPP-32⁺ (A), BrdU⁺/calbindin (CB)⁺ (B), BrdU⁺/parvalbumin (PV)⁺ (C), BrdU⁺/somatostatin (SOM)⁺ (D), or BrdU⁺/choline acetyltransferase (ChAT)⁺ (E) cells were found in the ipsilateral striatum. **F:** A BrdU⁺/NeuN⁺/CR⁺ cell (arrow) in the ipsilateral striatum. **G,H:** Many BrdU⁺/CR⁺ cells (arrows) were found in the ipsilateral striatum. **I–M:** DAP retroviruses encoding the marker gene human placental alkaline phosphatase (AP) were injected into lateral ventricles 1 day before inducing H/I to label dividing cells in the SVZ, and AP immunofluorescence was combined with immunostaining for neuronal markers at 5 weeks after H/I. Only AP⁺/CR⁺ interneurons were found in the ipsilateral striatum. Scale bar = 20 μ m.

Fig. 2.

Photomicrographs of newborn neurons in the rat striatum. **A–C:** BrdU was injected on days 3–5 of recovery from H/I (P9–P11), and BrdU immunofluorescence was combined with immunostaining for neuronal markers at 5 weeks (P41) after H/I. BrdU⁺/NeuN⁺ cells in the ipsilateral striatum were observed. A2 depicts eight consecutive 0.5-μm confocal images in the z-dimension and one projection image showing BrdU and NeuN immunostaining, separately and in merged images. **D–G:** BrdU was injected on embryonic days 14–17, and BrdU combined with neuronal markers immunostaining was performed on P42. BrdU⁺/NeuN⁺ medium-sized neurons (D and higher magnification in E1; E2 depicts eight consecutive confocal images and one projection image of the cell in E1), BrdU⁺/DARPP-32⁺ cells (F), and BrdU⁺/parvalbumin (PV)⁺ cells (G) in the striatum were found. Note that NeuN protein is restricted in the nucleus in A–C, whereas in D,E NeuN protein is distributed both in the nucleus and in the perinuclear cytoplasm. Scale bars = 10 μm in A1 (applies to A1,B,C,E1); 10 μm in A2 (applies to A2,D,E2); 10 μm in F1 (applies to F1–G2).

Fig. 3.

Pbx is expressed both in projection neurons and in local interneurons in the striatum. **A,B:** Images showing DARPP-32⁺ and calbindin (CB)⁺ cells expressed Pbx in the striatum of a 6-week-old rat. **C1–C4:** Some parvalbumin (PV)⁺ cells also express Pbx (arrows). Note that a PV⁺ cell did not express Pbx (arrowhead in C4). **D1–D3:** A calretinin (CR)⁺ cell expressed Pbx (arrow). **E1–E4:** Five weeks after H/I, many BrdU⁺/Pbx⁺ cells (arrows) were found in the ipsilateral striatum. **F–H:** Most of the doublecortin (Dcx)⁺ cells expressed Pbx in the ipsilateral striatum. Note that Pbx immunoreactivity was stronger in BrdU⁺ and Dcx⁺ cells compared with the other cells in the striatum. **I1–L2:** Many Dcx⁺ cells expressed CR (arrows) in the ipsilateral striatum 5 weeks after H/I. Scale bars = 20 μm in L2 (applies to A–C,D1,E1,F–L2); 5 μm in E4 (applies to D2,D3,E2–E4).

Fig. 4. Doublecortin (Dcx)⁺/NeuN⁺ cells are more abundant in the ipsilateral striatum 5 months after H/I. **A,B:** Dcx/NeuN double immunostaining in the contralateral (A) and ipsilateral (B) striatum showing that Dcx⁺ cells were abundant in the ischemic penumbra of the ipsilateral striatum but rare in the contralateral striatum 5 months after H/I. **C:** Higher magnification of the boxed area in A. **D:** Higher magnification of the boxed area in B showing only NeuN staining. Note the many small-diameter NeuN⁺ cells in the ischemic penumbra. **E:** Higher magnification of the boxed area in B showing many Dcx⁺/NeuN⁺ cells in the ischemic penumbra. **F,G:** Parvalbumin (PV)⁺ cells in the contralateral (F) and ipsilateral striatum (G) 5 months after H/I. **H,I:** Higher magnification of the boxed areas in F. **J:** Higher magnification of the boxed areas in G. Scale bars = 200 μ m in G (applies to A,B,F,G); 20 μ m in J (applies to C–E,H–J).

Fig. 5. DARPP-32⁺ cells and tyrosine hydroxylase (TH)⁺ terminals are rare in the ischemic penumbra 5 months after H/I. **A,B:** DARPP-32 immunostaining in the contralateral (A) and ipsilateral (B) striatum 5 months after H/I. **C,D:** TH immunostaining in the contralateral (C) and ipsilateral (D) striatum 5 months after H/I. **E-H:** Higher magnification of the boxed areas in A–D. Note that DARPP-32⁺ cells and TH⁺ terminals are rarely observed in the ischemic penumbra. Scale bars = 200 μm in B (applies to A–D); 20 μm in H (applies to E–H).

Fig. 6. Doublecortin (Dcx)⁺ cells continually migrate into the damaged area of the brain after neonatal H/I. **A:** NeuN immunostaining in the ipsilateral hemisphere 2 months after H/I. **B:** Dcx immunostaining in the adjacent section. **C–E:** Higher magnification of the boxed areas in B, showing Dcx⁺ cells migrating into the neocortex (C,D) and striatum (E). **F–I:** Higher magnification of the boxed areas in C–E, showing morphologies of Dcx⁺ cells in the ipsilateral neocortex and striatum 2 months after H/I. Scale bars = 500 μ m in B (applies to A,B); 100 μ m in E (applies to C–E); 20 μ m in G (applies to F–I).

Fig. 7.

The density of calretinin (CR)⁺ cells is significantly increased in the ipsilateral (IL) striatum 5 months after H/I. **A,B:** CR⁺ cells in the contralateral (CL) and IL striatum 5 months after H/I. **C,D:** Higher magnification of boxed areas in A and B. **E:** Quantitative analysis of the density of CR⁺ cells in the CL striatum and intact area and damaged area of the IL striatum. Data are means $\pm$ SEM; asterisk indicates significant difference ($P \leq 0.05$) from another two groups; one-way ANOVA with Fisher LSD post hoc test; $n = 3$. **F,G:** Sampling scheme used for quantification of striatal CR⁺ cells. For each coronal section, six fields in the contralateral striatum and one to four fields in the intact area or damaged area (penumbra) of the ipsilateral striatum were analyzed. Scale bars = 200 μ m in B (applies to A,B); 20 μ m in C (applies to C,D).

Fig. 8. Glial cells in the striatum 5 months after H/I. **A,B:** DAPI nuclear staining is shown in the contralateral (A) and ipsilateral (B) striatum 5 months after H/I. **C,D:** Higher magnification of the boxed areas in A,B. **E,F:** NeuN/GFAP double immunostaining in the contralateral (E) and ipsilateral (F) striatum. **G,H:** NeuN/Iba-1 double immunostaining in the contralateral (G) and ipsilateral (H) striatum. **I,J:** GST- π /RIP double immunostaining in the contralateral (I) and ipsilateral (J) striatum. Scale bars = 200 μ m in B (applies to A,B); 20 μ m in J (applies to C–J).

TABLE 1

Primary Antibodies

Target protein	Antigen	Species	Dilution	Source
Human placental alkaline phosphatase (AP)	Purified human placental AP	Rabbit antiserum	1:30	Accurate Chemical (Westbury, NY) catalog No. YSRTAHP537
5-Bromo-2'-deoxyuridine (BrdU)	BrdU	Rat monoclonal IgG2a	1:20	Accurate Chemical catalog No. OBT0030S
Calbindin (CB)	Purified bovine kidney calbindin-D-28K	Mouse monoclonal IgG1	1:400	Sigma (St. Louis, MO) catalog No. C9848
Calretinin (CR)	Recombinant rat calretinin	Mouse monoclonal IgG1	1:600	Chemicon (Temecula, CA) catalog No. MAB1568
Calretinin (CR)	Recombinant rat calretinin	Rabbit antiserum	1:2,000	Chemicon catalog No. AB5054
Choline acetyltransferase (ChAT)	ChAT purified from rat brain	Mouse monoclonal IgG1	1:100	Chemicon catalog No. MAB305
DARPP-32	A synthetic peptide (sequence CVEMIRRRRPTPAML) surrounding Thr34 of human DARPP-32	Rabbit polyclonal	1:100	Cell Signaling (Beverly, MA) catalog No. 2302
DARPP-32	A synthetic peptide sequence corresponding to aa 2–20 of DARPP-32 of human origin	Goat polyclonal	1:50	Santa Cruz (Santa Cruz, CA) catalog No. SC-8483
Doublecortin (Dcx)	Peptide: aa 385–402 of human Dcx	Goat polyclonal	1:100	Santa Cruz catalog No. SC-8066
Glia1 fibrillary acidic protein (GFAP)	GFAP isolated from cow spinal cord	Rabbit antiserum	1:500	Dako (Carpinteria, CA) catalog No. Z0334
Glutathione S-transferase- π (GST- π)	Purified human GST- π	Rabbit antiserum	1:500	MBL (Woburn, MA) catalog No. 312
Iba1	C-terminal end (sequence PTGPPAKKAISELP) of Iba1	Rabbit antiserum	1:500	Wako (Richmond, VA) catalog No. 019-19741
NeuN	Mouse brain cell nuclei	Mouse monoclonal IgG1	1:400	Chemicon catalog No. MAB377
Parvalbumin (PV)	PV purified from frog muscle	Mouse monoclonal IgG1	1:400	Chemicon catalog No. MAB1572
Pbx	Peptide: aa 411–430 of Pbx1 of human origin	Rabbit polyclonal	1:50	Santa Cruz catalog No. SC-888
Rip	Rat olfactory bulb oligodendrocytes	Mouse monoclonal IgG1kappa	1:25	Developmental Studies Hybridoma Bank (Iowa City, IA)
Somatostatin (SOM)	Peptide: aa 1–106 of SOM of human origin	Rabbit polyclonal	1:50	Santa Cruz catalog No. SC-13099
Tyrosine hydroxylase (TH)	TH purified from PC12 cells	Mouse monoclonal IgG1kappa	1:400	Chemicon catalog No. MAB318