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Neurogenesis and neuronal commitment following ischemia in a new mouse model for neonatal stroke

S.D. Kadam^{1,3}, J.D. Mulholland³, J.W. McDonald^{1,3,4}, and A. M. Comi^{1,2,3}

¹Department of Neurology, Johns Hopkins University School of Medicine

²Department of Pediatrics, Johns Hopkins University School of Medicine

³Department of Neurology and Developmental Medicine, Kennedy Krieger Research Institute, Baltimore, MD -21205

⁴The International Center for Spinal Cord Injury, Kennedy Krieger Research Institute, Baltimore, MD -21205

Abstract

Stroke in the neonatal brain is an important cause of neurologic morbidity. To characterize the dynamics of neural progenitor cell proliferation and maturation after survival delays in the neonatal brain following ischemia, we utilized unilateral carotid ligation alone to produce infarcts in postnatal day 12 CD1 mice. We investigated the neurogenesis derived from the sub-ventricular zone and the sub-granular zone of the dentate gyrus subsequent to injury. Newly produced cells were labeled by bromodeoxyuridine at ~1week (P18-20) after the insult by 5 I.P. injections (each 50mg/kg). Subsequent migration and differentiation of the newborn cells was investigated at postnatal day 40 by immunohistochemistry for molecular neuronal and glial cell-lineage markers and BrdU incorporation. Cresyl violet stain demonstrated massive loss of neurons in the ipsilateral septal hippocampus in the CA3 and CA1 regions associated with atrophy. Total counts of new cells were significantly lowered not only in the ipsilateral injured but also the contralateral uninjured hippocampi and correlated with the lesion induced atrophy. Bilateral percent neuronal commitments in the dentate gyri however, were not significantly different from control. New cell densities in the neocortex and striatum increased bilaterally after neonatal stroke. The predominantly non-neuronal commitment of the SVZ derived new cells was similar to the percentage of non-neuronal commitment in controls. In conclusion, neurogenesis occurring at 1 week after neonatal ischemia in the model maintained cell-lineage commitment patterns similar to sham controls. However, the total number of hippocampal SGZ-derived new neurons was reduced bilaterally; in contrast, the SVZ-derived neurogenesis was amplified.

Keywords

Neurogenesis; Bromodeoxyuridine; Neuronal commitment; Hippocampus; Neocortex; Striatum

Corresponding Author: Dr. Anne M. Comi, Tower 100A, 707 N. Broadway, Baltimore MD 21205, Phone: 443-923-9150, fax: 443-923-9160, email: comi@kennedykrieger.org.

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1. Introduction

Neonatal stroke occurs in approximately 1 per 4000 term births and along with pediatric strokes is an important cause of neurologic morbidity (Lynch et al., 2002). Although the majority of children survive their stroke, about 75% have sequelae including cerebral palsy, epilepsy, and a range of cognitive impairments, including learning and memory problems (Delsing et al., 2001; Koelfen et al., 1995). Stroke in the developing brain is therefore an important cause of cognitive impairment that often persists into adulthood. Neurogenesis arising from populations of neural progenitors that persist in neurogenic niches of the sub-ventricular zone (SVZ) and sub-granular zone (SGZ) of the dentate gyrus (DG) are now a universally accepted feature of the postnatal and adult mammalian brain (Gould, 2007). The brain has also been shown to have some capacity for endogenous regeneration after various kinds of insults that lead to loss of neurons in adult mammalian brains like hypoxia-ischemia, acute seizures and trauma (Arvidsson et al., 2002; Goings et al., 2004; Parent et al., 2006; Parent and Lowenstein, 2002a; Parent and Silverstein, 2007). Studies have also shown that endogenous neurogenesis after ischemic injuries produce new cells that integrate into neural networks and participate in the recovery from neurological deficit (Hayashi et al., 2005; Nakatomi et al., 2002). This finding raises the possibility of repairing damaged circuits by recruiting such latent regenerative potential. Although most neurogenesis occurs prenatally or in the early postnatal period (Kuhn, 1996; Qiu, 2006), interest has been generated in studies investigating neuroregeneration after disease or injury during neonatal periods that lead to neuronal loss and depletion of the known neurogenic niches.

In neonatal brains hypoxia-ischemia has been shown to either increase or decrease hippocampal precursor cell survival (Bartley et al., 2005; Chang et al., 2006). It is known that endogenous neurogenesis can be modulated by various extrinsic and intrinsic factors. Therefore harnessing capabilities of such factors to amplify neurogenesis is a goal of post-neonatal stroke recovery therapy. This however will require an understanding of what role endogenous neurogenesis plays in post-neonatal stroke plasticity after survival delays. For example an acute manifold increase in post-stroke proliferation of neural progenitors does not necessarily mean that all the newborn cells will survive differentiation and maturation in an injured brain. This study allows for quantification of post stroke born neurons after survival delays for the purpose of addressing this question. In the present study newborn cells were labeled by bromodeoxyuridine (BrdU) incorporation at 1 week following a neonatal stroke induced by unilateral carotid ligation at postnatal day 12. The cells that survived the process of maturation and were able to differentiate over the period of a few weeks were quantified by immunohistochemistry at postnatal day 40. Another important issue was investigating whether acute insults like neonatal strokes alter inherent cell-lineage commitment patterns in SGZ and SVZ derived cells, compared to control over the period of immature brain development. Using cell lineage markers for neurons and macroglia, and quantifying the co-localization of such markers with the BrdU labeled nuclei we addressed the question in the ipsilateral injured and the contralateral uninjured hemispheres and compared them to control.

Hypoxia-ischemia models in neonatal mice (P7) have found the CD1 strain to be particularly susceptible to brain damage (Sheldon et al., 1998). Additionally at P12 the mouse brain has been reported to correspond to that of a full-term infant (Slinko et al., 2007). One of the hallmarks of neonatal susceptibility to ischemia is the age dependant sensitivity to excitotoxic injuries due to the developmentally regulated increased expression of AMPA and NMDA receptors in both rodents and humans (Talos et al., 2006a and b). In a pilot study we ligated CD1 mice at P7, P10, P12 and P21 and found that there is a peak period of vulnerability to injury in CD1 mice at P10-P12 with a higher incidence at P12. Associated with the vulnerability to injury we found a higher percentage of ligated P12 mice (compared with P7, P10 or P21) to have subsequent acute seizures when monitored for 4h following the ligation (unpublished

data). When neonatal stroke presents in the newborn period, it does so with seizures. Thus, the P12 CD1 mouse mimics the vulnerability of the neonate to acute ischemic seizures and grey matter injury and this is why this age was selected for these studies.

2. Results

The experimental paradigm and time points at which histological studies were conducted and regions of interest (ROIs) quantified and reported here in this section are described in detail in the experimental procedures section (i.e., section 4). Additionally the schematics of the experimental protocol are represented by Figure 7.

2.1. Histological outcomes

Unilateral carotid ligation resulted in an ipsilateral infarct predominantly in the middle and posterior cerebral artery perfusion territories. The most consistent injury occurred in the hippocampus and cerebral cortex, as elicited by the porencephalic cyst formations predominantly involving the watershed zones between the middle and posterior cerebral arterial perfusion territories of the neocortex and massive cell losses in the excitotoxic injury sensitive hippocampus. The striatum and thalamus were also consistently injured but comparatively less severely. In the hippocampus CA1 and CA3 pyramidal neurons from ipsilateral hippocampi showed marked susceptibility to ischemic cell death (Figure 1 compare A to B). 68% (n/n= 13/19; from 4 litters) of the ligated mice developed cystic infarcts. Ipsilateral hemispherical atrophy (Figure 1C and Figure 2C) included tissue loss due to formation of the porencephalic cyst in addition to the atrophy due to gliosis (i.e., GFAP up-regulation in atrophied scar tissue seen at P40). Average ipsilateral hemispherical atrophy scores (see Experimental Procedures 4.2) for post-stroke and sham controls were significantly different ($35.9 \pm 10\%$ and $4.5 \pm 4.2\%$ respectively, $p < 0.0001$). Average hippocampal atrophy scores (Figure 2C) were significantly different for stroke injured mice compared to sham controls ($68.8 \pm 15.1\%$ and $10.7 \pm 20.4\%$ respectively, $p < 0.0001$). The corresponding mean areas (mm^2) of the coronal sections from the series of sections in which total counts of BrdU positive cells were done also showed significant differences. These area measurements were used to study correlations between area and BrdU cell counts by location and treatment for statistical analyses. The mean ipsilateral hemispherical area (i.e., right hemisphere; Figure 2A) of ligation injured mice was $17.6 \pm 3 \text{ mm}^2$ and significantly lower compared to $27.6 \pm 3 \text{ mm}^2$ for the contralateral hemisphere ($p < 0.0001$). The contralateral hemisphere areas however did not show significant difference from control groups and were similar to areas of the left and right hemispheres from sham control brains (i.e., 29.1 ± 2 and $27.7 \pm 1.4 \text{ mm}^2$ respectively, $p = 0.2$). The mean ipsilateral hippocampal area (i.e., right hippocampus; Figure 2B) of ligation-injured mice was $1.1 \pm 0.4 \text{ mm}^2$ compared to $2.8 \pm 0.6 \text{ mm}^2$ for the contralateral hippocampus ($p < 0.0001$). Contralateral hippocampal areas were not significantly different from areas of left and right hippocampi from sham control brains (i.e., 3.2 ± 0.9 and $2.7 \pm 0.3 \text{ mm}^2$ respectively, $p = 0.16$). Thus areas of contralateral hemispheres and hippocampi of ligation injured mice from sections in which BrdU cells were quantified were similar to control and did not show evidence of any discernable ischemic injury with cresyl violet stain.

2.2. Post-stroke Neurogenesis

BrdU incorporation detected by green fluorescence (Alexa 488, Chemicon) was distinct in the nuclei of cells in the dentate gyrus subgranular zone and within the granule cell layer itself even at low magnifications (10X, Figure 3A). Few BrdU positive cells were also labeled in the hilar regions. In the neocortex and striatum, BrdU labeled cells presumably having migrated from the SVZ were seen dispersed randomly in control brains and in constellations around gliotic patches and along the infarct borders in ligation injured brains. Very few BrdU labeled cells remained in the neurogenic niche of the SVZ at ~3 weeks after cell birth. This small group

of non-migrant BrdU cells that often co-labeled with GFAP may represent the neural progenitors labeled during asymmetric cell division at 1 week after the stroke (Liu et al.; 2006)

Total counts of BrdU positive cells in the dentate gyrus (i.e., ROI 3) revealed significant differences between ligation injured and sham control brains (Figure 3 and Figure 4). Mean ipsilateral counts in ligation injured brains were 12.9 ± 6.3 compared to 32 ± 6.1 cells in sham controls and therefore significantly lower (Figure 4A, $p < 0.01$). Interestingly, counts of BrdU positive cells in non-injured contralateral hippocampi of ligation injured brains were 21.2 ± 4.7 compared to 33.1 ± 3.4 cells in controls and therefore also significantly lower (Figure 4B, $p < 0.01$) than sham controls. The ipsilateral reduction of endogenous post-stroke neurogenesis could be attributed directly to the ipsilateral atrophy of the hippocampi (Figure 4E) revealed by the strong correlation of ipsilateral DG counts to the respective ipsilateral hippocampal areas (mm^2) in which the counts were made ($r = 0.83$, $p < 0.05$). However the contralateral reduction in new cells found no direct correlation with respective hippocampal areas (Figure 4F, $r = 0.35$, $p = 0.12$). Despite similar hippocampal areas on serial coronal sections, total counts of BrdU positive cells was consistently lower in the uninjured contralateral hippocampi in ligation injured brains compared to contralateral (i.e., left side) hippocampi from sham controls.

In contrast to the overall lower new cell counts in the DG, BrdU positive cell counts increased significantly in the ligation injured brains in the striatum and neocortex (i.e., ROIs 1 and 2 respectively, $p < 0.01$) indicating an amplification and survival of post-stroke neurogenesis in bilateral SVZs. In the ipsilateral striatum of ligation injured mice mean new cell counts were significantly higher (50.53 ± 9.8 ; $p < 0.001$) compared to controls (22.7 ± 8.9). Ipsilateral neocortices also showed similar increases that were also significantly higher than controls (24.8 ± 10.5 cells in ligation injured compared to 12.8 ± 10.8 cells in controls; $p < 0.001$). Cell densities counts of BrdU positive cells were calculated to account for the areas of the anatomical contours of ROIs 1 and 2 (Figure 7, Paxinos and Franklin, 2001) in ligation injured and control mice. These cell density counts (Figure 5) showed an increase that was not only significant in the ipsilateral striatum and neocortex (Figure 5 A and C; 19.2 ± 10.9 cells/ mm^2 vs. 4.4 ± 1.7 cells/ mm^2 for controls; and 10.8 ± 5.3 cells/ mm^2 vs. 3.6 ± 2.9 cells/ mm^2 for controls respectively; $p < 0.001$) but also in the contralateral striatum and neocortex (Figure 5B and D) of ligation injured brains (6.8 ± 2.4 cells/ mm^2 vs. 4.4 ± 1.9 cells/ mm^2 for controls; $p = 0.02$ and 5.6 ± 2.1 cells/ mm^2 vs. 3.6 ± 2.5 cells/ mm^2 in controls; $p = 0.04$ respectively).

2.3. Neuronal commitment

Neuronal commitment of BrdU positive cells labeled after the neonatal stroke was investigated by quantification of co-labeling with the neuronal nuclear marker NeuN for mature neurons and percent neuronal commitment reported for the three regions of interest investigated (i.e., DG, striatum, neocortex). GFAP and NG2 co-localization was quantified in the DG for non-neuronal commitment. At 3 weeks after BrdU incorporation GFAP and NG2 staining of cell morphologies was consistent with non-pyramidal cell types and was consistently seen co-localized with the BrdU positive cells in the hilar zone of area 3. The percent neuronal commitment in the DG in general was strong as has been previously reported in rats (Porter, 2004). Percent BrdU positive cells that co-labeled with NeuN in ligation injured hippocampi (Figure 6A) was $80.32 \pm 20\%$ compared to $91 \pm 4.8\%$ in the ipsilateral control DG and not significantly different assuming unequal variances between the two groups. Contralateral percent neuronal commitment was very similar between ligation injured and sham control groups (89.3 ± 5.3 and $91.3 \pm 2.5\%$ respectively). The non-neuronal commitment as assessed by GFAP co-localization in the DG was $11 \pm 8\%$ in ipsilateral injured hippocampi compared to $4 \pm 2.2\%$ in controls and was significantly different ($p = 0.02$). Contralaterally GFAP co-localization with BrdU positive cells was not significantly different between the two groups

(i.e., 5.5 ± 3.5 and $5 \pm 2.2\%$ respectively; $p=0.7$). With quantification of NG2 co-localization, the study again found significant increase in ipsilateral ligation injured hippocampi compared to control ($19.7 \pm 10\%$ and $4.1 \pm 2.4\%$ respectively; $p=0.001$). However they were also significantly different contralaterally between ligation injured and control groups ($7.7 \pm 3.7\%$ and $3.8 \pm 2.1\%$ respectively, $p=0.02$).

Cell lineage commitment in the striatum and neocortex (Figure 6B) was essentially non-neuronal and therefore significantly different from the hippocampal SGZ related neurogenesis both in ligated injured and sham control brains. Percent neuronal commitment in the ipsilateral striatum in ligation injured brains was $1.3 \pm 1.5\%$ and significantly lower compared to $4 \pm 2.4\%$ in the corresponding region of interest in the control brains. Contralaterally the percent neuronal commitment was similar between the two groups ($3.7 \pm 2.8\%$ and $3.3 \pm 2.5\%$ respectively). In the neocortex (sensorimotor cortex) percent neuronal commitment was not significantly different bilaterally in ligation injured brains (i.e., $5.3 \pm 3.3\%$ ipsilaterally and $7.2 \pm 5.5\%$ contralaterally) compared to control brains (i.e., $4.8 \pm 3.6\%$ ipsilaterally and $6.9 \pm 5.2\%$ contralaterally).

2.4. No influence of animal gender on post-stroke injury and neurogenesis

The mice in the ligated injured group did not show significant sex differences (male $n=4$; female $n=6$) in their overall mean hippocampal ($62 \pm 9.4\%$ and $70.9 \pm 18.4\%$ respectively) and hemispherical ($37 \pm 8.3\%$ and $33.2 \pm 11.2\%$ respectively) percent atrophy scores. Hippocampal areas in the sections from which counts were made were also not significantly different by sex. Percent neuronal commitment was not significantly different among the sexes in the ligation injured group of mice for the new cell counts in all three ROIs. No significant gender differences for all the above mentioned parameters was detected between male ($n=5$) and female ($n=5$) sham control mice.

3. Discussion

The aim of the present study was to characterize the post-stroke neurogenesis after long-term survival in a mouse model of neonatal ischemic strokes. The areas of interest were the newborn cells in the ipsilateral injured and contralateral uninjured DG originating from the SGZ niche of neural progenitors and newborn cells in the ipsi- and contralateral striatum and neocortex most likely originating from the SVZ. Although we cannot exclude the possibility that some of the newly generated cells in the striatum and neocortex are derived from local precursors, previous work using retroviruses in rats suggested that the SVZ is the main source of new cells in the neocortex and striatum (Yang, 2007). There are three important findings in this study. First, neonatal ischemia significantly reduced new cell counts as seen after long survival delays in the injured brains in the ipsilaterally atrophied hippocampi. Importantly new cell counts were also significantly reduced in the uninjured contralateral hippocampi. Second, overall percent neuronal commitment of SGZ derived new cells did not change significantly in spite of the lower counts. Third, SVZ derived neurogenesis in the injured brains, as assessed by new cell densities in the striatum and neocortex, were significantly amplified in injured brains both ipsi- and contralaterally as quantified by counts of new cells in the striatum and neocortex compared to control. However, cell lineage commitment after injury was predominantly non-neuronal and therefore remained similar to control. The comparatively lower percent neuronal commitment observed in the ipsilateral striatum of ligation injured mice (Results 2.3) may among other possibilities indicate a trend towards increased non-neuronal commitment in SVZ derived cells that migrate towards the site of injury in the striatum. Although we do not have direct evidence that new cells quantified in the neocortex and striatum solely migrated from the SVZ, previous research indicates that local progenitor populations are minimal at best and SVZ proliferation is responsible for most of the cells that migrate into the striatum and neocortex after ischemic injuries (Parent et al., 2002b, Gould et al., 1999). Our main

observation for the study is that postnatally, striatal and neocortical neurogenesis is non-neuronal in sham controls and remains non-neuronal after ischemia (i.e., new cell counts go up significantly but cell commitment fates do not change).

These results indicate that neonatal stroke clearly has a significant effect on post stroke neurogenesis. If pools of restricted and unrestricted precursors exist within the neurogenic niches of the SGZ and SVZ then neonatal stroke in this mouse model did not alter overall cell-lineage commitment profiles. However the two neurogenic zones underwent differential post-stroke plasticity. The cell proliferation in the striatum and neocortex showed amplification both ipsilaterally as was expected but also contralaterally in the week following the injury. The SGZ on the other hand showed marked suppression of neurogenesis derived new granule cell populations during the same time period. This result presents two alternatives for the SGZ derived new cells; 1) the ischemic injury directly affects the rate of neural precursor cell proliferation, or 2) the ischemic injury and the resulting lesion allows fewer newborn cells to survive the process of maturation and circuit integration. Further studies to look at rate of proliferation of new cells following the neonatal stroke at temporally spaced out time points after the stroke are underway. This additional data will help understand whether the rate of proliferation is amplified or suppressed within the SGZ after the neonatal stroke. If the rates of proliferation are indeed amplified like seen in the SVZ, it would mean from the findings of the current study that most of them degenerate over time. Since counts of newborn granule cells were also significantly lower in the contralateral SGZ derived cells, the unilateral ischemia negatively modulated contralateral endogenous neurogenesis that was independent of the ischemia related massive cell loss and atrophy noted in the ipsilateral hippocampi.

Very few of the SVZ derived cells that migrated to the striatum and neocortex became neurons. Specifically new BrdU labeled cells that had migrated to infarct borders of the neocortical and striatal lesions were not found to co-label with NeuN. This finding is interesting in lieu of studies that have shown SVZ related amplification of neurogenesis associated with a substantial increase in doublecortin (DcX) and neuron specific β -tubulin (TUJ1) positive new cells within the SVZ and in migratory mode moving towards infarct borders when immunohistochemistry was done at more acute time points after ischemic insults in neonatal rats (Yang and Levison, 2006). Paucity of SVZ derived neurons after long survival delays may indicate that DcX and TuJ1, that are considered to be markers of committed immature neurons, either have a transient phase of upregulation in the post-stroke phase or that the neuronal progenitors begin to migrate towards the striatum and neocortex but do not survive the process of maturation and circuit integration over time. Studies quantifying temporal patterns of expression of immature and mature neuron markers with new cells identified by BrdU incorporation after stroke in neonatal rats have shown a similar trend (Yang et al., 2007). With only ~5% of the new cells co-labeling with NeuN both in the ligation injured and control mice our findings are consistent with data from other studies that suggest that most newly generated neuronal precursors derived from SVZ neurogenesis do not survive maturation (Arvidsson, 2002 ;Gould, 1999;Plane, 2004;Thored, 2006). Interestingly similar trends noted in a MCAO model of unilateral ischemia revealed that erythropoietin (EPO) treatment following the insult significantly changed new cell commitment profiles in the striatum towards neuronal commitment without changing the overall counts of new cells (Gonzalez et al., 2007). This data indicate that exogenous factors can potentially alter cell commitment fates after ischemic injuries which may also have a possible therapeutic value.

In conclusion we have demonstrated that neonatal stroke due to an ischemic insult in CD1 mice results in gross perturbation of SGZ and SVZ derived neurogenesis that are paradoxical to each other. The results of this study will help determine long-term effects of commonly used pharmacological agents on post-stroke neurogenesis in future studies.

4. Experimental Procedures

4.1. Ischemic stroke at P12, bromodeoxyuridine labeling and tissue preparation

All litters of CD-1 mice were purchased from Charles River Laboratories Inc (Wilmington, MA). All research was conducted according to a protocol approved by the Johns Hopkins University School of Medicine Animal Care and Use Committee (IACUC). Newly born litters of pups arrived at postnatal five days old (P5) and were allowed to acclimate for seven days. Animals were housed in polycarbonate cages on a 12 hour light dark cycle and food provided ad libitum. On P12, animals were subjected to permanent unilateral double ligation of the carotid artery. Briefly, animals were anesthetized with isoflurane carried by a 50-50 mixture of O₂ and N₂O. The right common carotid artery was double ligated with 6-0 surgisilk and the outer skin closed with 6-0 monofilament nylon. Sham control animals were treated identically except for the carotid was not dissected or ligated. This model of ligation of the unilateral carotid artery is based on the Rice-Vannucci model (Rice et. al., 1981) in which the ligation alone does not decrease cerebral perfusion below critical levels, and the addition of hypoxia is required to cause an infarct (Vannucci and Vannucci, 2005). In this new model of neonatal strokes, ligation alone was found to be sufficient to result in infarcts in the watershed zone of the perfusion territories of the cerebral arteries and the middle cerebral artery perfusion territory. The variability in severity of ischemic lesions in the model possibly arises from variability in drops of perfusion pressure of the internal carotid artery. Since we do not transect the carotid between the two permanent ligatures there is the possibility of increasing patency of the ligated but intact carotid artery over time leading to reperfusion injury. Losses of constrictive efficacy of the ligatures occur as a result of degradation in the tensile strength of silk sutures against the pulsating carotid artery. Also establishment of efficient collateral circulation after the initial edema of the excitotoxic injury subsides is akin to human stroke conditions. Similar trends have been reported (Wainwright et al., 2007) after quantification of cerebral blood flow (CBF) using laser doppler after an HI insult in rat pups induced by permanent unilateral carotid ligation and 70 min of hypoxia. CBF in the ipsilateral hemispheres showed a gradual improvement in the first 12 h after the insult that was associated with increased CBF levels on the contralateral side and at 7 days there was no difference between the ischemic and contralateral hemispheres.

In order to identify the newborn cells in the post-stroke period, the bromodeoxyuridine (BrdU) labeling method was used. BrdU (Sigma, St. Louis, MO) dissolved in saline was intraperitoneally injected (total 5 injections; 50 mg/kg each) from P18 to P20 (i.e., 9am and 6pm starting on P18). With this method, we labeled the new cells that were synthesizing DNA about 1 week after the acute stroke over a period of 60h. Previous studies in neonatal rats have shown peak post-stroke neurogenesis to occur at this time point (Hayashi et al., 2005 ;Iwai et al., 2006) All the mice in the study were survived for 3 weeks following the injection protocol at which time they were anesthetized with chloral hydrate (90 mg/ml; intraperitoneal) before being transcardially perfused with 4% paraformaldehyde in phosphate buffer (pH 7.4). The whole brain was removed and submerged in the same fixative overnight. The brains were cryoprotected by first immersing in 15% sucrose for 24 hours, followed by 30% sucrose for 24 hours following which they were rapidly frozen using dry ice and placed in -80°C storage. Coronal brain sections 20µm thick were cut on a cryostat in serial order to create 10 series of sections that were mounted on super frost plus glass slides and stored at -20°C in preparation for immunohistochemistry.

4.2. Computerized brain asymmetry analysis

Using MCID 7.0 Elite (InterFocus Imaging Ltd, Cambridge, UK) we developed a method of computer-assisted comparison of brain tissue area in ipsilateral versus contralateral hemispheres of fixed cresyl violet stained mouse brain slices, photographed after calibration

using an AxioCam color camera and AxioVision 2.05 software. Brain atrophy scores (of the affected right side compared to the contralateral side) were measured for evenly spaced brain sections ($n=10.8\pm2.7$) spanning between the levels of the anterior horn of the lateral ventricle and the caudal hippocampus. The hippocampal atrophy asymmetry was calculated as follows:

$$[1-(\text{ipsilateral area}/\text{contralateral area})] \times 100 = \text{percent ipsilateral hippocampal atrophy}$$

For each brain, hippocampal and hemispherical atrophy scores from the series of equidistant sections were combined to calculate an average atrophy score which for the developing brain represent both the lesion induced direct atrophy and the probable injury induced stunt in developmental brain growth. Hippocampal and hemispherical areas reported were calculated for sections in which actual total counts were done to make direct area vs BrdU cell count correlations.

4.3. Double fluorescent immunohistochemistry

After blocking for unspecific reactivity, adjacent series of 20 μ m thick coronal brain sections were sequentially stained for a cell-lineage marker followed by BrdU detection and a nuclear stain (Hoechst, Chemicon 33342). Individual series were incubated in the primary antibodies [1.NeuN (1:1000, Chemicon, MAB377), 2.GFAP (1:10, Immunostar) or 3.NG2 (1:200, Chemicon, AB5320)]. For detection of BrdU incorporation, DNA was first denatured by incubation of slides with HCl (2 mol/L) at 37°C for 1/2 an hour, and rinsed for 10 minutes in 0.1 M boric acid (pH 8.5). After washing in PBS, sections were incubated in mouse anti-BrdU (1:200, Roche, 1170376). Sections were fixed with 4% paraformaldehyde between markers. The secondary antibodies used were Alexa 594 (1:100, Invitrogen) for the progenitor cell markers and Alexa 488 (1:400, Invitrogen) for BrdU. Triple labeling was done with nuclear stain (1:2000, Hoechst). Slides were examined with an Olympus (Fluoview) confocal microscope using the FV 1000 confocal system based on an Olympus IX81 inverted microscope stand and wide field fluorescence optics equipped with filter cubes for fluorophores absorbing in the UV, blue, green and near IR ranges.

4.4. Total cell counts

In order to visualize the co-localization of lineage markers with BrdU labeling, confocal Z stack images were taken with 1 μ m step sizes in sections that remained 18–20 microns thick after immunohisto-staining and orthogonal views used to confirm co-localization. Total counts of BrdU positive cells were done in the DG (area 3, Figure 7C) and co-localization with NeuN, NG2 and GFAP confirmed in bilateral hemispheres for 4 equidistant coronal sections for each brain (ligation injured $n=11$; sham control $n=10$). Counts for striatum and sensorimotor neocortex were done in areas 1 and 2 (Figure 7A and B) respectively by marking the anatomical location of the region of interest (i.e., ROI; Paxinos and Franklin, 2001) in the coronal sections using closed contours in Stereo Investigator software (MicroBrightField Inc. VT, USA) at lower magnification (2.5X). Total counts of BrdU and BrdU/NeuN positive cells were done at higher magnification (10X) using pre-assigned hemisphere and region specific markers (i.e., left striatum, left neocortex, right striatum, and right neocortex) within the software. The macro-viewing window within the software was used for navigation within the defined contours of the sensorimotor neocortex and striatum (Figure 7A and B; Paxinos and Franklin, 2001). Cell counts were done in 4 consecutive sections per brain series [coordinates of which ranged from Bregma 0.86mm to Bregma -0.70mm (Paxinos and Franklin, 2001)] and average cell densities calculated for each area bilaterally. Thus 100% sampling (i.e., equivalent to a sampling grid of counting frames adjacent to one another covering the entire ROI) was done through entire ROIs in the maximum number serial coronal sections ($n=4$) in which the ROIs could be clearly defined in the ipsilateral injured hemisphere (Manaye et al., 2007) by closed contours. This method is a modification of systematic random sampling was done as described previously by Manaye et al., where every cell of interest (i.e., BrdU positive cell) in the ROIs

was counted which is equivalent to a grid that has sampling windows adjacent to one another covering the entire ROI. Since the aim of random sampling is to give every cell of interest an equal chance of being counted; therefore counting all the cells of interest in the ROI does not bypass this very important rule. In the DG this was an obvious choice because of the low counts and a well defined ROI. In the striatum and neocortex there was a non-random distribution of the BrdU positive cells on the lesioned side and variability in the areas of the anatomical contours of the ROIs in each brain and between sections. Therefore a large number of counting frames and extensive sampling would be required to get an accurate approximation of true counts. Total counts of the cells of interest within the anatomical contours defining the striatum and neocortex were not beyond reasonable counts for a manual counting protocol. Therefore total cell counts in entire ROIs were done and densities (cells /mm²) reported to account for ipsilateral injury related atrophy in the neocortex and striatum.

4.5. Data analysis

Statistical analyses were run in SPSS for Windows (SPSS Inc., Chicago, Illinois, USA). Pair wise *t* tests were carried out to analyze the area and count data for comparisons between the ipsi- and contralateral sides in the ligation injured group. Comparisons with the control groups were done with independent sample *t* tests for means and 2-tailed Mann-Whitney U-tests for medians. Correlations were reported whenever statistical significance was noted. A probability below 0.05 was considered significant.

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Figure 1.

Cresyl violet stained coronal sections at P40 (20 μ m thick; scale bar- 250 μ m in B applies to A and B) from a mouse with a neonatal stroke induced at postnatal day 12. Ipsilateral injured hippocampi (A) showed massive loss of neurons in the CA3 and CA1 regions as compared to the contralateral uninjured hippocampus (B). C. Cresyl violet stained sections from P12 ligated CD1 mice (left column) and sham controls (right column) showing typical infarct injury at P40. Green (ipsilateral) and red (contralateral) contours show how hippocampal atrophy was measured in MCID.

Figure 2.

Histograms of unilateral stroke induced brain atrophy data compared to sham controls using MCID software. **A.** Average cerebral hemisphere areas (mm²) of ipsilateral (right) and contralateral (left) hemispheres in coronal sections of control and ligated injured mice. **B.** Average hippocampal areas (mm²) of ipsilateral (right) and contralateral (left) hemispheres in coronal sections. **C.** Mean ipsilateral percent atrophy compared to contralateral in the hippocampi and cerebral hemispheres.

Figure 3.

I. Immunohistochemistry labeling at P40 for BrdU (green) and NeuN (red) positive cells in the subgranular and granule cell layer of the hippocampi (20 μ m thick; 10X magnification) ipsi- and contralateral (**A** and **B** respectively) to the neonatal stroke. Co-labeled cells appear yellow and were confirmed to be the same cell using orthogonal views of stacked images on a confocal (**C**). They represent cells born after the stroke that have survived differentiation and matured into neurons.

II. Immunohistochemistry labeling for BrdU (green), NG2 (red) and Hoechst. (**A**) BrdU positive cells in the subgranular zone of the hippocampi (20 μ m thick; 60X magnification). Triple co-labeled cell seen in center of panel A, confirmed to be the same cell using orthogonal views of stacked images. **B.** Shows the same cell with BrdU and NG2 co-labeling and **C** shows the same BrdU positive nucleus with nuclear stain (Hoechst) co-localization.

III. Immunohistochemistry labeling for BrdU (green), GFAP (red) and Hoechst (**A**) BrdU positive cells in the subgranular zone of the hippocampi (20 μ m thick; 60X magnification). Triple co-labeled cell seen in center of panel A, confirmed to be the same cell using orthogonal views of stacked images. **B.** Shows the same cell with BrdU and GFAP co-labeling and **C** shows the same BrdU positive nucleus with nuclear stain (Hoechst) co-localization.

Figure 4.

Neurogenesis in the post-stroke DG. **A** and **B** - Both ipsi- and contralateral hippocampi in injured mice had significantly lower counts of BrdU positive cells. **C** and **D** — Ipsilateral hippocampi average area scores for the corresponding sections in which the counts were done were significantly lower than controls whereas contralateral hippocampi were not. **E** and **F** — Correlations of total counts of BrdU positive cells in area 3 (● - ligated injured and ○ - controls) and their respective hippocampal areas found a tight correlation ipsilaterally but not contralaterally. Contralaterally (F) the dotted line demarcates lower counts of BrdU positive cells (● - ligated injured and ○ - controls) in ligated injured brains as compared to control brains over the same range of hippocampal areas. This difference between the two groups resulted in the loss of correlation of total hippocampal areas to corresponding cell counts for the contralateral hippocampi.

Figure 5.

Neurogenesis in the post-stroke neocortex and striatum. **A** and **B** - significantly increased BrdU positive cell density counts in the ipsilateral and contralateral spared neocortex (total BrdU positive cells/mm² of sensorimotor neocortex). **C** and **D** - significantly increased BrdU positive cell counts in the ipsilateral and contralateral striatum.

Figure 6.

A. Percent cell lineage commitment of cells in the DG that were BrdU labeled 1 week after the neonatal stroke and quantified after a survival delay of 3 weeks. Mean percent neuronal commitment did not change significantly in the hippocampi of injured brains; however percent non-neuronal commitment increased significantly both for NG2 and GFAP positive new cells in ipsilateral hippocampi compared to control. Additionally %NG2 positive new cells showed a significant increase in the contralateral hippocampi of injured brains compared to control (**). A pair wise *t* test showed that % NG2 commitment was also significantly higher in the ipsilateral compared to contralateral hippocampi within the ligation injured group of mice (*). **B.** Percent cell lineage commitment was significantly neuronal in the SGZ derived cells (i.e.,

ROI 3 of the hippocampi) compared to the SVZ derived cells (i.e., ROI 1 and 2 of the striatum and sensorimotor neocortex respectively) in the ipsilateral striatum and neocortex (*) both in control and ligation injured mice. Additionally neuronal commitment was further significantly lowered in the ipsilateral striatum (i.e., ROI 1) in injured mice compared to the sham controls (**).

Figure 7.

A. Schematics of the experimental protocol for study. **B and C.** Schematics of regions of interest (ROI) for quantification of neurogenesis at P40. **B.** Representative coronal section at Bregma -0.02mm (Paxinos and Franklin 2001) marking ROIs 1 and 2 for quantification of neurogenesis in the striatum and sensorimotor neocortex respectively. **C.** Representative coronal section at Bregma -0.7mm marking ROI 3 for quantification of SGZ neurogenesis for the hippocampus. Magnified view shows area of dentate gyrus (DG) where total counts for ipsi- and contralateral hippocampi were done. Total counts were done in series of 4 sections for each area and brain bilaterally and averages calculated. CC - Corpus callosum; AC - Anterior commissure; LV - Lateral ventricle; IC - Internal capsule.