Stem Cell Research (2013) 11, 669–686

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# Hypoxic-preconditioning enhances the regenerative capacity of neural stem/progenitors in subventricular zone of newborn piglet brain

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Received 29 October 2012; received in revised form 2 April 2013; accepted 20 April 2013
Available online 28 April 2013

**Abstract** Perinatal hypoxia–ischemia (HI) results in brain injury, whereas mild hypoxic episodes result in preconditioning, which can significantly reduce the vulnerability of the brain to subsequent severe hypoxia–ischemia. Hypoxic-preconditioning (PC) has been shown to enhance cell survival and differentiation of progenitor cells in the central nervous system (CNS). The purpose of this study was to determine whether pretreatment with PC prior to HI stimulates subventricular zone (SVZ) proliferation and neurogenesis in newborn piglets. One-day-old piglets were subjected to PC (8% $O_2$/92% $N_2$) for 3 h and 24 h later were exposed to HI produced by combination of hypoxia (5% $FiO_2$) for a pre-defined period of 30 min and ischemia induced by a period of 10 min of hypotension. Here we demonstrate that SVZ derived neural stem/progenitor cells (NSPs) from PC, HI and PC + HI piglets proliferated as neurospheres, expressed neural progenitor and neurodevelopmental markers, and that greater proportion of the spheres generated are multipotential. Neurosphere assay revealed that preconditioning pretreatment increased the number of NSP-derived neurospheres in SVZ following HI compared to normoxic and HI controls. NSPs from preconditioned SVZ generated twice as many neurons and astrocytes *in vitro*. Injections with 5-Bromo-2-deoxyuridine (BrdU) after PC revealed a robust proliferative response within the SVZ that continued for one week. PC also increased neurogenesis *in vivo*, doublecortin positive cells with migratory profiles were observed streaming from the SVZ to striatum and neocortex. These findings show that the induction of proliferation and neurogenesis by PC might be a positive adaptation for an efficient repair and plasticity in the event of a hypoxic–ischemic insult.


## Introduction

Hypoxic–ischemic (HI) injury to the prenatal and perinatal brain is a major contributor to global child mortality and morbidity (Volpe, 2001). Perinatal hypoxic–ischemic injury affects between 1 and 8 per 1000 full-term infants and nearly 60% of low birth-weight (premature) infants (Vannucci, 2000; Wagner et al., 1999). Birth asphyxia is the cause of 20 to 50% of all neonatal deaths worldwide. Approximately 25% of children who survive birth asphyxia develop permanent neurological

*Abbreviations:* BrdU, 5-bromo-2-deoxyuridine; Dcx, doublecortin; GFAP, glial fibrillary acidic protein; PC, hypoxic-preconditioning; HI, hypoxia–ischemia; SVZ, subventricular zone; NSPs, neural stem/progenitor cells

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http://dx.doi.org/10.1016/j.scr.2013.04.007

dysfunctions including cerebral palsy, mental retardation, learning disabilities, and epilepsy (Ashwal, 1993; Vannucci, 2000; Volpe, 2001; Wagner et al., 1999). Although the exact cause of HI encephalopathy is not always identified, antecedents include prolapsed umbilical cord, uterine rupture, placental damage, maternal hypotension and acute neonatal and maternal hemorrhage. The outcome from HI injury is further influenced by a variety of factors that include the gestational age as well as the nature, severity, and duration of hypoxic–ischemic insult. Despite advances in supportive care, no effective treatment strategies for HI brain injury are available at present and only partial benefit from hypothermia for neonates with moderate hypoxic–ischemic injury has been realized (Edwards et al., 2010).

Hypoxic-preconditioning (PC) is a phenomenon in which mild episodes of hypoxia induce a significant increase in resistance of neurons to subsequent damaging influences of severe hypoxia–ischemia (Dirnagl et al., 2003; Gidday, 2006; Ran et al., 2005). This initial PC stimulus triggers a cascade of endogenous adaptive mechanisms resulting in the development of tolerance. Although the molecular mechanisms of PC-induced neural hypoxic–ischemic tolerance are not completely understood, recent studies showed that PC in adult and neonatal rodents induced by brief carotid or middle cerebral artery occlusions afford neuroprotection when it precedes the lethal ischemic insult by 1–7 days (Gidday et al., 1994; Kitagawa et al., 1991; Stagliano et al., 1999; Vannucci et al., 1998). We have recently demonstrated for the first time the protective efficacy of hypoxic-preconditioning against hypoxic–ischemic injury in newborn piglet model (Ara et al., 2011).

In the last two decades, our knowledge concerning the underlying molecular basis of PC has substantially grown, and there is hope to potentially imitate the induction of an endogenous neuroprotective state in patients with a high risk of cerebral ischemia. Research into PC has led to the idea of prophylactically inducing protection in patients such as those undergoing brain and heart surgery and those with transient ischemic attack or subarachnoid hemorrhage. An even greater potential of the PC paradigm lies in its role as an experimental strategy to identify the mechanisms of endogenous brain neuroprotection. The major goal of such research is to understand how the brain protects itself against shortages in substrate delivery or other injurious challenges, with the explicit purpose of therapeutically exploiting such mechanisms by boosting or exogenously substituting endogenous protectants following HI.

Neural stem/progenitor cells (NSPs) are multipotent precursors that self-renew and retain the ability to differentiate into neurons, astrocytes and oligodendrocytes (Okano, 2002a, b). They reside throughout life in neurogenic zones such as the subventricular zone (SVZ) and subgranular zone (SGZ) of the hippocampal dentate gyrus (Palmer et al., 1995; Reynolds et al., 1992; Sanai et al., 2004). Recent advances in stem cell research, including selective expansion of neural stem/progenitor cells (NSPs) *in vitro*, identification of NSPs in the brain and detection of neurogenesis in brain, have laid the groundwork for the development of novel therapies aimed at inducing regeneration in the damaged central nervous system (CNS). Two main strategies have evolved from our recent understanding of stem cell biology. The first one consists of grafting NSPs to replace the dying cells; the second aims at recruiting endogenous stem cells or progenitors to the lesion sites (Emsley et al., 2005; Scheffler et al., 2006).

There is increasing evidence to support the existence of endogenous compensatory mechanisms, which are activated in response to injury and disease (Li and Chopp, 1999; Li et al., 1997; Lindvall and McKay, 2003). Brain injury caused by cerebral ischemia, seizures, or traumatic injury (Dempsey et al., 2003; Gould et al., 1997; Gu et al., 2000; Jin et al., 2001; Liu et al., 1998; Parent et al., 1997) can stimulate neurogenesis in SVZ and dentate gyrus (DG) of hippocampus, and direct the migration of newly produced neurons towards affected regions (Arvidsson et al., 2002; Hicks et al., 2007; Jin et al., 2003; Nakatomi et al., 2002; Parent et al., 2002a,b; Sun et al., 2007). Several studies demonstrate that newborn neurons migrate toward ischemic lesions, where they might participate in brain repair and functional recovery (Jin et al., 2003; Nakatomi et al., 2002; Parent et al., 2002a; Sun et al., 2007). However, the number of newly generated neurons remains insufficient to restore normal brain function (Arvidsson et al., 2002; Bjorklund and Lindvall, 2000). To overcome this limitation, the manipulation of NSPs has developed into a key strategy for brain repair. The proliferation potential of progenitor cells can be enhanced by external stimulators, e.g. environmental enrichment (Komitova et al., 2002), caloric restriction, exercise and growth factors (Dempsey et al., 2003; Jin et al., 2002; Sun et al., 2003) and by hypoxic preconditioning (Li et al., 2010; Liu et al., 1998; Naylor et al., 2005). PC triggers an adaptive response that prepares the brain to minimize damage and promote regeneration in the event of future ischemic insult. The investigation of endogenous pathways by which the brain protects itself from ischemia represents a novel paradigm for research into cerebral hypoxia–ischemia — one that holds promise for identifying unique hypoxic–ischemic therapeutics.

In the present study, we used a piglet model of cerebral hypoxia–ischemia; a powerful *in vivo* model which emulates important clinical similarities to human situation of birth asphyxia, to evaluate the NSP cell proliferation, differentiation and neurogenesis in SVZ of newborn piglets subjected to PC and severe hypoxia–ischemia subsequent to PC. Although no animal model can perfectly replicate the complexity of HI events that occur in the human neonate, the piglet model used in this study offers distinct advantages in replicating specific mechanisms, morphology and maturational stages relevant to age dependent injury responses. Piglets, with features of a neonatal human brain such as overall shape, gyral pattern, gray/white matter distribution, degree of myelination, and brain growth spurt (Armstead and Kurth, 1994; Dickerson and Dobbing, 1966; Hoehner et al., 1994), offer an appropriate model to study injury mechanisms and treatment approaches for cerebral ischemic injury. A major challenge facing translational efforts with respect to transplantation of stem/progenitor cells to the CNS are the need for more predictive animal models. The use of a non-rodent, non-inbred model, such as the piglet, may better predict success for NSP transplantation in the human patients. Piglets are advantageous as models of NSP therapy because they have heterogeneous genetic backgrounds, are developmentally equivalent to brain growth, maturation and differentiation to that of the human at birth, and have larger body sizes, providing better opportunity for long-term safety studies and surgical manipulations (Flecknell et al., 1982; Haaland et al., 1997). Porcine neural progenitors have been used as donor cells and are

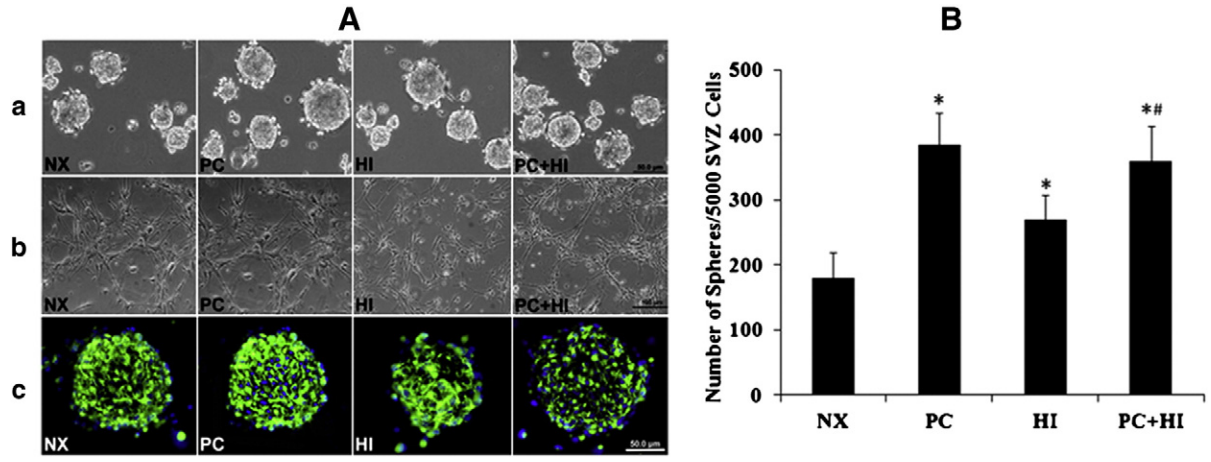


**Fig. 1** (A) Morphological and phenotypic properties of primary neural stem/progenitor cells derived from subventricular zone (SVZ) of newborn piglet brain. (a) Phase contrast image of primary SVZ neurosphere formation in culture from normoxic (NX), preconditioning (PC), hypoxic–ischemic (HI) and preconditioning + hypoxic–ischemic (PC + HI) piglet brain. (b) Differentiated neurospheres from NX, PC, HI and PC + HI piglet SVZ. (c) Nestin immunoreactivity (green) and Hoescht-labeled nuclei (blue) in undifferentiated neurospheres. (B) Hypoxic-preconditioning increases the number of colony-forming neural stem/progenitor cells derived from SVZ of neonatal piglet brain. Bar graph shows the quantification of secondary spheres generated from NX, PC, HI and PC + HI. $^{*}P < 0.05$ NX vs PC, NX vs HI and NX vs PC + HI; $^{\#}P < 0.05$ HI vs PC + HI using ANOVA and Bonferroni's post-hoc tests. Values are the mean number of spheres generated/5000 SVZ cells ± SD of four independent experiments, derived from four different neural stem/progenitor cell preparations from four individual piglet SVZ's/group.

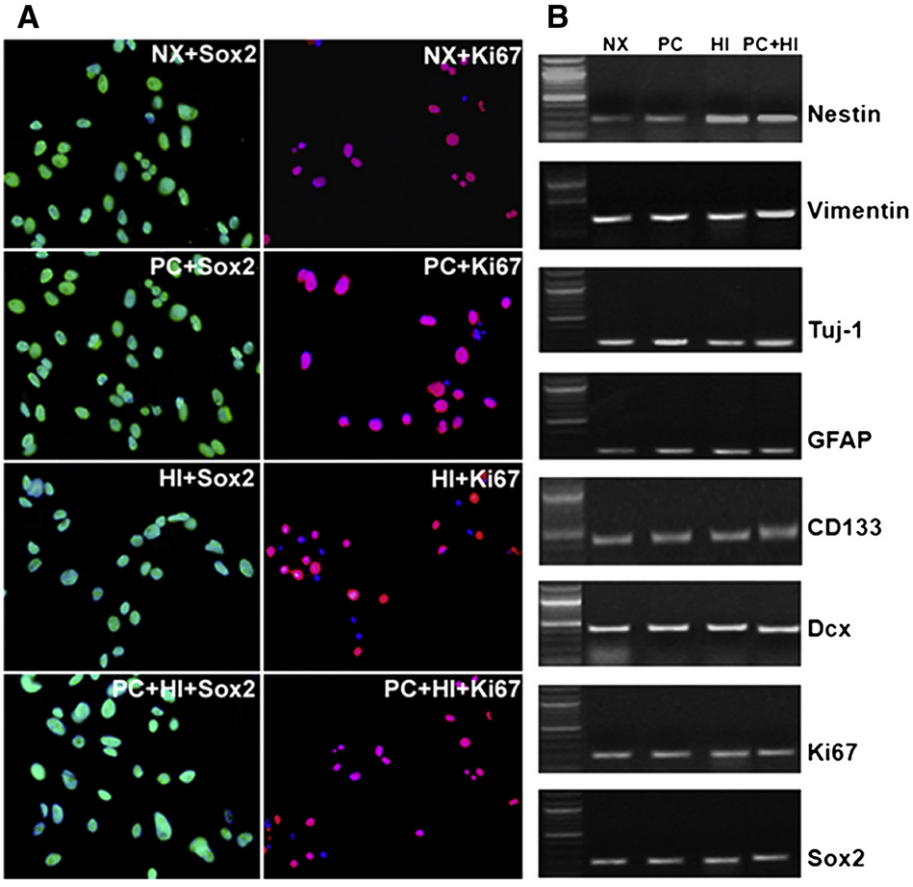


**Fig. 2** Neural stem/progenitor cells derived from preconditioning and hypoxic–ischemic piglet SVZ express neural progenitor and neural differentiation markers. (A) Immunofluorescence staining of neurospheres showing expression of stem/progenitor cell marker, Sox2 and proliferation marker Ki67. (B) RT-PCR of undifferentiated and differentiated neurospheres showing gene expression of CD133, Dcx, Ki67, Sox2, nestin, Tuj1 and GFAP in NX, HI and PC + HI piglet NSPs. A 100 bp ladder is at the left of the gel.

capable of engrafting into mammalian CNS after transplantation (Armstrong et al., 2003; Isacson and Deacon, 1996). These cells, therefore, provide a large animal comparison for human NSPs and have been considered as an alternative source for donor material. Thus, the studies conducted in piglet brains, which resemble those of humans in size and developmental trajectory, offer an important translational bridge from *in vitro* and rodent models to human patients.

Here, we report for the first time in a large animal model that PC pretreatment prior to hypoxia–ischemia increases proliferation, differentiation, and neurogenesis in SVZ of newborn brain. Our studies show that PC performed 24 h prior to hypoxia–ischemia significantly increased NSP cell proliferation both *in vitro* and *in vivo* and increased neurogenesis *in vivo*. These results suggest that the stimulation of proliferation and neurogenesis by PC might be a positive adaptation for an efficient repair in the event of an hypoxic–ischemic insult and this model might be a useful paradigm for the preclinical evaluation of novel cell based therapies for hypoxic–ischemic brain damage.

**Table 1** Percentage of neurons, astrocytes and oligodendrocytes generated from NSPs of newborn piglet brain.

| Groups | Percentage of cells | | |
|---|---|---|---|
| | Neurons (Tuj-positive) | Astrocytes (GFAP-positive) | Oligodendrocytes (O4-positive) |
| Normoxia | 42.0 ± 5.0 | 32.0 ± 4.9 | 13.0 ± 2.1 |
| PC | 66.0 ± 8.0 [a] | 40.0 ± 7.5 [a] | 10.0 ± 1.8 |
| HI | 55.0 ± 10.2 [a] | 35.0 ± 5.0 [a] | 9.0 ± 1.5 |
| PC + HI | 64.0 ± 12.0 [a b] | 42.0 ± 7.2 [a b] | 10.0 ± 1.2 |

[a] $P < 0.05$ Nx vs PC, NX vs HI and NX vs PC + HI.
[b] $P < 0.05$ HI vs PC + HI.

## Materials and methods

### Animals

All experimental animal protocols were approved by the Drexel University College of Medicine IACUC Committee and performed in accordance with US National Institutes of Health guidelines as outlined in the Policy on Humane Care and Use of Laboratory Animals (NIH publication August, 2002). All efforts were made to minimize animal suffering and to reduce the number of animals used.

### Hypoxic-preconditioning

Hypoxic-preconditioning was performed as described by Ara et al. (2011). Briefly, one-day-old female piglets were placed in a large plexiglass hypoxia chamber (Coy Laboratory products Inc., Grass Lake, MI) through which 8% $O_2$/92% $N_2$ was circulated for 3 h. Female piglets were chosen for these studies because in

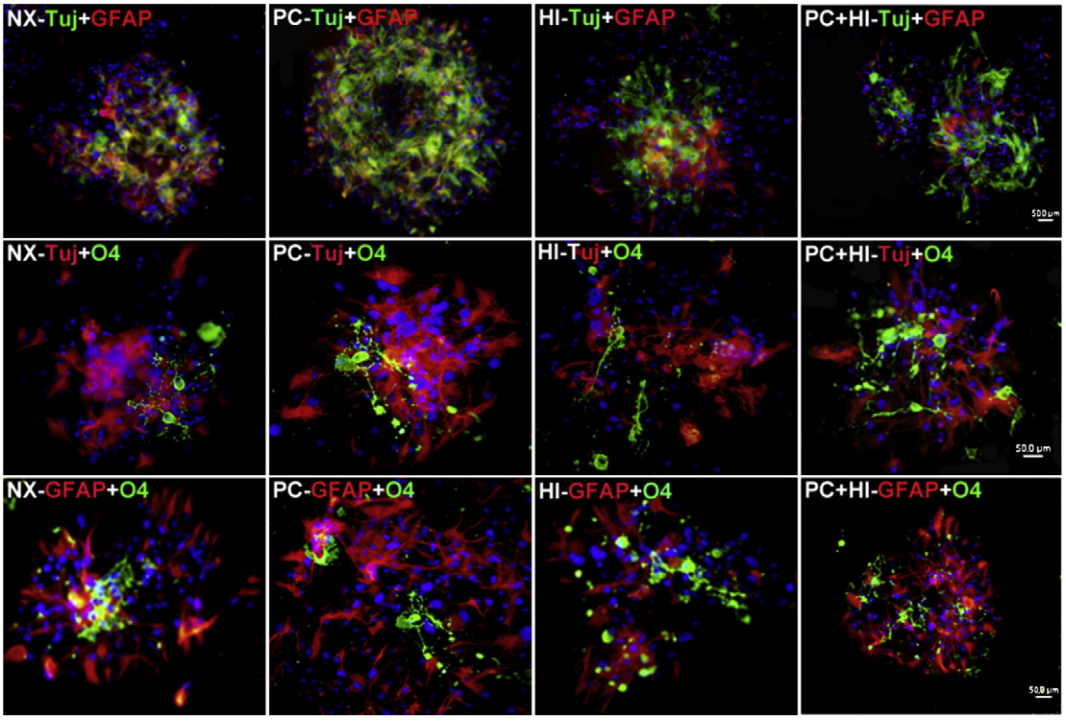


**Fig. 3** Piglet SVZ neural stem/progenitor cells derived from preconditioned and hypoxic–ischemic neonatal piglet SVZ are multipotent. Single primary EGF and bFGF2-generated spheres were stained with lineage-specific markers, Tuj1 (a marker for neurons), GFAP (a marker for astrocytes) and O4 (a marker for immature oligodendrocytes). Double immunolabeling of clonally derived spheres with Tuj1 (green) and GFAP (red) or Tuj1 (red) and O4 (green) or GFAP (red) and O4 (green) reveals that differentiated NSPs are multipotent with populations of neurons, astrocytes and oligodendrocytes. Majority of these cells were immunoreactive for a single marker (80%).

our preliminary studies we have found that females are more resistant to hypoxia–ischemia than males. Control piglets were exposed to 21% $O_2$. After hypoxic-preconditioning or normoxia, all animals were returned to the animal room for 1, 3 and 7 days.

## Cerebral hypoxia–ischemia

Cerebral hypoxia–ischemia was induced in newborn female piglets (one-day-old and average weight of 1.5 kg) as described previously (Ara et al., 2011). Briefly, piglets were anesthetized with 4% isofluorane, then intubated and ventilated. Inspired oxygen ($FiO_2$) and peak inspiratory pressure (PIP) were adjusted to maintain arterial oxygen saturation ($SaO_2$) 95–98% and arterial $pCO_2$ 35–45 mm Hg. An umbilical artery was aseptically cannulated for monitoring of blood pressure and arterial blood gases. Core body temperature was maintained at 38–39 °C. Heart rate (HR), mean arterial blood pressure (MABP), temperature and $SaO_2$ were monitored and recorded for the duration of the experiment. A digital electroencephalogram (EEG) device was used to monitor EEG amplitude and frequency. After intubation, the use of isofluorane was discontinued, and fentanyl (0.05 mg/kg) and pancuronium (0.3 mg/kg) were given as needed to maintain anesthesia. Hypoxia–ischemia was induced by decreasing $FiO_2$ to 5% and continued for 40 min. $FiO_2$ was decreased or increased by 1% increments during the insult to maintain HR (>130 beats/min) and MABP (>70% baseline). Hypotension was induced for the final 10 min of the insult by decreasing $FiO_2$ until the MABP was <70% of baseline. Hypoxia was terminated by resuscitation with 100% oxygen for 10 min to mimic the clinical situation. Furthermore, we chose 100% oxygen because in asphyxiated newborn piglets, microcirculation in the brain and normalization of metabolic markers as glutamate are restored faster with 100% than 21% $O_2$ (Solas et al., 2001). Following 10 min of 100% $FiO_2$, the ventilator rate and $FiO_2$ were reduced to maintain $PaO_2$ within the normal range until the piglet was able to breathe spontaneously, at which time the piglet was extubated. Animals were recovered for 1, 3 and 7 days. During the recovery period each piglet was neurologically assessed using the method described by Thoresen et al. (1996) approximately every 8 h for the first 24 h and then at 48 h, 72 h and 7 days. After reoxygenation periods, animals were anesthetized and perfused transcardially for histological and immunohistochemical analysis.

## Experimental groups

Piglets were divided into four groups (n = 4 or 5 in each group at each time point): (1) control normoxic group (NX): piglets were exposed to 21% $O_2$ for 3 h; (2) hypoxic preconditioned (PC) group: piglets were subjected to ($8\%O_2$/ $92\%N_2$) for 3 h; (3) hypoxic–ischemic (HI) group: animals were subjected to 5% $FiO_2$ for 40 min with 10 min hypotension; and (4) hypoxic preconditioned + hypoxic–ischemic (PC + HI) group: the piglets were preconditioned with $8\%O_2$/ $92\%N_2$ 24 h prior to hypoxia–ischemia (5% $FiO_2$, 40 min with 10 min hypotension). The piglets in all these four groups were recovered for 1, 3 and 7 days.

## Neurosphere preparation

Neurospheres were prepared from one-day-old piglets according to Ara et al. (2010). Briefly, piglets from above mentioned four groups were anesthetized after 24 h recovery using 4% isofluorane followed by intravenous injection of euthesol solution at a dose of 1.75 mg/kg. The piglets were sacrificed by decapitation and their brains were removed using aseptic techniques. Decapitation under anesthesia using intravenous injection of euthesol euthanasia solution at an overdose was performed to ensure rapid loss of consciousness combined with the ability to collect brain tissue that is anatomically undamaged and chemically uncontaminated. This method of euthanasia is consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association and was approved by the Drexel University College of Medicine IACUC committee.

The SVZ was isolated, placed in chilled sterile PBS with 0.6% glucose, and mechanically minced (one SVZ per preparation for each group). The tissue pieces were incubated in Hank's balanced salt solution containing 0.1% trypsin with 0.04% DNAase for 20 min at 37 °C. Following three washes in 0.04% DNAase, the tissue was triturated, passed through a 40 μm cell strainer and the number of viable cells was determined with a hemocytometer by exclusion of 0.1% Trypan Blue dye. The cells were then plated as a single cell suspension at a density of $3.75 \times 10^6$ cells into 75 $cm^2$ cell culture flasks in 10 ml DMEM/HAMS F12 supplemented with 20 ng/ml epidermal growth factor (EGF), 20 ng/ml human basic fibroblast growth factor 2, (bFGF-2), 2% B-27 supplement (Invitrogen Corporation, San Diego, CA) and penicillin G/streptomycin sulphate/amphotericin (1:100; Mediatceh Inc., Manassas, VA). After 20 days in culture, neurospheres were generated and floated in the culture. Cultures were passaged every 2 weeks by mechanical tituration of the spheres to yield a single cell suspension. The mixture of single cells was then replated using identical conditions.

## Neurosphere differentiation

Neurospheres were collected and plated onto poly-L-ornithine (15 μg/ml) coated sterile coverslips (Nunc, Naperville, IL) in EGF and bFGF-2 free media at a density of 10 spheres per well for 5–7 days. In some experiments, neurospheres were dissociated into single cells and plated at a density of $2.5 \times 10^4$ cells/$cm^2$ in EGF and bFGF-2 free media per well for 5–10 days with media replenished every 2 days. Differentiated cells were fixed and processed for immunofluorescence or RT-PCR studies. Experiments were performed at passage 2 through 4 for immunofluorescence and RT-PCR studies.

## Clonal generation of neurospheres

To demonstrate the multipotency of neural stem/progenitor cells, we performed clonal analysis according to Ara et al. (2010). Single-cell suspensions made from passage 3 neurospheres derived from piglet SVZ were diluted in EGF and bFGF-2 medium and plated in 96-well plates (1 cell per well). Plates were scored 24 h later. All wells that contained only one viable cell were marked and these wells were rescored 16–20 days later for the presence of spheres. Every 48 h, 50%

of the growth medium was exchanged for fresh medium. The spheres were transferred onto poly-L-ornithine (15 μg/ml) coated sterile coverslips (1 sphere/coverslip) and differentiation was induced in medium by omitting EGF and bFGF-2. The differentiated spheres were processed for triple and double labeling immunofluorescence.

For subcloning experiments, primary neurospheres were extracted, individually dissociated and the resulting single cell suspension from each neurosphere (200 cells/ml/well) were plated into individual wells of a six-well plate for quantitative determination of the frequency of secondary spheres. Some of the secondary spheres were processed for second subcloning step to generate tertiary spheres.

## Neurosphere quantitation

Cells were cultured for the *in vitro* neurosphere assay (Reynolds and Weiss, 1996) under clonal conditions as described above. Cells were plated at 10 cells/μl in 24-well (0.5 ml/well) plates in DMEM/HAMS F12 supplemented with 20 ng/ml epidermal growth factor (EGF), 20 ng/ml human basic fibroblast growth factor 2, (bFGF-2), 2% B-27 supplement and penicillin G/streptomycin sulphate/amphotericin. The total number of spheres that formed in each well was counted after 10 days; only colonies >100 μm in diameter were counted as spheres. Ten random fields were counted per well. The frequency of sphere forming cells (i.e. NSPs) was calculated from the average number of spheres per field, the area of the field, and the area of the well.

## Detection of gene expression by RT-PCR analysis

For RT-PCR analysis, undifferentiated and differentiated neurospheres derived from four different NSP preparations were collected, washed twice with phosphate buffered saline (PBS), pelleted and subjected to RNA isolation according to Ara et al. (2010). Total RNA was isolated using QIAGEN RNeasy Mini reagent kit (Qiagen, CA). The purity and integrity of the RNA preparation was checked using spectrophotometer and by 1% formaldehyde-agarose gel electrophoresis. First-strand

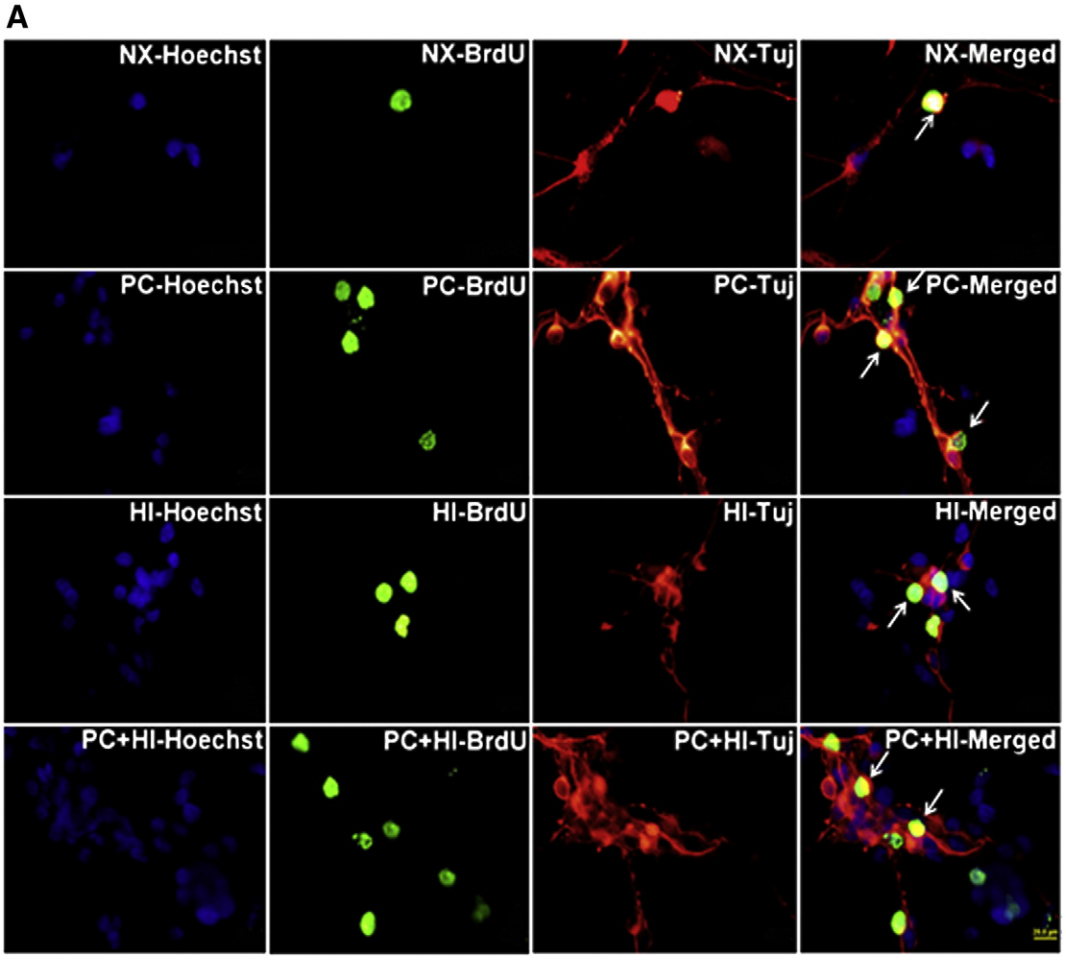


**Fig. 4** Hypoxic-preconditioning increases the proliferation of neural stem/progenitor cells *in vitro*. Cells positive for the neuronal marker Tuj1 (A) or astrocytic marker GFAP (B) incorporated BrdU indicating that both neuronal and glial precursors are *de novo*-generated from proliferating NSP cells. (C) Quantitative analysis of BrdU-positive progenitor cells showed over 45% of NSPs from PC + HI piglets positive for BrdU in early passages, whereas only 30% cells were BrdU positive in HI piglets. Data are reported as means ± SD of four independent experiments, derived from four different NSP preparations from four individual piglet SVZ's/group. $^{*}P < 0.05$ NX vs PC, NX vs HI and NX vs PC + HI; $^{\#}P < 0.05$ HI vs PC + HI.

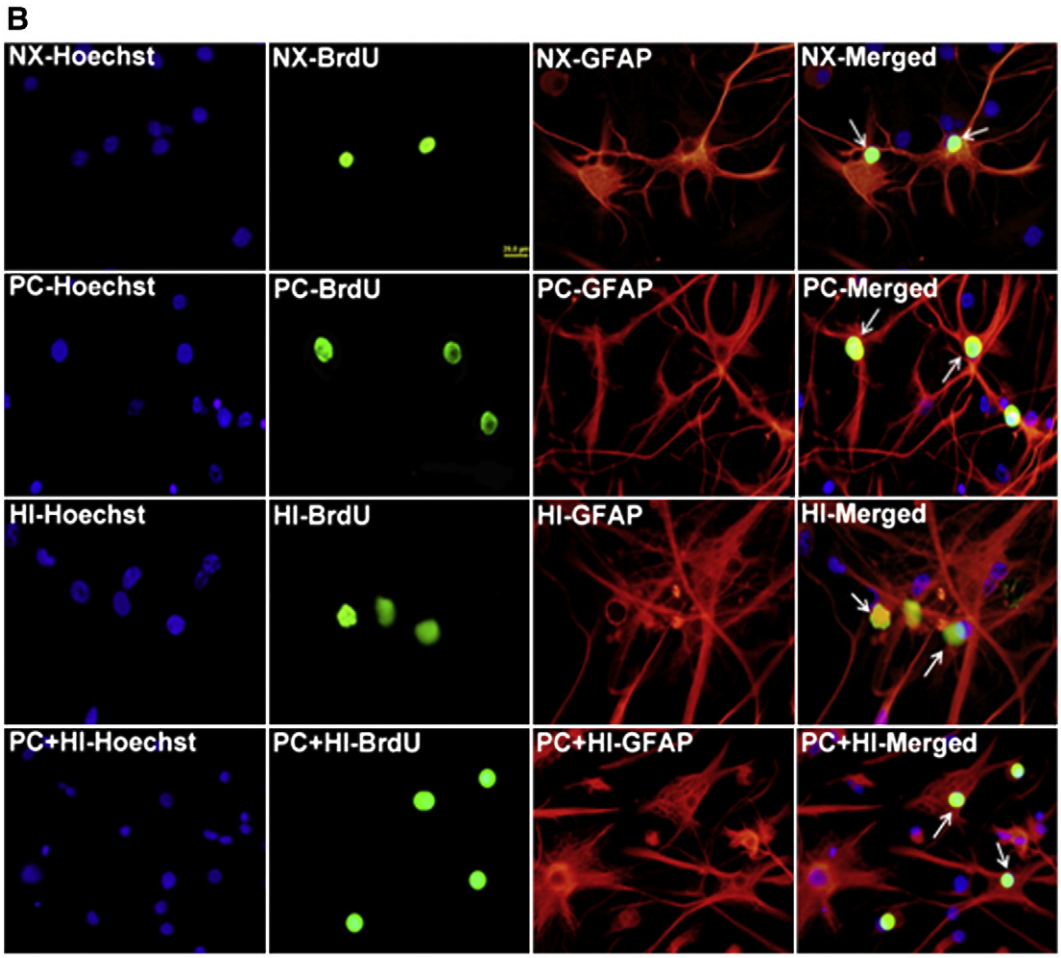


**Fig. 4** (*continued*).

complementary DNA (cDNA) was synthesized from 1 μg of total RNA using SuperscriptTM II reverse transcriptase and oligo (dT) 18 primers (Invitrogen Corporation, San Diego, CA). The transcripts that encode CD133 (prominin 1), proliferation marker Ki67, nestin, vimentin, Sox2 (SRY-box containing gene 2), Tuj1 (class III β-tubulin), microtubule-associated protein Dcx (double cortin), and GFAP (glial fibrillary acidic protein) were amplified by polymerase chain reaction (PCR). PCR products were run on 1.5% agarose gels and their size was estimated using a 100-bp ladder.

## Immunocytochemistry

For immunocytochemical studies, EGF and bFGF-2 generated individual primary neurosphere was transferred onto poly-L-

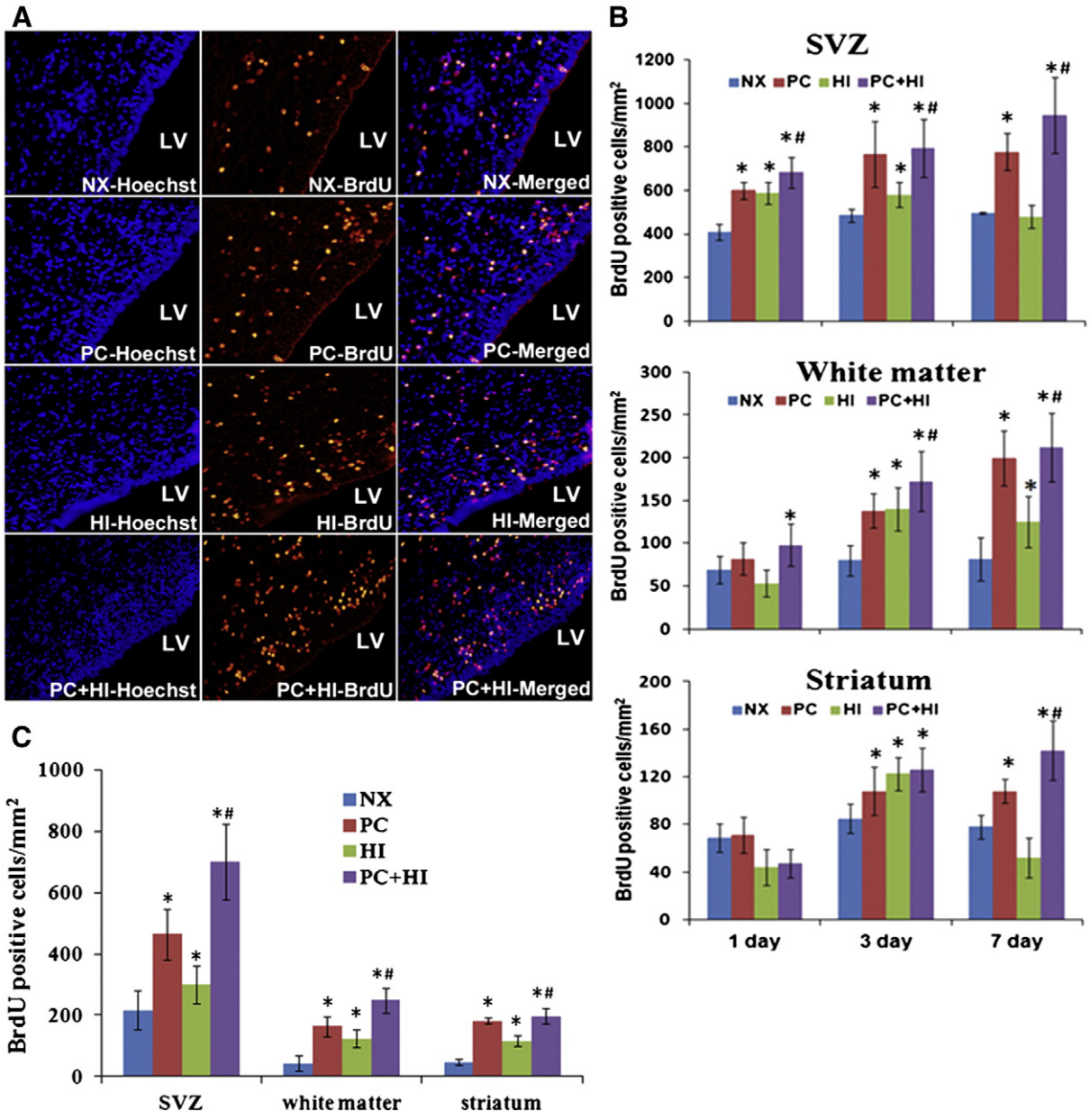


**Fig. 5** Hypoxic-preconditioning increases the number of BrdU positive cells in newborn piglet brain. (A) Shows the BrdU incorporation of neural stem/progenitor cells in SVZ after one week of recovery following repeated administration of BrdU injections. (B) The number of BrdU-positive cells/mm$^2$ (following a single injection of 50 mg/kg, 2 h before sacrifice) increased in the subventricular zone (SVZ), White matter and Striatum of newborn piglet brain at 1, 3 and 7 days of recovery post hypoxic-preconditioning. (C) Following repeated administrations; BrdU-labeled cells were significantly increased in the SVZ, striatum and white matter at 7 days of recovery following hypoxic-preconditioning. Data are reported as means ± SD in four sections from each piglet brain with four animals per group. $^*P < 0.01$ NX vs PC; $^*P < 0.01$ NX vs HI; $^*P < 0.01$ NX vs PC + HI and $^\#P < 0.05$ HI vs PC + HI.

ornithine coated 13 mm coverslips as previously reported (Ara et al., 2010). The neurospheres were fixed, washed and blocked with 5% normal goat serum in PBS with 0.1% triton X-100 and processed for immunocytochemistry with antibodies to intermediate filament nestin (Millipore, MA, 1:100 dilution). Cells were washed and incubated with biotinylated goat anti-rabbit IgG followed by streptavidin. The cells were counterstained with nuclear dye Hoechst 33258 (2 μg/ml) for 5 min and mounted in Vectashield (Vector laboratories, Burlingame, CA). For assessment of nonspecific staining, the primary antibody was omitted and replaced with rabbit primary antibody isotype control (Life Technologies, Grand Island, NY).

Neurospheres were also processed for neural stem/progenitor markers Sox2 and a proliferation marker Ki67. Neurospheres were immunostained for Sox2 (mouse IgG, Abcam Inc. Cambridge, MA, 1:100 dilution) and Ki67 (Santa Cruz Biotechnologies, Santa Cruz, CA, 1:100 dilution) for 30 min at room temperature. Cells were washed and incubated with biotinylated secondary antibodies followed by streptavidin.

To assess the differentiation potential of neurospheres, single primary neurosphere was transferred onto poly-L-ornithine (15 μg/ml) coated sterile coverslips and differentiation was induced in EGF and bFGF-2 free medium for 5–10 days. Differentiated neurospheres were immunostained live for oligodendroglial progenitor marker O4 (hybridoma supernatants, mouse IgM, a gift from Dr. Judy Grinspan, 1:20 dilution) under non-permeabilized conditions, or after fixation with 4% paraformaldehyde and permeabilization with 0.1% Triton X-100 for anti-Tuj1(rabbit IgG, Covance, CA, 1:500 dilution) or anti-GFAP (mouse IgG, Millipore, MA, 1:200 dilution) to identify neurons and astrocytes respectively. For double labeling of Tuj1 and O4 or GFAP and O4, live cells were incubated with O4 antibody, washed and incubated with anti-FITC-conjugated anti-mouse IgM. Cells were washed, blocked with 5% normal goat serum in PBS with 0.1% triton X-100 and incubated in primary antibody against Tuj1 or GFAP. A rhodamine-conjugated goat anti-rabbit IgG secondary antibody was used to detect Tuj1 staining and rhodamine-conjugated anti-mouse IgG was used to detect GFAP staining. For Tuj1 and GFAP double staining, cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and then blocked with 5% normal goat serum for 15 min at room temperature. The cells were incubated in primary antibodies against Tuj1 and GFAP together followed by a secondary antibody for FITC-conjugated-anti-rabbit IgG and rhodamine-conjugated anti-mouse IgG to detect Tuj1 and GFAP, respectively. The cells were washed in PBS and counterstained with nuclear dye Hoechst for 5 min and mounted in Vectashield. Secondary antibodies for all immunofluorescent staining were obtained from Jackson Immunoresearch laboratories, West Grove, PA. For control immunochemistry, primary antibody was replaced with a specific immunoglobulin isotype control antibody. The expression of each antigen was examined in separate experiments at least three times.

## 5-Bromo-2-deoxyuridine incorporation and detection

Cell proliferation was estimated by using 5-Bromo-2-deoxyuridine (BrdU). Neurospheres were trypsinized and plated on poly-L-ornithine coated coverslips and were cultured for 20 h in the presence of 30 μg/ml of BrdU prior to immunostaining. Cells were fixed with 70% ethanol for 30 min at −20 °C and then denatured by incubation in 2 N HCl for 30 min at room temperature followed by extensive rinses in 0.1 M borate buffer, pH 8.5. The cells were then incubated with monoclonal anti-BrdU (1:100; Santa Cruz Biotechnology Inc, Santa Cruz, CA) alone or double stained for either BrdU/Tuj1 or BrdU/GFAP or BrdU/O4 for 40 min at room temperature. Cells were washed and incubated with secondary antibodies conjugated to appropriate fluorochromes (Jackson Immunoresearch, West Grove, PA) for 30 min. After five washes with PBS, cells were counterstained with nuclear dye Hoechst 33258 and mounted with Vectashield. BrdU positive cells were counted in 10 random fields of three coverslips and percent positive was calculated by dividing the positive cells by the total cell number.

Progenitor cell proliferation *in vivo* was estimated by injecting above-mentioned groups of piglets (n = 4) with BrdU (Sigma, 50 mg/kg body weight, 10 mg/ml in 0.007 N NaOH in 0.9% NaCl) intraperitoneally. Two BrdU staining paradigms were used. Paradigm one was used to identify the phenotype and location of S-phase cells at 1, 3, and 7 days by pulsing single BrdU injections at the time point of interest followed by sacrifice 2 h later. The second paradigm involved BrdU injections at 8 h intervals twice daily starting day 1 of recovery for 5 consecutive days followed by sacrifice at 7 days after initial injection to determine cell fate, phenotype, survival and migration of cells. The piglets were anesthetized and transcardially perfused for single and double immunofluorescence labeling.

For immunohistochemical analysis of BrdU, the 5-μm paraffin sections were pretreated with 2 N HCl followed by extensive rinses in 0.1 M borate buffer. Sections were incubated overnight with anti-BrdU antibody. Sections were also stained using a rabbit anti-Dcx antibody (1:100; Santa Cruz Biotechnology Inc, Santa Cruz, CA) for a marker of migrating neuroblasts. After washing, sections were incubated for 1 h with biotinylated secondary antibodies followed by streptavidin for 30 min at room temperature. The sections were counterstained with Hoechst and mounted in Vectashield. For double labeling of BrdU/Dcx, BrdU/GFAP and BrdU/nestin, sections were pretreated in 2 N HCl for 60 min, rinsed in 0.1 M borate buffer and incubated in primary antibody against BrdU. The sections were washed, and incubated with rhodamine-conjugated anti-rat IgG (Jackson Immunoresearch, West Grove, PA) for 1 h at room temperature. Sections were then subsequently stained using anti-Dcx to identify immature neurons, anti-GFAP to identify astrocytes and anti-nestin to identify NSPs. After washing, sections were incubated with FITC-conjugated anti-rabbit or anti-mouse-IgG. The cells were counterstained with Hoechst for 5 min and mounted in Vectashield.

## Quantification of BrdU immunostaining

The BrdU-positive cells were counted in tissue sections counterstained with Hoechst 33258 and quantification was performed in four sections from each piglet brain by an independent investigator. In each case, the area of the SVZ, white matter and striatum was traced and measured in square millimeters using image J software (NIH, Bethesda, MD). The total number of BrdU-positive cells in the selected areas were counted and divided by the area to obtain a measurement of cells per $mm^2$. The values of cell counts are presented as means ± SD and statistical analysis was conducted using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple-comparisons post-test.

## Statistical analysis

For quantification of the percentage of cells producing a given marker, in any given experiment, the numbers of immunoreactive cells were determined relative to the total number of Hoechst-labeled nuclei. Three coverslips were evaluated for each type of marker and five random fields of each coverslip were analyzed. Each field contained 100–200 cells. Results were analyzed for statistical significance using analysis of variance (ANOVA), and all error bars representing SDs of four independent experiments, derived from four different neural stem/progenitor cell preparations from four individual piglet SVZ's/group. Post hoc test (Bonferroni's multiple-comparison

test) was carried out to analyze means between four independent experiments of cell preparations. *P* values $<0.05$ were considered significant.

## Results

### Hypoxic-preconditioning increases the number of colony-forming neural stem/progenitor cells derived from SVZ of neonatal piglet brain

We first characterized NSPs at 24 h recovery after NX, PC, HI, and PC + HI in newborn piglet brain as described in Ara et al. (2010). SVZ derived NSPs proliferated and formed free floating neurospheres, which grew in size over time in response to mitogen EGF and bFGF-2 (Fig. 1A, Panel a). During subsequent subcultures, the floating spheres could be dissociated into single cells and proliferated to generate new spheres. These cells could also generate neurospheres when cultured at a density of one cell per well, confirming that neurospheres resulted from expansion of individual clones rather than from aggregation of cells. Clonal analyses performed on NX, PC, HI and PC + HI NSPs yielded between 4.0 and 9.5% primary sphere-forming efficiency. The frequency of secondary and tertiary neurosphere generation was also between 5 and 9%. When single spheres were plated onto poly-L-ornithine coated glass coverslips in 24-well culture plates in the absence of mitogen EGF and

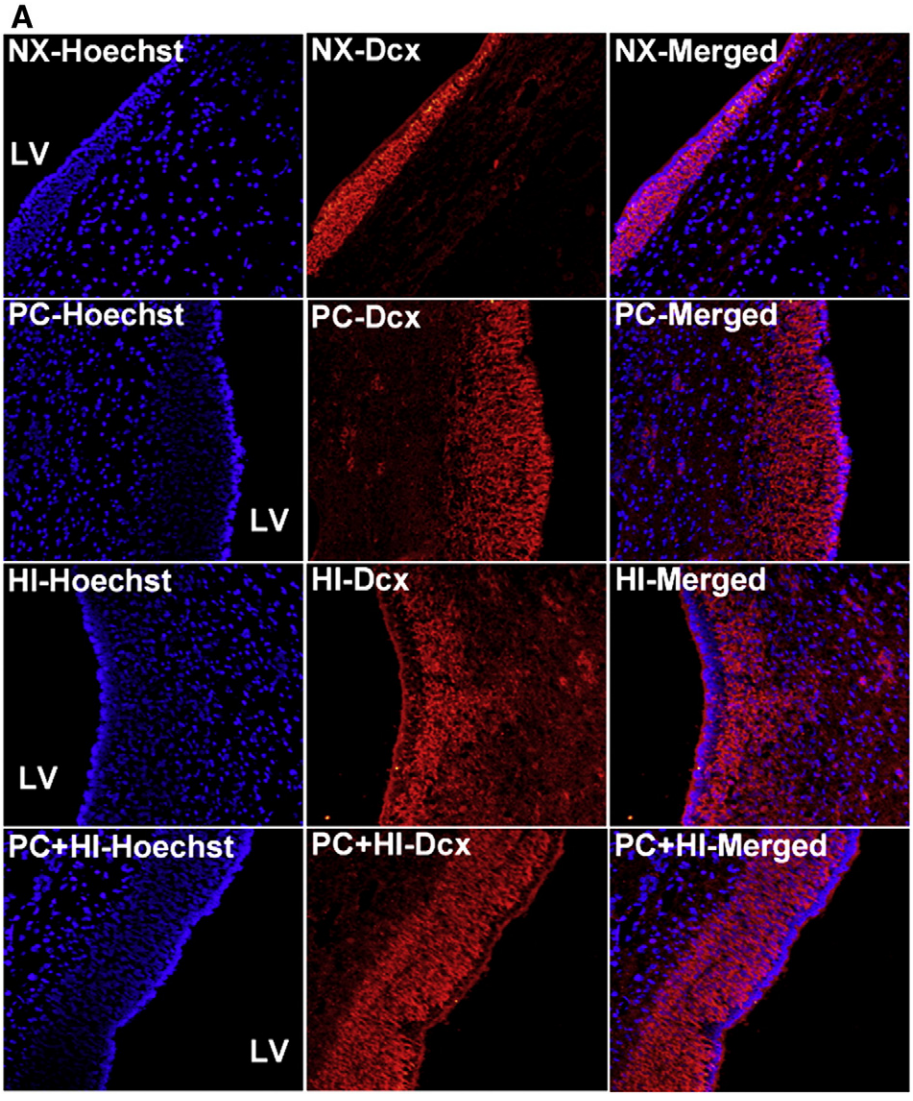


**Fig. 6** (A) Doublecortin immunoreactivity in SVZ is increased in hypoxic-preconditioned newborn brain at 7 days of recovery. Dcx-positive cells (red) were more abundant in the SVZ of preconditioned (PC) and preconditioning followed by hypoxia–ischemia (PC + HI) piglets compared with the hypoxic–ischemic (HI) and normoxic (NX) piglets at 7 days. LV = lateral ventricle. Scale bar 50 μm. (B) Doublecortin immunoreactivity in cortex is increased in hypoxic-preconditioned newborn brain at 7 days of recovery. Dcx positive cells were more abundant in the neocortex of preconditioned animals after one week of recovery compared to that of NX and HI piglets. Scale bar 50 μm.

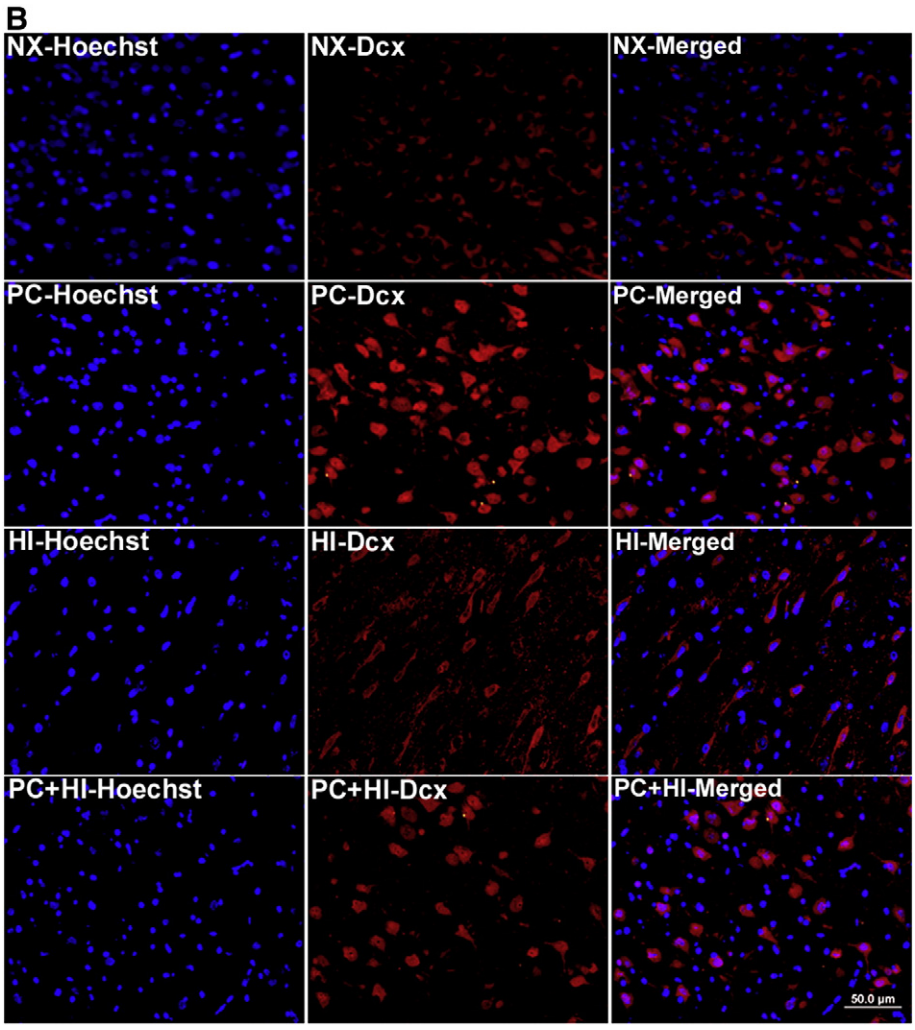


**Fig. 6** (*continued*).

bFGF-2, the spheres exhibited little proliferation and differentiated within a few days (Fig. 1A, Panel b). These cells could also be grown as adherent monolayers. Both adherent cells and spheres exhibited morphological characteristics consistent with other mammalian neural progenitor cultures.

We next studied whether NSPs derived from preconditioned and hypoxic–ischemic SVZ express markers that are indicative of an undifferentiated state. Using a rabbit polyclonal antibody against nestin, an intermediate filament protein which is an early marker for nondifferentiated neural progenitors, we found that more than 80% of cells forming the neurosphere were positive for nestin. Fig. 1A (Panel c), shows the nestin positive neurospheres (green) and Hoechst labeled nuclei (blue) generated from NX, PC, HI and PC + HI SVZ in the presence of growth factors, EGF and bFGF-2. These results verify that EGF and bFGF-2 induces the proliferation of undifferentiated neural stem/progenitor cells derived from SVZ of newborn piglet brain.

Cells were isolated from SVZ of PC, HI and PC + HI piglet brain for the *in vitro* neurosphere assay under clonal conditions to determine whether there was a concomitant increase in the number of sphere forming cells after PC compared to normoxic controls. Cells were plated at 10 cells/μl in 24-well (0.5 ml/well) plates in DMEM/HAMS F12 supplemented with EGF and bFGF-2. The total number of spheres that formed in each well was counted after 10 days. Quantitative analysis of the neurospheres revealed a significant increase in the number of spheres derived from PC and PC + HI piglet SVZ after 24 h recovery compared with the number of spheres derived from control SVZ ($P < 0.05$, Fig. 1B). Twice as many neurospheres were generated from the SVZ of piglets following PC and from piglets that were preconditioned 24 h prior to HI than from normoxic SVZ (PC, 395 ± 32; PC + HI, 380 ± 55 compared with NX, 190 ± 30) (Fig. 1B). The number of neurospheres generated from the SVZ of PC + HI piglets was 1.4-fold higher than that of HI SVZ (380 ± 55 compared with 275 ± 35) and the number of spheres generated from HI SVZ was 1.5 fold higher than normoxic controls (275 ± 35 compared with 190 ± 30) (Fig. 1B).

## Neural stem/progenitor cells derived from preconditioned and hypoxic–ischemic neonatal piglet SVZ express neural progenitor and neural differentiation associated markers and genes

Passage three individual neurospheres were transferred onto poly-L-ornithine coated sterile coverslips and one

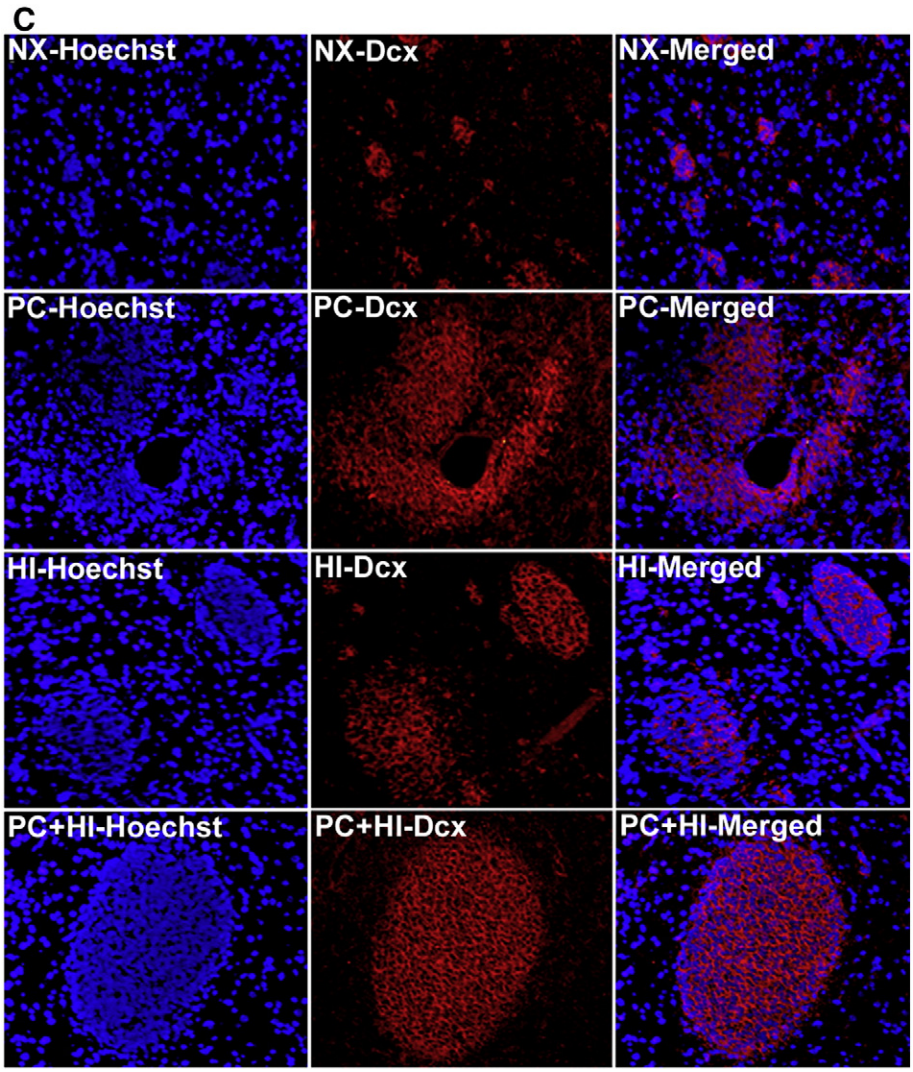


**Fig. 6** (*continued*).

week after plating, cells were stained with neural stem/progenitor marker Sox2 and a proliferation marker Ki67. Immunofluorescence studies showed the expression of Sox2 as well as the presence of proliferation marker Ki67 in NSPs derived from PC, HI and PC + HI SVZ (Fig. 2A).

Total RNA from primary neural stem/progenitor cell cultures derived from NX, PC, HI and PC + HI neonatal piglet SVZ was examined by RT-PCR for the expression of established neurodevelopmental markers. We investigated the expression of the stem/progenitor cell markers and neural differentiation markers in these cells. The piglet NSP cultures exhibited the expression of nestin, vimentin, CD133 and nuclear transcription factor Sox2, as well as the proliferation marker Ki67 (Fig. 2B). These cells also expressed cytoskeletal associated proteins double cortin (Dcx), an immature neuron marker Tuj1, and astrocyte marker GFAP. In each case after PCR, a single product of a size appropriate for the target RNA was present, indicating that neonatal piglet SVZ derived neurospheres express each of these neural stem/progenitor cell and neural differentiation associated genes (Fig. 2B).

## Neural stem/progenitor cells derived from preconditioned and hypoxic—ischemic neonatal piglet SVZ are multipotent

In order to demonstrate the multipotency of piglet SVZ derived NSP cells from PC, HI and PC + HI piglet, we performed a clonal analysis. We examined whether NSPs had the potential to differentiate into cells with neuron, astrocyte, and oligodendrocyte characteristics. Single primary EGF and bFGF2-generated spheres were stained with lineage-specific markers, Tuj1 (a marker for neurons), GFAP (a marker for astrocytes) and O4 (a marker for immature oligodendrocytes). Double immunolabeling of clonally derived spheres with antibodies to Tuj1 and GFAP or Tuj1 and O4 or GFAP and O4 revealed that differentiated NSPs are multipotent with populations of neurons, astrocytes and oligodendrocytes (Fig. 3).

The percentage of neurons and astrocytes generated from NSPs from PC alone or PC + HI was significantly greater than the percentages generated from NSPs from

HI or normoxic SVZs ($P < 0.05$, Table 1). The percentage of neurons was ~1.6 and 1.5 fold higher in NSPs from PC and PC + HI respectively compared to that of normoxic SVZ (PC, 66 ± 8%; PC + HI, 64 ± 12% compared with NX, 42 ± 5%; $P < 0.05$). The percentage of astrocytes was ~1.3 fold higher in PC and PC + HI NSPs compared to normoxic controls (PC, 40 ± 8%; PC + HI, 42 ± 7% compared with NX, 32 ± 5%; $P < 0.05$). The percentage of neurons and astrocytes in PC + HI NSPs increased ~1.2 fold over that of HI NSPs (PC + HI, 64 ± 12% compared to HI, 55 ± 10% (neurons) and 42 ± 7% compared to 35 ± 5% (astrocytes) respectively ($P < 0.05$, Table 1). We did not observe significant differences in the percentages of oligodendrocytes between the four groups (Table 1).

## NSP proliferation increases in newborn brain following hypoxic-preconditioning

To analyze the proliferative capacity of NSPs isolated from PC, HI and PC + HI piglet SVZ, we used bromo-deoxyuridine (BrdU), which is selectively incorporated by cells during S phase of mitosis. Cells positive for the neuronal marker Tuj1 or astrocytic marker GFAP incorporated BrdU (Figs. 4A and B), indicating that both neuronal and glial precursors are *de novo*-generated from proliferating progenitor cells. There was a significant increase in BrdU positive cells in PC and PC + HI animals compared to normoxic controls. NSPs from PC piglets showed 30% higher BrdU positive cells than normoxic NSPs (36 ± 4.5% compared with 25.0 ± 5.0%; $P < 0.05$) (Fig. 4C). There was a 40% increase in BrdU positive NSPs from PC + HI piglets compared to normoxic controls in early passages (44 ± 6.0% compared with 25.0 ± 5.0%; $P < 0.05$), and 25% increase in BrdU positive NSPs in HI piglets (33.0 ± 6.0% compared with 25.0 ± 5.0%; $P < 0.05$) (Fig. 4C). The percent of BrdU/Tuj1 (16 ± 2.0%) and BrdU/GFAP (12 ± 2.0%) double-labeled cells from PC + HI SVZ were significantly increased ($P < 0.05$) compared to the percent from HI (9 ± 1.0% for BrdU/Tuj1 and 8 ± 1.0% for BrdU/GFAP).

## Hypoxic preconditioning increases progenitor cell proliferation *in vivo*

BrdU labeling studies were performed to assess PC-induced SVZ cell proliferation *in vivo*. Pulsing single BrdU injections at the time point of interest followed by sacrifice 2 h later or injecting BrdU at 8 h intervals twice daily for 5 consecutive days resulted in BrdU incorporation into the replicated DNA molecules and labeling of proliferating progenitor cells. After 1, 3 days or one week of recovery from PC or PC performed 24 h prior to HI insult; there was an increased proliferation of cells in the SVZ, striatum and white matter as revealed by increased BrdU uptake (Fig. 5). Fig. 5 shows the numbers of BrdU-positive cells in NX, PC, HI and PC + HI animals in SVZ, white matter and striatum following single (Fig. 5B) and repeated administration (Fig. 5C) of BrdU injections. The number of BrdU positive cells increased significantly ($P < 0.05$) in SVZ of PC group at 24 h, 3 days and 7 days compared to normoxic controls with single BrdU injection paradigms. In HI group, the number of BrdU positive cells were significantly higher at 24 h and 3 days compared to normoxia, however, at 7-day recovery, there was no difference in BrdU positive cells between the two groups. In PC + HI group the number of BrdU positive cells in SVZ increased by 1.66 fold, 1.6 fold and 2.0 fold at 24 h, 3 and 7 days of recovery respectively compared to normoxia and by 1.2 fold, 1.4 fold and 2.0 fold compared to HI group (Fig. 5B). In subcortical white matter and striatum, there was a significant increase in BrdU positive cells in PC group at 3 and 7 days compared to normoxia. In PC + HI group, the BrdU positive cells were significantly higher ($P < 0.05$, Fig. 5B) at 3 and 7 days in white matter and striatum compared to normoxic group and significantly higher ($P < 0.05$) at 3 and 7 days in white matter and at 7 days in striatum compared to HI group.

Following repeated BrdU administration, the number of BrdU labeled cells in SVZ, white matter and striatum increased significantly after PC and PC + HI compared to normoxia (Fig. 5C). Quantitatively, BrdU positive cells in SVZ, white matter, and striatum increased by 2.1 fold, 3.9 fold and 4 fold respectively in PC group compared to normoxic group. In HI group there was 1.4 fold, 3 fold, and 2.5 fold increase in BrdU positive cells in SVZ, white matter and striatum respectively (Fig. 5C). The number of BrdU positive cells in SVZ, white matter and striatum of PC + HI group increased by 3.3 fold, 6 fold, and 4.2 fold compared to normoxic controls at 7 days of recovery. Furthermore, the BrdU-labeled cell numbers in the SVZ, white matter and striatum of PC + HI animals was significantly higher (2.5 fold, 2 fold, and 1.7 fold respectively, $P < 0.05$, Fig. 5C) than that of non-preconditioned (HI) animals. These results suggest that hypoxic-preconditioning performed 24 h prior to HI may promote progenitor cell proliferation in newborn piglet brain, contributing to the repair of the injured brain.

## Hypoxic preconditioning increases neurogenesis *in vivo*

To investigate whether there is an increase in neurogenesis *in vivo* after PC and HI, we evaluated Dcx expressing cells in the SVZ, neocortex (Figs. 6A and B) and striatum (Supplementary Fig. S6C) of newborn piglet brain. After 1 week of recovery, there were significantly more Dcx-immunostained cells in the SVZ of PC and PC + HI piglets compared with the HI and normoxic SVZ (Fig. 6A). Statistical analysis showed that the number of Dcx-positive cells in the SVZ of PC and PC + HI groups (387 ± 92 and 420 ± 102/mm$^2$) displayed a significant increase (1.5 fold and 1.6 fold respectively; $P < 0.05$) compared with the normoxic group (264 ± 65/mm$^2$) one week after recovery. Furthermore, the Dcx-positive cell numbers in the SVZ of PC + HI animals was significantly higher (420 ± 102/mm$^2$; $P < 0.01$) than that of non-preconditioned (HI) animals (311 ± 76/mm$^2$). In PC and PC + HI animals, a large number of Dcx positive cells with morphologies of migrating neuroblasts were found in striatum, which appeared to be migrating from the SVZ (Fig. S6C). Dcx positive cells with migratory profiles were rare in the normoxic striatum. A large number of the Dcx positive cells in PC (62 ± 20/mm$^2$; $P < 0.05$) and PC + HI animals (78 ± 27/mm$^2$; $P < 0.05$) migrated into the neocortex compared to normoxic (38 ± 10 mm$^2$) and HI animals (50 ± 15 mm$^2$) (Fig. 6B). After BrdU injections, the numbers of BrdU/doublecortin (Fig. 7A), BrdU/nestin (Fig. 7B), and

BrdU/GFAP (Fig. S7C) double-labeled cells also increased after PC (Table 2). The proportion of BrdU$^+$-Dcx$^+$, BrdU$^+$-Nestin$^+$ and BrdU$^+$-GFAP$^+$ double-labeled cells among BrdU-positive cells in the PC and PC + HI animals was significantly higher compared to normoxic or HI groups (Table 2; $P < 0.05$). These results suggest that PC may contribute to postnatal neurogenesis by modulating the proliferation and fate of SVZ neural/progenitor cells.

## Discussion

The present investigation demonstrates a comprehensive evaluation of the effects of hypoxic-preconditioning and hypoxia–ischemia subsequent to preconditioning on proliferation, differentiation and neurogenesis of NSPs resident in subventricular zone both *in vitro* and *in vivo*. Our data establishes that NSPs derived from SVZ of newborn piglets pretreated with preconditioning prior to hypoxia–ischemia formed greater number of neurospheres, generated twice as many neurons and astrocytes *in vitro*, and increased proliferation and neurogenesis in SVZ, striatum and white matter of newborn piglet brain *in vivo*. To the best of our knowledge this is the first study that utilized an *in vivo* piglet model of hypoxic-preconditioning-induced ischemic tolerance to demonstrate that PC induces proliferation and neurogenesis in newborn brain after hypoxic–ischemic injury.

Neurogenesis by NSPs and survival of newly differentiated cells can contribute to self-repair after neuronal loss (Arvidsson et al., 2002; Jin et al., 2003; Nakatomi et al., 2002; Sun et al., 2007). The process can be stimulated in response to CNS injury such as ischemic, traumatic, and inflammatory brain injury (Dempsey et al., 2003; Gould et al., 1997; Gu et al., 2000; Jin et al., 2001; Liu et al., 1998; Parent et al., 1997) and by signaling from astroglia (Song et al., 2002). However, spontaneous brain regeneration is inefficient to fully compensate for the neuronal loss observed in CNS disorders (Bjorklund and Lindvall, 2000). Activating the pathways governing restorative responses with select stimulants might enhance this process such that substantial functional tissue regeneration can occur. One of the strategies to activate endogenous repair systems is hypoxic-preconditioning, which activates an adaptive response that prepares the brain to minimize damage and promote regeneration in the event of future ischemic insult. Hypoxic-preconditioning, a sublethal hypoxic stimulus can also reduce infarct size and ameliorate neurologic deficits and, being sub threshold, does not induce cell death (Stenzel-Poore et al.,

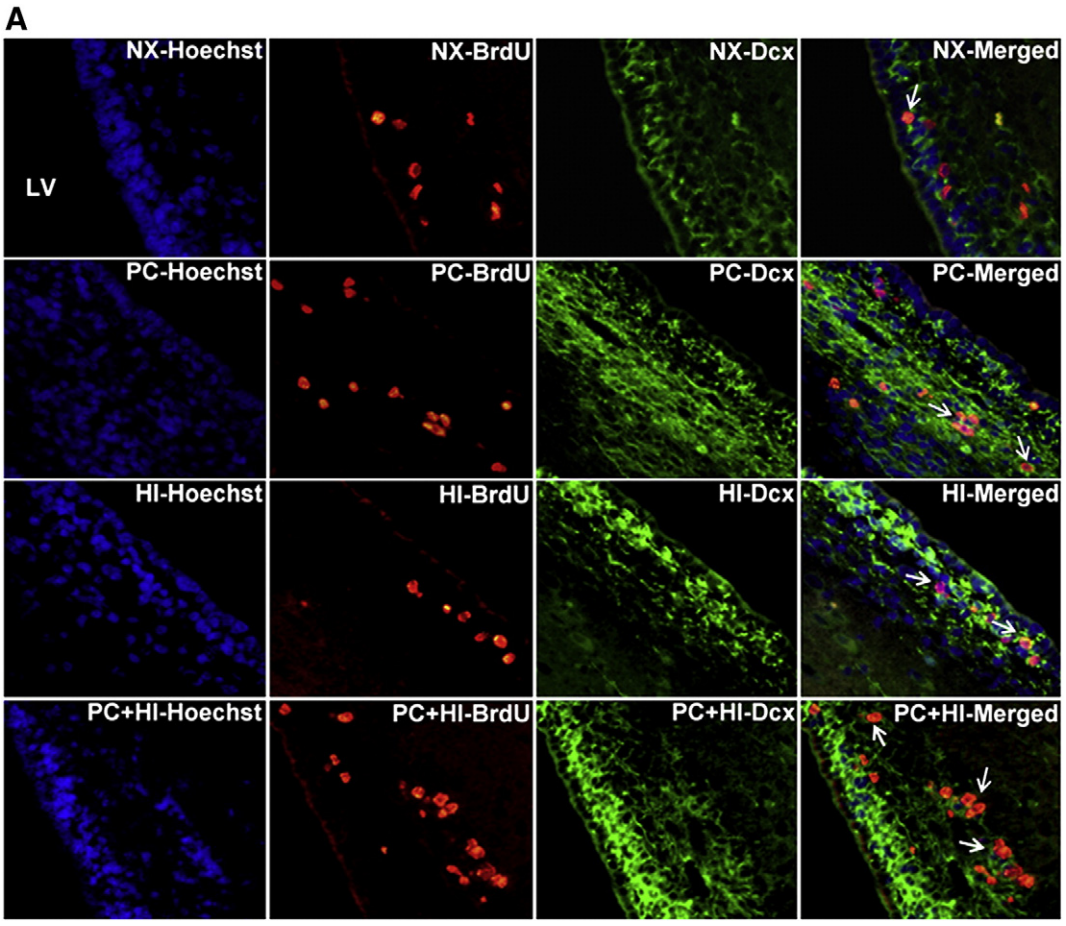


**Fig. 7** Double immunolabeling of BrdU positive cells with double cortin (A) and nestin (B) in NX, PC, HI and PC + HI piglet brain after 7 days of recovery. Images from the same panel showing BrdU (red), Dcx (green, 7A), and nestin (green, 7B) immunoreactivity separately and as merged images for the control, PC, HI and PC + HI groups. Scale bar 50 μm. LV = lateral ventricle.

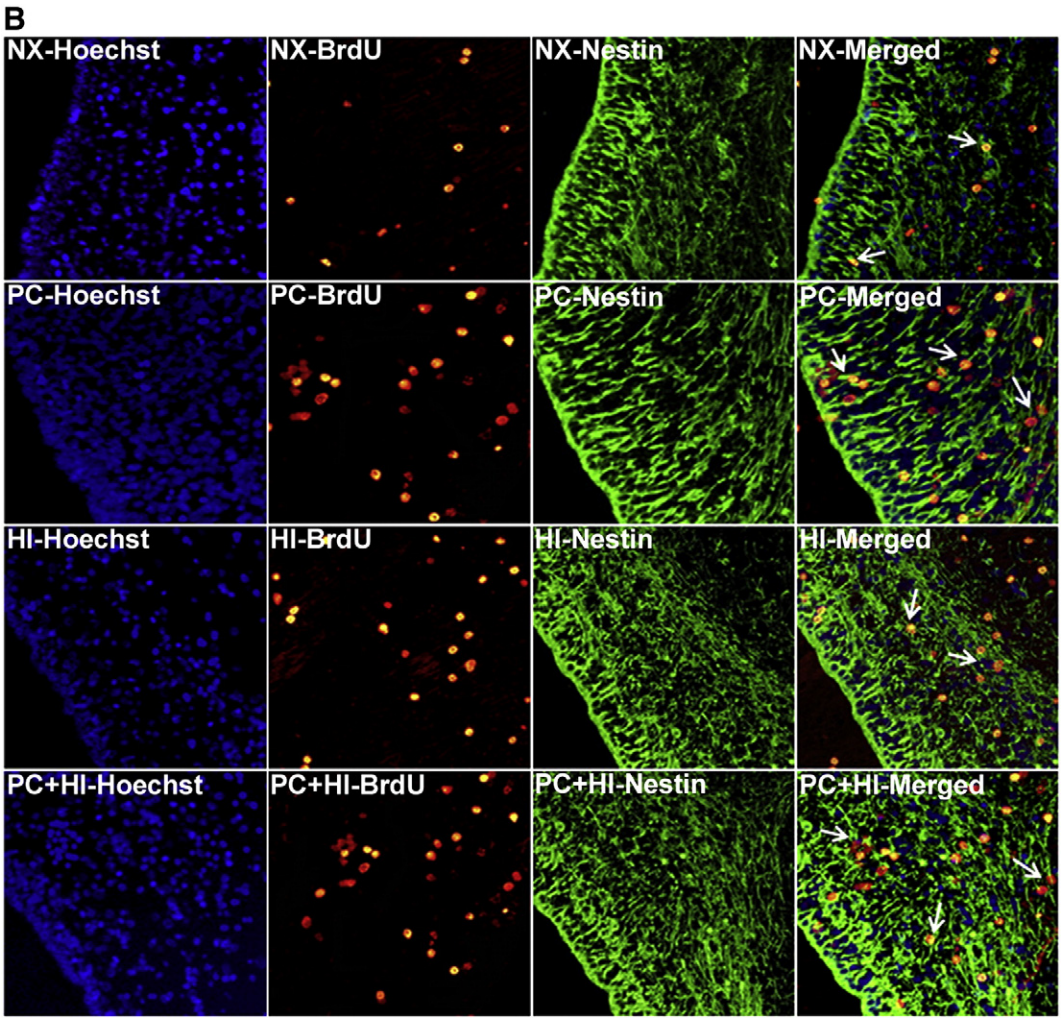


**Fig. 7** (*continued*).

2003). Such preconditioning also increases the number of proliferating precursors in the brain (Jin et al., 2001). The presence of newly generated neuronal and glial precursors (Naylor et al., 2005) after transient or prolonged ischemia has been interpreted as a restorative response of the brain to injury (Martino and Pluchino, 2006). PC can be used as an experimental strategy to identify mechanisms for brain protection with the purpose of therapeutically exploiting such mechanisms by boosting or exogenously substituting endogenous protectants following ischemia.

Our previous study (Ara et al., 2011) demonstrated the protective efficacy of hypoxic-preconditioning against hypoxic–ischemic injury in newborn piglet model. We showed that hypoxic exposure for 3 h, 24 h before hypoxia–ischemia, was effective in reducing brain damage in newborn piglets while itself causing no morphologic evidence of neuronal injury. Preconditioning performed 24 h prior to severe hypoxia–ischemia reduced the neuronal loss in all the brain regions studied at 3 and 7 days after cerebral hypoxia–ischemia (Ara et al., 2011). Here, we studied the induction of neurogenesis in response to hypoxic-preconditioning treatment at 1 day, 3 days and one week after neonatal HI brain injury.

To address the question of the potential role of ischemic tolerance in progenitor cell proliferation, we used BrdU, an analog of thymidine. After 1, 3 days or one week of recovery from PC or PC performed 24 h prior to HI insult; there was an increased proliferation of cells in the SVZ, striatum and white matter as revealed by increased BrdU uptake, as well as by increased numbers of nestin-positive cells in the entire SVZ. These new cells were found to express neuronal and astrocytic markers, double cortin or GFAP. This increase in cells in S-phase within the region of the SVZ that harbors the stem/progenitor cells correlates with a doubling of the number of tripotential, self-renewing NSPs as measured using the neurosphere assay. BrdU incorporation assay *in vitro* demonstrated that hypoxic treatment enhanced BrdU incorporating into NSPs, and the cells positive for the neuronal marker Tuj1 or astrocytic marker GFAP incorporated BrdU during differentiation, indicating that both neuronal and glial precursors are *de novo*-generated from proliferating progenitor cells. Though BrdU may potentially indicate DNA repair (Rakic, 2002), the mildness of the hypoxic episode, the long delay between exposure to hypoxia and BrdU injection, the dose of BrdU administered as well as the marked augmentation of its incorporation rate into the germinative SVZ strongly suggest that BrdU labeling actually reflects proliferating cells.

The present study shows that PC also induces neurogenesis in newborn piglet brain. Our data show that dividing Dcx

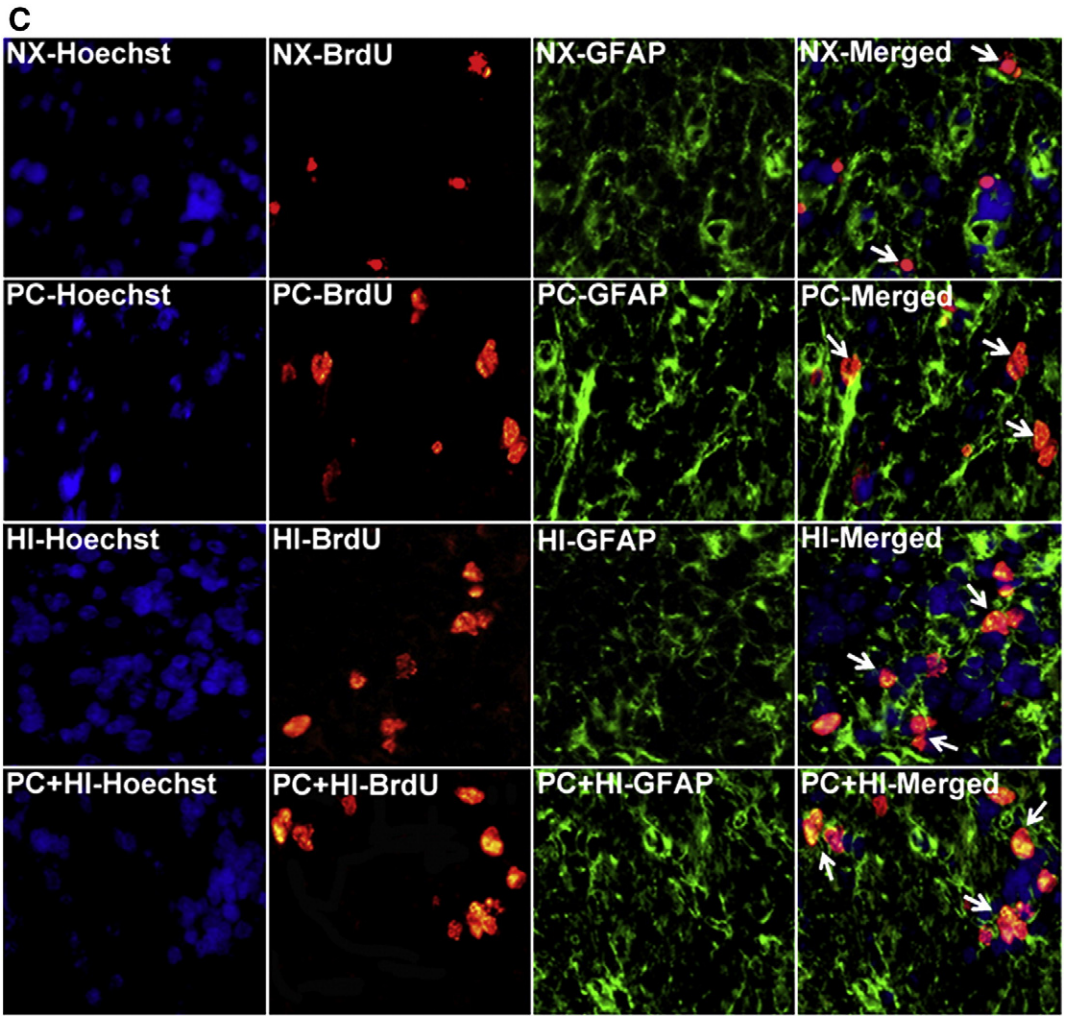


**Fig. 7** (*continued*).

positive cells are restricted to the SVZ, and that Dcx positive cells with morphologies of migrating neuroblasts were found in striatum and also migrated to neocortex, suggesting that the SVZ is the main source of new neurons in the neocortex. Nonetheless, we cannot exclude the possibility that some of the newly generated neurons are derived from local precursors or from glial progenitors that dedifferentiate and become respecified as neurons. Furthermore, prior PC augmented the increase in neurogenesis that occurred in the post-hypoxic–ischemic brain. These findings are largely consistent with previous studies unequivocally showing that various stresses, including hypoxia, can induce neurogenesis. Global and focal ischemic brain injuries in adult animals have been demonstrated to trigger compensatory neurogenesis from neural stem cells or progenitor cells located in germinative areas such as the hippocampal dentate gyrus or the SVZ (Kokaia and Lindvall, 2003). According to Nakatomi et al. (2002), these cells originating from the SVZ are capable to migrate and to incorporate the hippocampal circuitry. Further studies have shown that neonatal hypoxia for 5 min strongly stimulated the generation of new cells in the SVZ within the ensuing three weeks, and demonstrated that newly formed cells gave rise to additional functional neurons (Pourie et al., 2006). A study by Lee et al. (2007) also observed increased neurogenesis in adult Sprague–Dawley rat brain at 4–7 days of reperfusion following PC induced by a 10 min transient middle cerebral artery occlusion. Considering our findings that greater number of neurons were newly generated after PC treatment, PC-induced neurogenesis might offer a potential means of replacing damaged neurons. In addition, the presence of more robust neurogenesis in the PC + HI group despite a lower level of tissue injury than in the HI group indicates that neurogenesis may not be correlated with tissue injury. This is consistent with the conclusion drawn by Arvidsson et al. (2001) who outlined that enhanced neurogenesis in adult rats following stroke was not correlated to the extent of brain damage.

In conclusion, the present study utilized an *in vivo* piglet model of hypoxic-preconditioning-induced ischemic tolerance to demonstrate that PC induces proliferation and neurogenesis of endogenous NSPs up to 7 days after the hypoxic–ischemic insult. PC appears as a broad, non-invasive and reproducible strategy to prime the endogenous NSPs to a state of "readiness" by stimulation of their survival signaling pathways before introduction into ischemic environment. The signaling pathways involved in PC can be successfully employed for developing new therapeutic interventions to enhance endogenous neurogenesis after HI brain injury.

**Table 2** BrdU positive cells that co-labeled with each cell-type marker in the SVZ of newborn piglet brain.

| Groups | Number of cells/mm$^2$ (SVZ) | | |
|---|---|---|---|
| | BrdU$^+$-Dcx$^+$ | BrdU$^+$-nestin$^+$ | BrdU$^+$-GFAP$^+$ |
| Normoxia | 18 ± 7 | 36 ± 7 | 15 ± 5 |
| PC | 77 ± 20 [a] | 205 ± 34 [a] | 64 ± 20 [a] |
| HI | 39 ± 16 [a] | 107 ± 22 [a] | 39 ± 12 [a] |
| PC + HI | 112 ± 21 [a b] | 285 ± 45 [a b] | 95 ± 23 [a b] |

[a] $P < 0.05$ or $<0.01$ Nx vs PC, NX vs HI and NX vs PC + HI.
[b] $P < 0.05$ HI vs PC + HI.

Future advances in terms of identifying key molecular mediators present the possibility of improving functional outcome in hypoxic–ischemic and stroke patients. Growth factors and other proliferation-inducing molecules might be synthesized by the astroglial and the surviving neurons in the cortex and striatum and could diffuse into the neurogenic regions of the brain. This concept is strengthened by the recent observations from our laboratory showing increased vascular endothelial growth factor expression in the neurons and astrocytes throughout the newborn piglet brain subjected to hypoxic-preconditioning (Ara et al., 2011). Sun et al. (2003) showed that VEGF enhanced the delayed survival of newborn neurons in the dentate gyrus and subventricular zone after ischemia. Future studies involving the modulation of adaptive response of brain to ischemic injury by exogenous growth factor treatment or by altering the expression of growth factors will be required to determine more definitively whether above paradigms will help to increase cerebral ischemia or stroke-induced neurogenesis.

## Acknowledgments

This work was supported by the American Heart Association grant 0835233N, and March of Dimes Foundation grant #6-FY09-321 to J.A.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2013.04.007.

## References

Ara, J., et al., 2010. Characterization of neural stem/progenitor cells expressing VEGF and its receptors in the subventricular zone of newborn piglet brain. Neurochem. Res. 35, 1455–1470.

Ara, J., et al., 2011. Hypoxic-preconditioning induces neuroprotection against hypoxia–ischemia in newborn piglet brain. Neurobiol. Dis. 43, 473–485.

Armstead, W.M., Kurth, C.D., 1994. Different cerebral hemodynamic responses following fluid percussion brain injury in the newborn and juvenile pig. J. Neurotrauma 11, 487–497.

Armstrong, R.J., et al., 2003. Transplantation of expanded neural precursor cells from the developing pig ventral mesencephalon in a rat model of Parkinson's disease. Exp. Brain Res. 151, 204–217.

Arvidsson, A., et al., 2001. N-methyl-D-aspartate receptor-mediated increase of neurogenesis in adult rat dentate gyrus following stroke. Eur. J. Neurosci. 14, 10–18.

Arvidsson, A., et al., 2002. Neuronal replacement from endogenous precursors in the adult brain after stroke. Nat. Med. 8, 963–970.

Ashwal, S., 1993. Brain death in early infancy. J. Heart Lung Transplant. 12, S176–S178.

Bjorklund, A., Lindvall, O., 2000. Self-repair in the brain. Nature 405 (892–3), 895.

Dempsey, R.J., et al., 2003. Stroke-induced progenitor cell proliferation in adult spontaneously hypertensive rat brain: effect of exogenous IGF-1 and GDNF. J. Neurochem. 87, 586–597.

Dickerson, J.W., Dobbing, J., 1966. Some peculiarities of cerebellar growth in pigs. Proc. R. Soc. Med. 59, 1088.

Dirnagl, U., et al., 2003. Ischemic tolerance and endogenous neuroprotection. Trends Neurosci. 26, 248–254.

Edwards, A.D., et al., 2010. Neurological outcomes at 18 months of age after moderate hypothermia for perinatal hypoxic ischaemic encephalopathy: synthesis and meta-analysis of trial data. BMJ 340, c363.

Emsley, J.G., et al., 2005. Adult neurogenesis and repair of the adult CNS with neural progenitors, precursors, and stem cells. Prog. Neurobiol. 75, 321–341.

Flecknell, P.A., et al., 1982. Accurate measurement of cerebral metabolism in the conscious, unrestrained neonatal piglet. II. Glucose and oxygen utilization. Biol. Neonate 41, 221–226.

Gidday, J.M., 2006. Cerebral preconditioning and ischaemic tolerance. Nat. Rev. Neurosci. 7, 437–448.

Gidday, J.M., et al., 1994. Neuroprotection from ischemic brain injury by hypoxic preconditioning in the neonatal rat. Neurosci. Lett. 168, 221–224.

Gould, E., et al., 1997. Neurogenesis in the dentate gyrus of the adult tree shrew is regulated by psychosocial stress and NMDA receptor activation. J. Neurosci. 17, 2492–2498.

Gu, W., et al., 2000. Cortical neurogenesis in adult rats after reversible photothrombotic stroke. J. Cereb. Blood Flow Metab. 20, 1166–1173.

Haaland, K., et al., 1997. Posthypoxic hypothermia in newborn piglets. Pediatr. Res. 41, 505–512.

Hicks, A.U., et al., 2007. Enriched environment enhances transplanted subventricular zone stem cell migration and functional recovery after stroke. Neuroscience 146, 31–40.

Hoehner, P.J., et al., 1994. Dihydropyridine ligand binding decreases earlier in adolescent than in infant swine after global cerebral ischemia. Stroke 25, 2060–2065 (discussion 2066).

Isacson, O., Deacon, T.W., 1996. Specific axon guidance factors persist in the adult brain as demonstrated by pig neuroblasts transplanted to the rat. Neuroscience 75, 827–837.

Jin, K., et al., 2001. Neurogenesis in dentate subgranular zone and rostral subventricular zone after focal cerebral ischemia in the rat. Proc. Natl. Acad. Sci. U. S. A. 98, 4710–4715.

Jin, K., et al., 2002. Vascular endothelial growth factor (VEGF) stimulates neurogenesis *in vitro* and *in vivo*. Proc. Natl. Acad. Sci. U. S. A. 99, 11946–11950.

Jin, K., et al., 2003. Directed migration of neuronal precursors into the ischemic cerebral cortex and striatum. Mol. Cell. Neurosci. 24, 171–189.

Kitagawa, K., et al., 1991. 'Ischemic tolerance' phenomenon detected in various brain regions. Brain Res. 561, 203–211.

Kokaia, Z., Lindvall, O., 2003. Neurogenesis after ischaemic brain insults. Curr. Opin. Neurobiol. 13, 127–132.

Komitova, M., et al., 2002. Effects of cortical ischemia and postischemic environmental enrichment on hippocampal cell genesis and differentiation in the adult rat. J. Cereb. Blood Flow Metab. 22, 852–860.

Lee, S.H., et al., 2007. Ischemic preconditioning enhances neurogenesis in the subventricular zone. Neuroscience 146, 1020–1031.

Li, Y., Chopp, M., 1999. Temporal profile of nestin expression after focal cerebral ischemia in adult rat. Brain Res. 838, 1–10.

Li, Y., et al., 1997. Apoptosis and protein expression after focal cerebral ischemia in rat. Brain Res. 765, 301–312.

Li, Y., et al., 2010. Sublethal transient global ischemia stimulates migration of neuroblasts and neurogenesis in mice. Transl. Stroke Res. 1, 184–196.
Lindvall, O., McKay, R., 2003. Brain repair by cell replacement and regeneration. Proc. Natl. Acad. Sci. U. S. A. 100, 7430–7431.
Liu, J., et al., 1998. Increased neurogenesis in the dentate gyrus after transient global ischemia in gerbils. J. Neurosci. 18, 7768–7778.
Martino, G., Pluchino, S., 2006. The therapeutic potential of neural stem cells. Nat. Rev. Neurosci. 7, 395–406.
Nakatomi, H., et al., 2002. Regeneration of hippocampal pyramidal neurons after ischemic brain injury by recruitment of endogenous neural progenitors. Cell 110, 429–441.
Naylor, M., et al., 2005. Preconditioning-induced ischemic tolerance stimulates growth factor expression and neurogenesis in adult rat hippocampus. Neurochem. Int. 47, 565–572.
Okano, H., 2002. Neural stem cells: progression of basic research and perspective for clinical application. Keio J. Med. 51, 115–128.
Okano, H., 2002. Stem cell biology of the central nervous system. J. Neurosci. Res. 69, 698–707.
Palmer, T.D., et al., 1995. FGF-2-responsive neuronal progenitors reside in proliferative and quiescent regions of the adult rodent brain. Mol. Cell. Neurosci. 6, 474–486.
Parent, J.M., et al., 1997. Dentate granule cell neurogenesis is increased by seizures and contributes to aberrant network reorganization in the adult rat hippocampus. J. Neurosci. 17, 3727–3738.
Parent, J.M., et al., 2002. Prolonged seizures increase proliferating neuroblasts in the adult rat subventricular zone-olfactory bulb pathway. J. Neurosci. 22, 3174–3188.
Parent, J.M., et al., 2002. Rat forebrain neurogenesis and striatal neuron replacement after focal stroke. Ann. Neurol. 52, 802–813.
Pourie, G., et al., 2006. Mild, non-lesioning transient hypoxia in the newborn rat induces delayed brain neurogenesis associated with improved memory scores. Neuroscience 140, 1369–1379.
Rakic, P., 2002. Adult neurogenesis in mammals: an identity crisis. J. Neurosci. 22, 614–618.
Ran, R., et al., 2005. Hypoxia preconditioning in the brain. Dev. Neurosci. 27, 87–92.
Reynolds, B.A., Weiss, S., 1996. Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. Dev. Biol. 175, 1–13.
Reynolds, B.A., et al., 1992. A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes. J. Neurosci. 12, 4565–4574.
Sanai, N., et al., 2004. Unique astrocyte ribbon in adult human brain contains neural stem cells but lacks chain migration. Nature 427, 740–744.
Scheffler, B., et al., 2006. Merging fields: stem cells in neurogenesis, transplantation, and disease modeling. Brain Pathol. 16, 155–168.
Solas, A.B., et al., 2001. Cerebral hypoxemia-ischemia and reoxygenation with 21% or 100% oxygen in newborn piglets: effects on extracellular levels of excitatory amino acids and microcirculation. Pediatr. Crit. Care Med. 2, 340–345.
Song, H., et al., 2002. Astroglia induce neurogenesis from adult neural stem cells. Nature 417, 39–44.
Stagliano, N.E., et al., 1999. Focal ischemic preconditioning induces rapid tolerance to middle cerebral artery occlusion in mice. J. Cereb. Blood Flow Metab. 19, 757–761.
Stenzel-Poore, M.P., et al., 2003. Effect of ischaemic preconditioning on genomic response to cerebral ischaemia: similarity to neuroprotective strategies in hibernation and hypoxia-tolerant states. Lancet 362, 1028–1037.
Sun, Y., et al., 2003. VEGF-induced neuroprotection, neurogenesis, and angiogenesis after focal cerebral ischemia. J. Clin. Invest. 111, 1843–1851.
Sun, D., et al., 2007. Anatomical integration of newly generated dentate granule neurons following traumatic brain injury in adult rats and its association to cognitive recovery. Exp. Neurol. 204, 264–272.
Thoresen, M., et al., 1996. Posthypoxic cooling of neonatal rats provides protection against brain injury. Arch. Dis. Child. Fetal Neonatal Ed. 74, F3–F9.
Vannucci, R.C., 2000. Hypoxic–ischemic encephalopathy. Am. J. Perinatol. 17, 113–120.
Vannucci, R.C., et al., 1998. Hypoxic preconditioning and hypoxic–ischemic brain damage in the immature rat: pathologic and metabolic correlates. J. Neurochem. 71, 1215–1220.
Volpe, J., 2001. Hypoxic–ischemic encephalopathy. In: Volpe, J.J. (Ed.), Neurology of the Newborn. WB Saunders, Philadelphia, PA.
Wagner, C.L., et al., 1999. The use of hypothermia: a role in the treatment of neonatal asphyxia? Pediatr. Neurol. 21, 429–443.