In vivo MRI of endogenous stem/progenitor cell migration from subventricular zone in normal and injured developing brains

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Introduction

Subventricular zone (SVZ) is a continual germinal zone surrounding the ventricles, which expands prominently during the latter third trimester of prenatal period and remains constant thereafter throughout the mammalian life. It is the largest source of neural stem cells and transit-amplifying progenitor cells that can generate progeny during mammalian forebrain development (Brazel et al., 2003; Marshall et al., 2003). Since currently it is not possible to clearly discern neural stem cells from the multi-potential progenitors that possess limited self-renewal especially in neonatal SVZ, the term neural stem/progenitor cells (NSPs) has been adopted to encompass both populations (Felling et al., 2006; Felling et al., 2006; Iwai et al., 2004a; Yang et al., 2007; Yang and Levison, 2006). Previous studies have demonstrated that the NSPs within SVZ provide a great potential to regenerate not only the neurons but also various glial cells required to reconstitute a fully functional developmental brain (Felling et al., 2006; Iwai et al., 2006; Ong et al., 2005; Romanko et al., 2004a; Yang et al., 2007; Yang and Levison, 2006, 2007). Thus the ability to identify and track NSPs in vivo could greatly help us understand the brain responses to injuries and design therapeutic interventions for exploration of their curative potential.

At present, electron microscopy, immunohistochemistry and fluorescence imaging are the standard techniques for identifying the SVZ generate the olfactory interneurons and most of glia cells in the forebrain (Brazel et al., 2003; Marshall et al., 2003). While enormous progress has been made in recent decades in uncovering the contributions of NSPs in SVZ to normal brain development, it is becoming increasingly imperative to investigate the alterations of NSP behavior after brain injuries during early postnatal period. Perinatal hypoxia–ischemia (HI) is the leading cause of neurological disorders resulting from birth complications. Approximately 50% of survivors will develop neurological sequelae such as cerebral palsy, epilepsy, and cognitive deficits (Felling et al., 2006; Wood et al., 2000; Yang and Levison, 2006). Previous studies have demonstrated that the NSPs within SVZ provide a great potential to regenerate not only the neurons but also various glial cells required to reconstitute a fully functional developmental brain (Felling et al., 2006; Iwai et al., 2006; Ong et al., 2005; Romanko et al., 2004a; Yang et al., 2007; Yang and Levison, 2006, 2007). Thus the ability to identify and track NSPs in vivo could greatly help us understand the brain responses to injuries and design therapeutic interventions for exploration of their curative potential.

Understanding the alterations of migratory activities of the endogenous neural stem/progenitor cells (NSPs) in injured developing brains is becoming increasingly imperative for curative reasons. In this study, 10-day-old newborn rats with and without hypoxic–ischemic (HI) insult at postnatal day 7 were injected intraventricularly with micron-sized iron oxide particles (MPIOs), followed by serial high-resolution MRI at 7 T for 2 weeks. MRI findings were correlated with the histological analysis using iron staining and various immunohistochemical double staining. The results indicated that in normal and HI-injured brains the NSPs from the subventricular zone (SVZ) were labeled by MPIOs, and migrated as newly created cells in normal and injured brains in both rostral and caudal directions. The NSP radial migratory pattern could be observed in some rats. In the HI-injured brains during the same developmental period, the NSPs mainly migrated towards the HI lesion sites. The tangential, rostrocaudal migrations could be observed but impaired. These findings suggest that the NSP migratory pathways in SVZ change in response to the HI insult, likely due to the self-repairing efforts known in the neonatal brains. The MRI approach demonstrated here is potentially applicable to the in vivo and longitudinal study of NSP cell activities in developing brains under normal and pathological conditions and in therapeutic interventions.

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migration of NSPs from SVZ. They are often detected dynamically by means of retroviruses (Suzuki and Goldman, 2003; Walsh and Cepko, 1988), thymidine (Altman and Das, 1966; Lois and Alvarez-Buylla, 1994) or bromodeoxyuridine (BrdU) labeling (Hayashi et al., 2005; Yang and Levison, 2006). Recently, the transgenic mice expressing green fluorescent protein (GFP) driven by nestin promoter and multiphoton microscopy for detecting nestin-GFP cells have been successfully demonstrated to study the NSPs in the rostral migratory stream (RMS) in both intact and ischemic brains in young mice (Zhao and Nam, 2007). Despite the numerous studies investigating the NSP migration from SVZ using ex vivo tissue processing, there is very limited knowledge regarding the in vivo NSP migration within the whole brain. Although positron emission tomography is an in vivo and non-invasive method for tracking cell metabolism, it alone cannot provide sufficient spatial information to characterize the migrational activities (Cicchetti et al., 2007).

Magnetic resonance (MR) cellular imaging is an emerging technique for detecting migration in vivo and non-invasively. It provides the opportunity to gain a sufficiently high spatial resolution, relate functional measures with presence of cells in target organ, and repeat analysis at multiple time points (Bulte and Kraitchman, 2004; Cicchetti et al., 2007; Hoehn et al., 2002; Hoehn et al., 2007; Modo et al., 2002). Superparamagnetic iron oxide nanoparticles are commonly used as the exogenous contrast agent for cell labeling, which can be endocytosed by a variety of cell types and produce a strong signal loss in T2*-weighted imaging (T2 WI) by virtue of their susceptibility difference from the adjacent environment (Bulte and Kraitchman, 2004; Hoehn et al., 2007; Panizzo et al., 2009). Recently, Shapiro et al. demonstrated that the micron-sized iron oxide particles (MPIOs) have certain advantages for MRI cell tracking (Shapiro et al., 2004, 2005, 2006a,b, 2007; Summer et al., 2009). These large and relatively stable MPIOs can induce substantial susceptibility effect so that each cell with a single MPIO can be detected by MRI (Shapiro et al., 2004, 2006b, 2007). Moreover, MPIOs can be impregnated with various fluorescent dyes, permitting further detection of labeled cells by ex vivo immunofluorescence analysis (Shapiro et al., 2004, 2006a; Summer et al., 2009).

There are three approaches to label cells for MRI tracking (Hoehn et al., 2007). The first is in vitro labeling of stem cells or tumor cells for their post-transplantation MRI visualization. Such cell labeling is a time-consuming process and maintaining stem cells in undifferentiated state in culture is sometimes difficult (Guzman et al., 2007; Hoehn et al., 2002; Modo et al., 2002; Shapiro et al., 2006a). The second approach is systemic administration of MRI contrast agent for macrophage imaging, which has been reported so far to observe inflammatory processes in stroke, experimental autoimmune encephalomyelitis, graft rejection and arthritis (Bulte and Kraitchman, 2004; Hoehn et al., 2007; Wiart et al., 2007; Wu et al., 2006). The third approach is in situ cell labeling for monitoring neurogenesis by direct injection of MPIOs or iron oxide nanoparticles into the ventricles near the SVZ. Even though the uptake of MPIOs or iron oxide nanoparticles by the precursors in the SVZ is small (~30%) in adult rodents (Summer et al., 2009), several MRI studies have successfully demonstrated the migration of labeled cells along the RMS into the olfactory bulb (OB) in normal adult rats (Panizzo et al., 2009; Shapiro et al., 2006a; Summer et al., 2009). This approach offers the unique advantage of selective in situ labeling for study of NSPs in vivo.

It is known that the NSPs from the SVZ in the early postnatal brains are distinct from those in the adult brains in their migratory pathways (Braeza et al., 2003; Marshall et al., 2003). Few MRI studies published so far have exclusively focused on the NSP migration in normal and adult rat brains. There has been no report of in vivo MRI for tracking NSP activities in normal or injured developing brains. The objective of the present study was to employ the in situ cell labeling approach to characterize the migration of the endogenous NSPs from the SVZ in the postnatal developing rat brains in normal and HI-injured developing conditions. We further hypothesized that the NSP migrating patterns in normal and HI-injured developing brains would differ and could be detected by serial high-resolution in vivo MRI in addition to postmortem immunohistochemical analysis. Such in vivo MRI analysis can improve our understanding of NSP behavior in the developing mammalian brains and its alterations during injuries.

Materials and methods

Animal preparation

Pregnant Sprague–Dawley rats were obtained approximately 2 days before parturition. Neonate rats were kept with their mother in regular light/dark cycles for 7 days after birth. Twelve postnatal day 7 (P7) rats (12–16 g) were divided into two groups, 6 in normal group and 6 in HI group. Neonatal rats in HI group underwent HI insult at P7. Briefly, unilateral ligation of left common carotid artery was performed in each pup under isoflurane anesthesia. The surgery usually lasted 5 min for each pup. Afterwards, the pups were kept in an incubator for observation at 34 °C for about 10–15 min. Once regaining normal movement, they were returned to their mother for nursing for 1 h. They were then placed in a hypoxic chamber with 8% O2 and 92% N2 maintained at ambient temperatures 36 °C for 2 h (Chan et al., in press; Qiao et al., 2001; Wang et al., 2008; Yang et al., 2008; Yang and Wu, 2008).

At postnatal day 10 (P10), all neonatal rats in normal and HI groups were stereotactically injected with 10 μl (1.25×107) of 0.9 μm diameter non-fluorescent MPIOs (Bangs Laboratories, Fishers, IN, USA) into the anterior left lateral ventricle. The position was chosen to be 1 mm caudal from bregma, left 1.5 mm and down 2 mm. During the MPIOs injection, the rats were anesthetized with 1% isoflurane mixed with room air. Afterwards, they were kept in an incubator at 30–32 °C for 1 h to recover before returning to feeding cage. To label the newly created cells in the developing brains, BrdU (50 mg/kg; Sigma, St. Louis, MO, USA) was injected intraperitoneally to each rat thrice daily, 8 h apart, for 2 days before the final sacrifice for postmortem analysis (see Fig. 1). All experiments were approved by the Institutional Animal Care and Use Committee.

MRI scanning

All 12 rats were scanned using a 7 T animal MRI scanner (70/16 Bruker BioSpin MRI PharmaScan, Germany) with a 23 mm mouse brain coil 1 day before MRI injection and 1, 3, 7 and 14 days after MRI injection at P10 (see Fig. 1). During MRI, rats were positioned in a plastic holder with a stereotactic head-frame and anesthetized by isoflurane (1–1.5% at 0.8–1 l/min air flow via a nose cone) with respiratory monitoring. Body temperature was maintained at 37 °C using a rectal thermometer feedback probe and a circulating water pad. 3D coronal T2 WI were acquired with a 3D gradient echo (GE) sequence with the following image parameters: TR/TE/T2* = 50/80 ms, flip angle = 10°, FOV = 2.5×2.5×1.2 cm3, spatial resolution = 106×106×100 μm2, NEX = 1, and acquisition time ≈23 min. 2D axial and sagittal T2* WIs were acquired using a 2D multi-echo GE sequence with TR/TE1/TE2/TE3 = 550/8/14/20 ms, NEX = 6, FOV = 2.5×2.5 cm2, slice thickness = 0.3 mm, spatial resolution = 98×98×98 μm2, number of slices = 20 and acquisition time ≈10 min. The 2D coronal and corresponding axial T2-weighted images (T2 WIs) were also acquired using a Turbo RARE sequence with TR = 6000 ms, TE = 60/200 ms, NEX = 2, slice thickness = 0.5 mm for coronal images and 0.3 mm for axial images, spatial resolution = 98×98 μm2, number of slices = 20 and acquisition time ≈6 min per orientation.
Histology

After the final MRI scan at 7 days post injection (dpi) or 14 dpi (see Fig. 1), animals were anesthetized with a dose of ketamine (64 mg/kg) and xylazine (7 mg/kg) intraperitoneally and were perfused transcardially with 10% formaldehyde. The brains were then removed, fixed and embedded in paraffin. All sections were cut in 8 μm thickness. Four brains in each group were all cut in axial sections. The other 2 brains in each group were first cut along sagittal orientation from the external to outer hippocampal region in both hemispheres, and the remaining samples in axial sections. The sections from each brain were processed for Prussian blue (PB) staining for iron to determine the distribution of MPIOs. The PB staining was performed by incubation in a 1:1 mixture of 6% hydrochloric acid and 2% potassium ferrocyanide (Sigma, St. Louis, MO, USA) for 30 min at room temperature (24 °C). After washing thoroughly in distilled water, the brain sections were counterstained with eosin for 30 s. PB plus several immunohistochemical staining was performed to identify the different migratory cell types labeled by iron. Note that this approach has been successfully employed and validated in a recent study (Panizzo et al., 2009). Four primary antibodies were used: anti-nestin for neuroblast, anti-BrdU for newly created cells, anti-GFAP for astrocytes and/or astrocyte-like progenitor cells and anti-NeuN for mature neurons (Yang et al., 2007; Yang and Levison, 2006). In PB plus immunohistochemical staining, the PB staining was first performed without eosin counterstain. The sections were then treated with 3% H2O2 for 20 min followed by antigen repair in 0.01M sodium citrate with microwave, then with 4% normal goat serum (Boster) and placed overnight in the primary antibodies against NeuN (Chemicon, 1:500), GFAP (Neumaker, 1:2000), nestin (Chemicon, 1:200) or BrdU (BD, 1:1000), respectively. After incubation, sections were washed in 0.01M PBS and placed for 30 min in biotinylated anti-mouse (for NeuN, GFAP and nestin) or anti-rat (for BrdU) IgG secondary antibodies at 37 °C. After the sections were placed in avidin–biotin complex (ABC) reagent (Boster) for 20 min, they were reacted in a solution of 0.012% H2O2 and 0.05% 3′,3′-diaminobenzidine (Sigma, St Louis, MO, USA) in tris buffer (TB) for 3–5 min, and finally covered.

The numbers of migratory MPIOs were semi-quantified as the mean PB-positive stains per rectangular section view (400μm × 320μm) in various regions of ipsilateral hemisphere, including SVZ, RMS, corpus callosum (CC), external capsule (EC), OB, cortex and striatum (Yang et al., 2007; Yang and Levison, 2006, 2007). In brief, 20 axial sections covering the top of CC downwards to the bottom of RMS with thickness of 8 μm at 60 μm intervals were assessed. For each selected region, three to six views (1024 × 1024 pixels) were acquired from these sections with ×400 magnification using an image acquisition system (Q550CW, Leica, Manheim, Germany). ImageJ (from NIH and available at http://rsb.info.nih.gov/j/) was employed to count the PB-positive stains in each view (Henry et al., 2006). The statistical analysis was performed for each investigated region of ipsilateral hemisphere between 7 dpi (n = 3) and 14 dpi (n = 3) in both normal and HI groups, and between normal (n = 3) and HI (n = 3) groups at 7 dpi and 14 dpi, using Student’s paired and unpaired t-tests, respectively, with p < 0.05 being considered significant.

Results

MRI of migrating NSPs and histological colocalization in the normal developing brains

At 1 day post injection (dpi), the dark contrast was seen to permeate into both sides of lateral ventricle in the 3 neonatal rats in the normal group while the remaining 3 had the dark contrast mainly in the left lateral ventricle. Between 3 and 14 dpi, the dark contrast in lateral ventricle pervaded into the 3rd ventricle (3V) and the arachnoid space, such as the hippocampal fissure (HF) (black arrows in 1st and 2nd rows in Fig. 2) in all rats. A tangential migratory pattern was observed mainly along the rostral and caudal orientations from the serial MR images.

Fig. 3 shows the tangential migratory pattern at the rostral side along the left RMS at different time points in a neonatal rat. By 3 dpi, the dark migration contrast extended from the anterior part of the SVZ (SVZa) to the RMS (diagonal arrow in Fig. 3B) in all rats. It clearly entered the RMS pathway by 7 dpi (upright arrow in Fig. 3C) in all rats. By 14 dpi, the dark contrast reached the end of the RMS and extended into the OB (upright arrow in Fig. 3D) in 4 out 6 rats while it only reached the middle of RMS in the remaining 2 rats.

At the caudal side, the dark contrast was observed by 3 dpi extending from the anterior part of the left lateral ventricle into the central part of CC in all rats, and further into the contralateral side of CC in 3 out of 6 rats. By 7 dpi, the caudal migration could be detected along the CC backwards into ipsilateral (3 out of 6) or both sides (3 out 6) of the EC. Moreover, the dark migratory pathways could be observed from posterior lateral ventricle into the EC at the hippocampal outer border in all rats (white arrows in 1st and 2nd rows in Fig. 2). No dark contrast was seen in hippocampus.

In the PB iron staining, the tangential, rostrocaudal migratory pathways were seen to spatially correlate with the MPIO-induced dark contrast in MR images. Fig. 4 shows the sagittal and axial T2*WIs (TE = 20 ms) acquired at 14 dpi, together with the corresponding PB iron staining sections. The dense chain-like blue spots extended from the left anterior lateral ventricle into SVZa (Fig. 4 panel A) and the left middle segment of RMS pathway (Fig. 4 panel B) spatially correlated.
with the dark contrast in MRI. Fig. 4 panel C shows the isolated blue spots in the glomerular layer (solid arrows) and external plexiform layer (dotted arrow). They indicated that some MPIO-labeled NSPs migrated to the OB, likely becoming the periglomerular interneurons (Marshall et al., 2003; Suzuki and Goldman, 2003). Moreover, the iron positive cells were found in the ependymal wall (dotted arrow in Fig. 4 panel D) and dorsolateral SVZ (solid arrow in Fig. 4 panel D), indicating that some MPIOs left the ventricular space and entered the SVZ (Marshall et al., 2003; Shapiro et al., 2006a; Suzuki and Goldman, 2003). On the caudal side, the chain-like blue spots in the EC (Fig. 4 panel E) was seen to correlate the dark pathway adjacent to posterior lateral ventricle in MRI. In 2 out of 6 rats, certain dark spots could be seen in MRI in the frontal lobe close to the MPIO injection site, spatially correlating with the iron positive staining in cortex (Fig. 4 panel F). This finding suggested the possibility that the MPIO-labeled NSPs migrated from the anterior lateral ventricle through the white matter towards the cortex using the local radial glial fibers as the migratory scaffold, thus presenting a radial migrating pattern (Suzuki and Goldman, 2003). However, MRI exhibited more and larger areas with dark contrast in this region. This discrepancy likely arose from the image distortion caused by the venous vessels and concentrated MPIOs in the region. No iron positive staining was found in hippocampal cells. Furthermore, sparse iron positive cells were observed in the ipsilateral striatum close to the medial SVZ (data not shown) in 2 out of 6 rats.

Fig. 5 shows the semi-quantitative analysis of the migratory MPIOs with positive PB staining in various regions of the ipsilateral hemisphere in normal and HI brains at 7 and 14 dpi. The average numbers of PB-positive stains per section view in SVZ, RMS, CC, OB, cortex and striatum of the normal brains at 7 and 14 dpi were 33.6±12.0, 51.2±17.0, 42.8±17.0, 28.8±12.0, 12.5±5.5, 4.0±3.8 and 5.0±4.3, respectively. No significant difference was observed between 7 dpi and 14 dpi in the normal brains.

In general, the histological findings from the PB plus immunohistochemical double staining were also found to spatially correlate with MRI findings. Fig. 6 shows a series of iron plus nestin and iron plus GFAP double staining sections with the multiplanar MRI views reconstructed from 3D GE images from a normal rat at 14 dpi. Note that the cells positive with PB plus immunohistochemical double staining were seen in dark brown in these sections. The dense chains of iron+/nestin+ (panel A) and iron+/GFAP+ (panel G) cells show the bilateral migration along the central bypass of CC on the inner side of lateral ventricle. The long-distance migration stopped at the most caudal tip of CC close to the hippocampal inner border by 14 dpi,
spatially corresponding to the thick dark contrast in top MRI view in Fig. 6 (an obliquely horizontal view). Panels C and I are the sagittal sections of iron/nestin and the iron/GFAP staining, respectively, that include the hippocampus and neighboring cortex in ipsilateral side. Chain-like iron+/nestin+ and iron+/GFAP+ cells were found in the whiter matter (EC) along the upper and posterior border of hippocampus to the caudal tip of the SVZ, correlating with a dark path in the sagittal MRI view. Moreover, the isolated iron+/nestin+ and iron+/GFAP+ cells were seen in the contralateral (panels E and K) and ipsilateral cortex, and the glomerular layer of the ipsilateral OB (panels F and L). Similar histological findings were observed in the iron/BrdU and iron/NeuN double staining sections by 7 and 14 dpi (data not shown). In summary, at 7 and 14 dpi, the iron+/nestin+, the iron+/BrdU+, iron+/GFAP+ and iron+/NeuN+ cells distributed mainly along the ependymal wall and adjacent SVZ, migrated in a chain-like manner in CC, RMS and EC in all normal rats, dispersed in isolated manner in OB in 4 out of 6 normal rats and cortex in 2 out of 6 normal rats, but rarely migrated into the ipsilateral striatum, and none into the hippocampus.

**MRI of migrating NSPs and histological colocalization in the HI-injured developing brains**

In the HI group, moderate and severe cerebral HI injuries were observed in 4 and 2 rats, respectively. At 10 days after HI insult (i.e., 7 dpi), the rats with moderate HI injuries exhibited atrophy in the
ipsilateral cerebral hemisphere with a local infarcted cyst (4th rows in Fig. 7) while those with severe HI injuries showed severe atrophy with an extensive loss of brain tissues as a result of the large porencephalic cyst formation in the cortex, striatum, and/or hippocampus, and the loss of part of the ependymal wall in the ipsilateral cerebral hemisphere (3rd and 4th rows in Fig. 2).

By 1 dpi, the dark contrast only fully permeated into the left lateral ventricle in 4 rats (Fig. 7) while the remaining 2 rats exhibited dark contrast on both sides of lateral ventricle. By 3 dpi, the dark contrast entered the CC and EC and aggregated near the lesion sites (3rd row in Fig. 7). After 7 dpi, the main migratory pattern was presented as the dark contrast surrounding the lesion sites (3rd and 4th rows in Fig. 2). Since the SVZ was partially damaged by the HI injury, the dominant migrating routes along rostral and caudal directions observed in the normal rats appeared to be disrupted (Fig. 7), and were almost completely absent in 1 severe HI rat (3rd and 4th rows in Fig. 2). Note that weak dark contrast also appeared at 7 dpi in the ipsilateral cortex (arrows pointing Ctx in the Fig. 6.

Multiplanar 3D GE MRI (1st column) with iron/nestin and iron/GFAP double staining sections at various locations (2nd to 6th columns) in a normal rat at 14 dpi. Panels A to F show the iron/nestin sections, and panels G to L show the corresponding iron/GFAP sections. In the top MRI view (an oblique axial view shown as red dashed lines in the sagittal and coronal MRI views below), the thick dark contrast spatially correlates with the positive iron/nestin (panel A) and iron/GFAP (panel G) staining in dark brown. The magnified views of the locations indicated by the big arrows in panels A and G are shown in panels B and H, respectively. Panels C and I are iron/nestin and iron/GFAP staining sections, respectively, that correspond to the outer hippocampal region in the sagittal MRI view (also shown as dashed green lines in other two MRI views). Panel D shows the magnified view of the tip of caudal SVZ (big arrow in panel C) with numerous iron+/nestin+ cells. Panel J shows the chain-like pattern of iron+/GFAP+ cells in white matter around hippocampus (big arrow in panel I). Panels E and K are the sagittal sections that illustrate the isolated iron+/nestin+ and iron+/GFAP+ cells observed in the contralateral cortex, respectively. Panels F and L depict the isolated iron+/nestin+ and iron+/GFAP+ cells in the glomerular layer of ipsilateral olfactory bulb. Note that the inserts in panels K and L are magnified views of the location indicated by the arrow. CC — corpus callosum; EC — external capsule; Str — striatum; RV — right ventricle; LV — left lateral ventricle; Hip — hippocampus; Ctx — cortex; WM — white matter; GL — glomerular layer.

ipsilateral cerebral hemisphere with a local infarcted cyst (4th rows in Fig. 7) while those with severe HI injuries showed severe atrophy with an extensive loss of brain tissues as a result of the large porencephalic cyst formation in the cortex, striatum, and/or hippocampus, and the loss of part of the ependymal wall in the ipsilateral cerebral hemisphere (3rd and 4th rows in Fig. 2).
bottom row of Fig. 7) and/or striatum, which likely resulted from the endogenous iron accumulation due to an abnormal iron metabolism in HI injury (Yang et al., 2008). Moreover, a dark contrast in the contralateral CC was observed in 4 out of 6 rats, among which one rat exhibited a clearly dark contrast further into the contralateral cortex due to the bilateral lesion in this severe HI injury (3rd row in Fig. 2).

In the PB staining, the dominant MPIO migration towards the ischemic regions and an incomplete migrating pattern along the rostrocaudal pathways were generally observed, and were spatially correlated to the MRI findings. The right-side panels in Fig. 4 illustrate one sagittal and two axial T2*WIs acquired from a rat with severe HI injury at 14 dpi, together with the corresponding iron staining sections in panels G to L. In panel G, when compared with normal young rat (left-side panels), the blue stains at the beginning of ipsilateral RMS were pronouncedly sparse in the HI rat, indicating that the HI insult affected the movement of NSPs within SVZa and resulted in an imperfect migrating pattern in the rostral direction. Panels H and I show the migrating MPIO-labeled cells in different regions of the white matter, typically presenting as a chain at the central part of CC and the EC close to the necrotic lesion on the ipsilateral side. Such observations indicate that the white matter, such as CC and EC, remains to be a major NSP migratory pathway in HI-injured brains. Panel K depicts the numerous iron spots dispersed in the peripheral region of the infarcted cyst in ipsilateral cortex, indicating that the newly produced cells labeled with MPIOs migrated to the ischemic regions possibly to compensate the neural cell loss. Surrounding the large porencephalic cyst, the chain-like iron positive cells were seen to distribute along the ependymal wall and partly infiltrate into the adjacent posterior SVZ in panel L. Similar to the normal group, no positive iron stain was found in the hippocampal cells in HI group.

The average numbers of PB-positive stains per section view in SVZ, RMS, CC, EC, OB, cortex and striatum in HI brains at 7 and 14 dpi were 39.8±20.0, 15.6±8.0, 37.8±23.0, 22.8±17.0, 79.9±6.8, 28.0±20.0 and 7.0±7.0, respectively, as shown in Fig. 5. No significant difference was observed between 7 dpi (n = 3) and 14 dpi (n = 3) groups in these regions. However, significant differences between HI and normal rat brains were seen at both 7 and 14 dpi in SVZ, RMS and cortex. These results demonstrated that the MPIO-labeled NSPs proliferated more in SVZ of the HI brains than the normal brains, and migrated mainly towards the lesion cortex.

Fig. 8 shows the MR images with the corresponding double staining sections (for iron+/nestin+, iron+/GFAP+, iron+/BrdU+ and iron+/NeuN+ cells) at 7 and 14 dpi. In general, the MPIO-labeled cells appeared mainly along the ependymal wall and the adjacent SVZ (panels A, C, F, H, K, M, P, T and U). Then they migrated mainly to the HI lesion sites, into the infarcted core (panels D, J, N and S) and into the boundary of infarcted regions (panels O and R). Note that some migrations directly arose from the SVZ to the lesion sites in the cortex (panels F, H and M) or to the ipsilateral striatum (panel T). Other migrations to HI lesion sites appeared from the white matter by a long pathway as the typical chain in the EC (panels C, D, G, K, N, Q and V). When SVZa was not injured by the HI insult, the chain-like migration occurred in the rostral RMS route (panel B). Moreover, NSPs with MPIOs were seen to migrate to the contralateral hemisphere through the CC (panels A, I, K and L), and sparse mature neurons with MPIOs (iron+/NeuN+) were present in granule cell layer of the ipsilateral OB (panel W).

In summary, these results demonstrated that the endogenous MPIO-labeled NSPs mainly migrate to the damaged sites. The normal tangential, rostrocaudal migration patterns were seen to be disrupted. The radial migration occurred less in the intact cortex and striatum. These findings indicated that the NSPs migration routes changed in response to the HI injury, likely as a result of the global and local activities to compensate for the local cell loss in the damaged sites. Note that strong positive staining for nestin, BrdU and NeuN was generally observed in the HI perilesional regions (Fig. 8 panels O and R). However, such staining was markedly less in the lesion core (Fig. 8 panels D, N and S). The strong GFAP staining observed in the HI lesion sites (except the infarcted cysts) was likely resulted from the extensive gliosis at 17 days after HI insult (Fig. 8 panel I).

**Discussion**

Previous studies have shown that the SVZ neural stem/progenitor (NSP) cell migration pathways differ between neonatal and adult periods (Brazel et al., 2003; Doetsch and Alvarez-Buylla, 1996; Levison et al., 1993; Marshall et al., 2003; Suzuki and Goldman, 2003), and alter after ischemic injuries (Aridsson et al., 2002; Brazel et al., 2004; Felling et al., 2006; Gotts and Chesnelet, 2005b; Macas et al., 2006; Romanko et al., 2004a; Yang et al., 2007). However, the methods used largely relied on immunohistological analyses of excised tissues. In this study, the migration of NSPs in both normal and HI-injured developing rat brains was investigated for the first time using in vivo MRI via in situ cell labeling upon intraventricular injection of MPIOs. The MRI findings were confirmed by histological analyses, indicating that the MPIO-labeled cells along the migratory routes comprised newly created cells (iron+/BrdU+), neuroblasts (iron+/nestin+), astrocytes and/or astrocyte-like progenitor cells (iron+/GFAP+), and mature neurons (iron+/NeuN+) observed in both normal and HI-injured brains. These suggested that the MPIOs injected into the lateral ventricle might be loaded into the endogenous NSPs in SVZ via transcytosis through ependymal cells (Panizso et al., 2009), subsequently migrated to different regions such as OB, neocortex, subcortical white matter and injured sites, and differentiated into other cell types such as neurons and glial cells. Several studies have shown that severe HI injury depletes the precursors in the neonatal rodent SVZ (Brazel et al., 2004; Levison et al., 2001; Romanko et al., 2004b), resulting in the SVZ regression during the first 48 h after the HI insult. However, one study has also demonstrated that, if the SVZ was not directly damaged by the HI injury, the cell proliferation increased after day 3 post HI insult, leading to a 100% increase in the number of regenerative NSPs (Felling et al., 2006). For this reason, the MPIOs were injected at P10 in the current study to examine these NSP activities in the neocortices injured by the HI insult at P7. P10 was also chosen partly to allow the HI injury stabilize for successful intraventricular MPIO injection.

**Migratory pattern of NSPs with MPIOs in the normal developing brains**

Two migratory patterns of NSPs in SVZ have been reported so far in the normal postnatal developing rodent brains, which are distinct from those in adulthood (Brazel et al., 2003; Marshall et al., 2003; Suzuki and Goldman, 2003). The first is a tangential migration, in which the NSPs in SVZ migrate in a bilateral, rostrocaudal pattern along the wall of the lateral ventricle. Many of these cells travel a relatively long distance, along the RMS into the OB and eventually give rise to periglomerular and granular interneurons (Marshall et al., 2003; Suzuki and Goldman, 2003). In this study, the migratory pathway to the rostral side after MPIO injection in P10 rats (Figs. 2, 3 and 4) was similar to previously reports in adult rats (Panizso et al., 2009; Shapiro et al., 2006a; Sumner et al., 2009). This demonstrated that the rostral migration of the NSPs from SVZa to OB via RMS continues throughout the rodent life, and that in vivo MRI cell tracking of NSPs in SVZ in normal neonatal rats via in situ cell labeling is feasible. In the current study, the tangential migration to the caudal side was detected by in vivo MRI for the first time. At 14 dpi, the MPIO-carrying NSPs migrated along the central bypass of the CC to the most caudal tip of the CC (Figs. 2 and 6), and from the posterior lateral ventricle into the EC towards the caudal tip of the SVZ (Figs. 2 and 4). The tangential, and bilateral, rostrocaudal migrations observed were consistent with an ex vivo report using time-lapse videomicroscopy of the SVZ cells labeled by GFP encoding retrovirus (Suzuki and
Such migrations at the caudal side were not observed in previous MRI studies of adult rodents (Panizzo et al., 2009; Shapiro et al., 2006a; Sumner et al., 2009). Biologically, the differences here may arise from the fact that postnatal brains exhibit more NSP cell production and migration activities, and larger SVZ than adult ones (Marshall et al., 2003). Technically, this discrepancy may be attributed to the distorting and ‘blooming effect’ caused by the higher number of large MPIOs injected (1.4–1.5 × 10⁸, 50 μl and 1.63 μm), or the use of smaller iron oxide nanoparticles of lower concentrations in those previous MRI studies.

The second migratory pattern of NSPs in SVZ is the radial migration. In neonatal rat brains, NSPs are known to radially migrate from the SVZ towards the white matter and cortex by utilizing the local radial glial fibers as migratory scaffold before the juvenile age (P14), and will give rise to astrocytes and oligodendrocytes (Levison et al., 1993; Suzuki and Goldman, 2003). In the current study, the radial

Fig. 8. MRI images with the corresponding double staining sections (for iron+/nestin+, iron+/GFAP+, iron+/BrdU+ and iron+/NeuN+ cells) at 7 and 14 dpi. The 1st row shows the axial T2WI (TE = 20 ms) from a rat with moderate HI injury and the iron/nestin double stain sections (panels A to F) from the same rat at 7 dpi. MRI shows the dark lines extending from the needle hole and left lateral ventricle towards the RMS, infarcted region, and passing through CC towards the contralateral hemisphere. Panel A is the low-magnification section of the region marked by the dashed white box in MRI, showing the migrating iron+/nestin+ cells as dark chains and spots in CC and the lateral wall of the LV. Panels B, C and D depict the iron+/nestin+ cell migration in the RMS (towards the OB), from PLV to EC and in EC towards the infarcted region, respectively. Panels E and F depict the iron+/nestin+ cells in granule cell layer of ipsilateral OB and the lateral wall of LV, respectively, with the inserts showing the magnified views of the sites pointed by arrows. The 2nd row shows the T2WI (TE = 60 ms; left) and T2*WI (TE = 20 ms; middle) acquired at different axial locations from a rat with moderate HI injury at 14 dpi. Various iron/GFAP double staining sections are shown in panels G to J for the regions marked by dashed white boxes (panels G and I) and arrows (panels H and J) in MRI. Panels G, H and J depict the EC in the ipsilateral side, the PLV wall close to the lesion core, and the necrotic region in the ipsilateral cortex, respectively. The inserts in panel H and J show the magnified view of the sites pointed by the arrows. Panel I depicts the migrating iron+/GFAP+ cells as dark chains and spots in CC and the middle wall of LV. The 3rd row shows the axial T2WI (TE = 60 ms) from a rat with moderate HI injury at 14 dpi and the iron/BrdU double staining sections (panels K to O). Panel K is the low-magnification iron/BrdU section of the region marked by the dashed white box in MRI. Various high-magnification views of this region show the iron+/BrdU+ cells residing along the lateral wall of the ventricle (panel M), migrating as dark brown chains in the CC (panel L) and EC (panel N), and interspersing in the margin of the infarcted regions (panels O and S). The insert in panel O shows the further magnified view of the site pointed by the arrow. The 4th row shows the axial T2*WI (TE = 8 ms) from a rat with moderate HI injury at 7 dpi and the iron/NeuN double staining sections (panel P to W). Panels P, Q, R and S correspond to the regions marked by white and black dashed boxes, and white and black arrows in MRI, respectively. Panel P shows the typical dark chains extending from the PLV and needle path. Panel T is the magnified view of the inner wall of LV (as marked in panel P). Panels U and V, the magnified views of two regions marked in panel Q, show the posterior SVZ close to the PLV and the posterior EC close to the lesion cortex, respectively. Panels R, S and W depict the isolated iron+/NeuN+ cells in the perilesional area, infarct core, and granule cell layer of the ipsilateral OB, respectively. The inserts in panel R, S and W show the magnified view of the sites pointed by the arrows. LV — left lateral ventricle; CC — corpus callosum; EC — external capsule; RMS — rostral migratory stream, OB — olfactory bulb; PCyst — porencephalic cyst; PLV — posterior lateral ventricle; NP — needle path; LC — lesion core; Hip — hippocampus.
migration presented a weak and scattered dark contrast at all time points in the cortex after MPIO injection at P10 (Fig. 4). This may be a result of the disappearing radial glia fibers, such that after P14 the SVZ cells tended to migrate more into the medial areas of the subcortical white matter, but less into the neocortex for colonizing the dorsal forebrain structures (Brazel et al., 2003; Levison et al., 1993).

In the current study, the radial migration to the striatum could not be clearly observed by MRI after MPIO injection at P10. This could be ascribed to the absence of strong radial migration at the SVZ/striatum interface in the postnatal brains (Brazel et al., 2003; Levison et al., 1993; Suzuki and Goldman, 2003), and the technical interference in MRI arising from intrinsic susceptibility effects along the striatum border. This issue remains to be investigated with more optimal MRI procedures in the future. In this MRI study, a strong dark contrast was observed to migrate into the central part of the CC by 3 dpi, further into the contralateral side of the CC in half of the normal rats, and into the EC by 14 dpi (Figs. 2 and 4). Such SVZ cell migration was also reported in P3 and P14 rats in earlier ex vivo studies (Kakita et al., 2003; Levison et al., 1993). Although the exact reason for the migration within the CC remains unclear, this progenitor behavior is presumably for colonizing the contralateral hemisphere in conjunction with unmyelinated axons in the developmental state (Brazel et al., 2003; Kakita et al., 2003).

Migratory pattern of NSPs with MPIOs in the HI-injured developing brains

It has been widely recognized that the neonatal central nervous system (CNS) possesses a remarkable capacity to recover from an injury (Kennard principle) (Kolb and Gibb, 2007; Villablanca and Hovda, 2000; Yang and Levison, 2007). In adult mammalian brains, the dramatic proliferation, migration and differentiation of the SVZ progenitor cells in ischemic states have been demonstrated in previous studies (Arvidsson et al., 2002; Gotts and Chesselet, 2005a; Macas et al., 2006; Romanko et al., 2004a; Tonchev et al., 2007; Zhang et al., 2006). In the neonatal rats with moderate HI injury in the current study, the routes of tangential migration were observed to be generally imperfect on both the rostral and caudal sides, more prominently decreased on rostral side (Figs. 2, 4, 5, 7 and 8), and were almost absent in the ipsilateral hemisphere in one severely injured rat (Fig. 2). In fact, careful observation revealed that the absence of or imperfect local migratory pathway was generally associated with the extent of local SVZ damages caused by HI insult. These findings suggest that the NSPs behavior and function are impaired, and vary with actual HI injuries. With moderate insults, the SVZ can recover, but it fails upon more severe injury (Romanko et al., 2004a).

In the current study, the MPIO-labeled NSPs appeared to be located more prevalently within the ependymal wall and SVZ of the ipsilateral hemisphere in the moderate HI-injured brains than in the normal developing brains at 7 dpi (Fig. 5 and 8). This coincided with the time window of the ipsilateral SVZ expansion and the peak NSF proliferation previously reported during the late HI injury phase (Felling et al., 2006; Hayashi et al., 2005; Iwai et al., 2006; Ong et al., 2005; Yang et al., 2007; Yang and Levison, 2006). Consistent with the findings by others (Hayashi et al., 2005; Yang et al., 2007; Yang and Levison, 2006), our results showed that most of the newly generated NSPs with iron particles (iron+/BrDU+) migrated more to the damaged cortical sites in both the infarcted core and perilesional area than to normal cortex (Fig. 5, Fig. 8 panels N and O) and presented as neuroblasts (iron+/nestin+) (Fig. 8 panel D), astrocytes and/or astrocytes-like progenitor cells (iron+/GFAP+) (Fig. 8 panels H and J) and mature neurons (iron+/NeuN+) (Fig. 8 panels R and S). These findings suggest that the NSPs in SVZ play an important role in the self-repairing processes after HI insult in developing brains.

A number of limitations exist in the current study. The first is the lack of the immunohistological staining for several other neural cell phenotypes, such as oligodendrocytes and microglia. The second is the absence of quantitative analysis of different MPIO-labeled cell phenotypes in the migratory pathways. As discussed in previous studies, this approach of in situ SVZ cell labeling suffers from several other intrinsic limitations, such as low labeling efficiency, non-selective cell labeling, inability to distinguish live and migrating cells from dead cells or particles within microglia, and the interference from the susceptibility effects near the inject site caused by the local iron oxide particles of high concentration (Panizzi et al., 2009; Shapiro et al., 2006a; Sumner et al., 2009). These are the technical issues that future development of in situ cell labeling and in vivo cell tracking should address.

Conclusion

In this study, we demonstrated that intraventricular injection of MPIOs at P10 allows the MRI visualization of the migrations of neural stem/progenitor cells (NSPs) in subventricular zone (SVZ) in normal and injured developing rat brains. In normal brains, the endogenous NSPs mainly exhibited a tangential, rostrocaudal migration pattern from P10 to P24. The NSF radial migratory pattern could be observed but less clearly. For the hypoxia-ischemia (HI) injured postnatal brains during the same development period, the endogenous NSPs were found to mainly migrate to the HI lesion sites. The tangential, rostrocaudal migration pathways could be observed but compromised. These findings suggest that the NSF migration routes alter in response to the HI insult, likely as part of the self-repairing efforts in the neonatal brains. The approach of in situ SVZ cell labeling and in vivo MRI cell tracking demonstrated in this study is potentially applicable and valuable to the study of NSF cell activities in developing brains under normal and pathological conditions and during the therapeutic interventions.

Acknowledgments

This work was supported in part by research grants from the Hong Kong Research Grant Council (HKU 7793/08M) and the Chinese National Science Foundation Council (30770673).

References


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