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Defining the Critical Period for Neocortical Neurogenesis after Pediatric Brain Injury

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Key Words
Subventricular zone · Stem cells · Neurons · Regenerative medicine · Traumatic brain injury · Brain damage

Abstract
Pediatric traumatic brain injury (TBI) is a significant and underappreciated societal problem. Whereas many TBI studies have evaluated the mechanisms of cell death after TBI, fewer studies have evaluated the extent to which regeneration is occurring. Here we used a cryoinjury model to damage the somatosensory cortex of rats at postnatal day 6 (P6), P10 and P21. We evaluated the production of new neocortical neurons using a combination of 5-bromo-2-deoxyuridine (BrdU) labeling combined with staining for doublecortin (DCX). BrdU+/DCX+ bipolar cells were observed adjacent to the neocortical lesion, with their processes oriented perpendicular to the pial surface. As the animals aged, both the overall proliferative response as well as the production of neocortical neuroblasts diminished, with P6 animals responding most robustly, P10 animals less strongly, and P21 animals showing a very modest proliferative response and virtually no evidence of neocortical neurogenesis. When BrdU was administered at increasingly delayed intervals after the injury at P6, there was a clear difference in the number of new neuroblasts produced as a function of age, with the greatest number of new neocortical neurons produced between 4 and 7 days after the injury. These studies demonstrate that the immature brain has the capacity to produce neocortical neurons after traumatic injury, but this capacity diminishes as the brain continues to develop. Furthermore, in contrast to moderate hypoxic/ischemic brain damage in the P6 rat, where neurogenesis persists for at least 2 months, the response to cryoinjury is quite different as the neurogenic response diminishes over time.

Introduction

Pediatric traumatic brain injury (TBI) is a significant and underappreciated societal problem. Approximately 475,000 children under the age of 14 years incur a TBI each year. Roughly 37,000 of these children require hospitalization and estimates are that 2,700 children die each year from TBI-related injuries. The causes of injury vary widely by age. In infants, assault (shaken babies) is one of the main causes of TBI, whereas bicycle and motor vehi-

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cle accidents are the principle causes of injury in adolescents [1]. It has been estimated that the financial burden of pediatric TBI is USD 1 billion per year in hospital costs alone [2]. Given this enormous financial burden, the emotional burden placed on caregivers and the fact that injury evolves differently in children than in adults, there is a strong rationale for additional studies of pediatric TBI.

Whereas changes in cell proliferation have been examined in the subventricular zone (SVZ) following mechanical brain injury, there is as yet an incomplete understanding of this process, making comparisons between studies difficult. Nonetheless, there is strong evidence that SVZ cell proliferation and total cell numbers increase in response to a variety of injuries in the rat. Most studies to date have examined unilateral aspiration or percussion injury of the adult cerebral cortex. In rats, the total number of cells in the adult SVZ increases 1 week following aspiration lesions of the somatosensory cerebral cortex [3]. In the fluid percussion injury model in adult rats, the number of 5-bromo-2-deoxyuridine (BrdU)-labeled SVZ cells increases 2 and 4 days after injury [4]. When assessed at 1 year of recovery, control rats show significant age-related decreases in the numbers of PCNA+ and Ki67+ (i.e. dividing) SVZ cells, whereas lesioned animals do not [5].

Whereas cells born in the ventricular zone and SVZ of the developing forebrain migrate to many nuclei, neuroblasts in the intact adult SVZ of most vertebrates migrate primarily to the olfactory bulb (OB) along the rostral migratory stream [6, 7]. While this appears to be the case in the intact brain, a number of studies indicate that brain injury can induce – or allow – SVZ cells to migrate to non-OB areas, especially towards the lesion.

In our studies of neonatal hypoxic/ischemic brain injury, we have documented the significant production of new neurons from the SVZ that begins a few days after the injury and persists for at least 2 months [8, 9]. As studies of traumatic injury in adult rats have rarely seen migration of SVZ cells into the neocortex, we hypothesized that the production of new neocortical neurons would be greater in the immature brain. In the studies reported here, we used a cryoinjury model to damage the somatosensory cortex of rats at postnatal day 6 (P6), P10 and P21. The P6 time point was chosen to allow a direct comparison with our studies of hypoxic/ischemic injury; the P10 animal was chosen to model injury to neonates, and the P21 rat was chosen to model traumatic injury in toddlers [10–12]. We modified the cryoinjury model that was originally developed by Klatzo et al. [13], which involves touching the brain with a supercooled metal rod. The cooled metal probe causes tissue necrosis and a failure of the blood-brain barrier, leading to vasogenic brain edema and secondary brain damage [14, 15]. This model creates a lesion in rodents that is histologically similar to that observed in cases of cortical contusions in humans [16]. Using this injury paradigm, we evaluated the cell proliferation within the SVZ and the production of neuroblasts subsequent to injury as a function of increasing age.

Methods

Cryoinjury Model

All experiments were performed in accordance with protocols approved by the institutional animal care and use committee of the University of Medicine and Dentistry of New Jersey-New Jersey Medical School. Timed pregnant Wistar rats (Charles River, Wilmington, Del., USA) were housed and cared for by the Department of Comparative Medicine. After normal delivery, the litter size was adjusted to 12 pups per litter. Cryoinjury was performed on P6–21 rat pups using a modification of methods previously used on adult mice [17]. The pups were anesthetized with isoflurane (4% induction, 2% maintenance). Once fully anesthetized, the scalp was cleansed and an incision along the midline created to expose the skull. The cryoinjury was induced by bringing a copper probe (2.0 mm in diameter) that was prechilled with liquid nitrogen into contact with the parietal skull 2.5 mm lateral to the midline at the bregma. Sham-operated animals were anesthetized, their skull exposed, but the cold probe was not applied. The scalp was then sutured and the animals were returned to the dam. The entire procedure was typically completed within 5 min.

BrdU Injections

Intraperitoneal injections of BrdU (50 mg/kg dissolved in 0.007 N NaOH in PBS; Sigma, St. Louis, Mo., USA) were given once daily at various times after cryoinjury according to experimental designs. At specific time points, the animals were anesthetized with a mixture of ketamine (75 mg/kg) and xylazine (5 mg/kg) and then fixed by intracardiac perfusion with normal saline followed by 4% paraformaldehyde in PBS. Each brain was removed from the skull, postfixed overnight and then cryoprotected for 24 h in 30% sucrose in water. The brains were frozen in optimal cutting temperature compound embedding medium (Sakura Finetek, Torrance, Calif., USA) on a dry-ice/ethanol slush.

Immunofluorescence

Immunofluorescence staining was performed on 40-μm free-floating sections. Sections for BrdU staining were pretreated with 2 N HCl for 1 h at 22°C to denature DNA. The sections were blocked for 1 h in TBS with 10% donkey serum and 2% BSA and then incubated with primary antibodies for 24 h at 4°C. The following primary antibodies were used: anti-doublecortin (DCX; goat polyclonal, DCX-COOH terminus, 1/100; Santa Cruz Biotechnology, Santa Cruz, Calif., USA); anti-NeuN (mouse monoclonal, 1/100; Chemicon, Temecula, Calif., USA); anti-BrdU (rat

monoclonal, 1/30; Accurate Chemicals, Westbury, N.Y., USA), and anti-IBA-1 (1/200; Wako Chemicals, Wako, Tex., USA). Secondary antibodies against the appropriate species were incubated for 2 h at room temperature (all from Jackson Immunoresearch, West Grove, Pa., USA; 1/200). All secondary antibody combinations were carefully evaluated to ensure that there was neither crosstalk between fluorescent dyes nor cross-reactivity between secondary antibodies. The sections were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI; Sigma; 1 μg/ml) for 5–10 min and then coverslipped with Gel/Mount (Biomeda, Foster City, Calif., USA).

Microscopy

The sections were analyzed using a Zeiss LSM 510 confocal microscope with filter sets for Cy2, Dylight488 and Rhodamine Red-X. Confocal Z sectioning at 0.5-μm intervals was performed to examine double labeling. Images were acquired with Zeiss LSM software and analyzed offline using Zeiss LSM image browser software. Additional immunofluorescence images were acquired using an Olympus AX70 fluorescence microscope equipped with a SenSys CCD camera and IPLab imaging software.

Cell Quantification and Statistical Analysis

Three sections sampled at 120-μm intervals were quantified per brain. For each section, 5 fields surrounding the cryoinjured area were analyzed (2 fields on both the left and right sides of the injury, and 1 field ventrally). Each field occupied an area of a 100 × 100 μm square. Within each region, a Z-stack was created at 0.5-μm intervals through the entire depth of the section. The numbers of BrdU+/DCX+ cells in each region of the damaged neocortex were then quantified and averaged. To assess cell proliferation in the SVZ, the cell number was assessed within 3 regionally distinct fields within the SVZ. Three fields within the SVZ were captured at ×60: a medial field, a mediolateral field and a lateral field. Each field represented an area of 50 × 50 μm. The total number of BrdU+ cells and DAPI-labeled cells for each field were quantified. The data are presented as the percentage of labeled cells per field after correction using the Abercrombie correction. This method has been described in greater detail in an earlier publication [18]. Results from the cell counts were analyzed for statistical significance using ANOVA followed by post hoc comparisons using Tukey's post hoc test. All data are presented as means ± SEM. Comparisons were interpreted as significant when associated with $p < 0.05$. Where the post hoc test was used for multiple comparisons, superscript letters indicate significant differences; that is, means with the same letter are not significantly different. For example, a bar with the letter 'a' is not statistically different from one with the letters 'ab', but is different from a bar with the letter 'b'.

Results

Reproducibility of Cortical Injury

To determine whether there is altered proliferation of precursors of the SVZ and subsequent production of new migratory neuroblasts to the site of injury (a process referred to as neurogenesis), we first needed to optimize the cryoinjury model for the immature rat. The cold probe was applied to the skull of P6 rat pups for 15, 30 or 60 s, and the injury evaluated 4 days later. Applying the probe to the skull for 15 s produced very mild brain damage (fig. 1a). Applying the probe for 30 s produced a larger injury (fig. 1b); however, the size of the lesion was inconsistent. Applying the probe for 60 s produced a cylindrical lesion that ended just superficial to the subcortical white matter. By 4 days of recovery, the lesion cavity was free of debris. Unlike the inconsistent lesions produced using the 30-second probe application, the lesion produced using the 60-second probe application was more reproducible, although there remained some variability (fig. 1c, d). A similar injury was produced in animals ranging from 6 days postnatal to 21 days postnatal. The cryoinjury elicited a robust accumulation of activated microglia/macrophages, as revealed by IBA-1 staining (fig. 1e, f).

Cell Proliferation in SVZ

To determine how brain maturation affects SVZ precursor responses to cryoinjury, animals at P6, P10 and P21 were subjected to the cryoinjury (60-second probe application) and BrdU was administered once daily on days 1–4 of recovery; they were sacrificed 7 days later to evaluate cell proliferation. A strong proliferative response was elicited in animals injured at P6 (fig. 2, 3). Interestingly, this increase in cell proliferation was bilateral as there were significantly more BrdU-labeled cells in both the hemisphere ipsilateral to and contralateral to the cryoinjury compared to sham-operated animals (fig. 2, 3). A quantitative analysis of BrdU-labeled cells revealed that the response to the cryoinjury was most pronounced in the medial aspect of the SVZ on the ipsilateral side, where approximately 55% of the cells were labeled with BrdU. There was a 2.7-fold increase in BrdU-labeled cells in the ipsilateral medial SVZ compared to sham-operated controls. The increase in BrdU-labeled cells in the medial aspect of the contralateral SVZ compared to the sham was 1.8-fold.

In animals injured on P10, there was again a bilateral increase in BrdU+ cells elicited by the injury; however, BrdU+ cells were less abundant in the P10 animals compared to P6 animals, and the fold increase after injury was also reduced (fig. 2). In animals injured on P10, the response to the cryoinjury was most pronounced in the lateral aspect of the SVZ on the ipsilateral side, where the labeling index was approximately 40%. Comparing the medial aspect of the ipsilateral SVZ to the sham-operated animals revealed that there was a 2-fold increase in BrdU-labeled cells compared to sham-operated controls. The

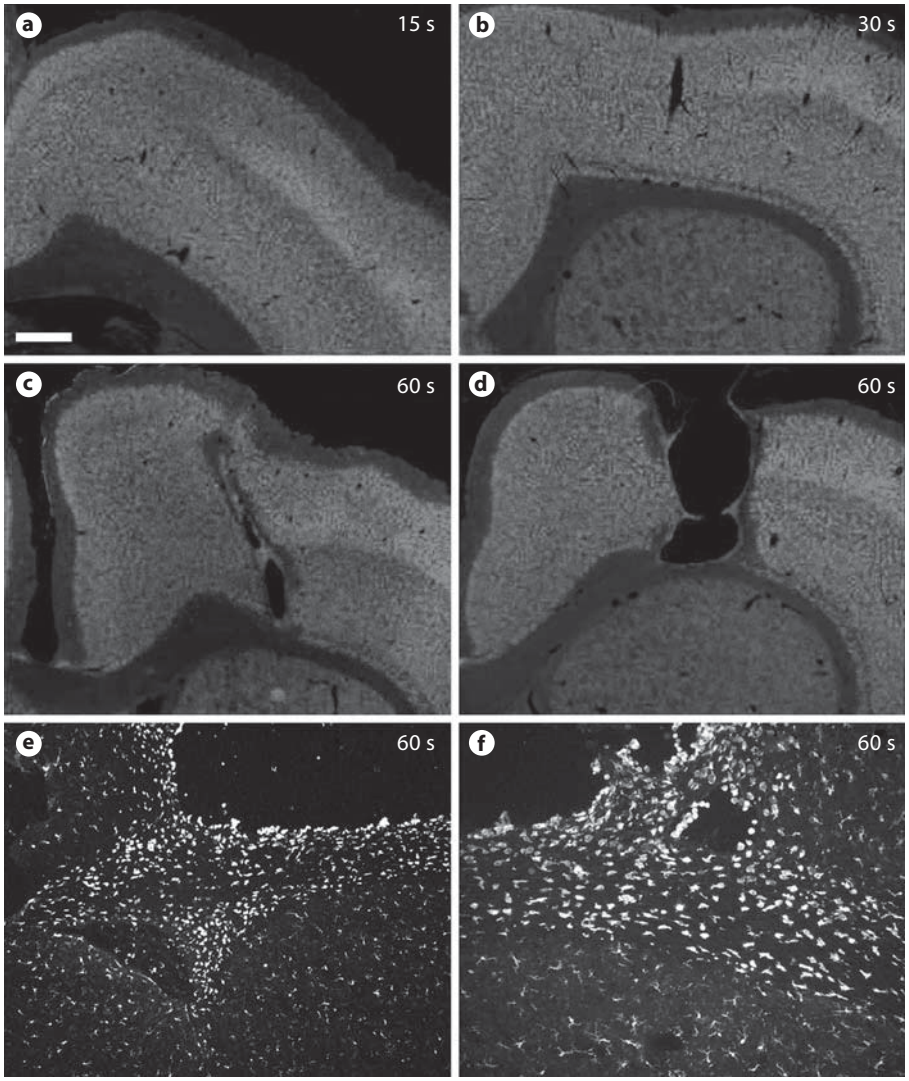


Fig. 1. A 60-second cryoinjury produces the most consistent and reproducible injury to the cerebral cortex. Scale bar = 1 mm (**a–c**) and 200 μm (**e**, **f**). **a**, **b** Damage to the cerebral cortex after 15 and 30 s of cryoinjury. **c**, **d** Damage to the cerebral cortex after 60 s of cryoinjury from 2 representative animals. **e**, **f** IBA-1 staining for microglia at 96 h of recovery after 60 s of cryoinjury.

increase in BrdU-labeled cells in the medial aspect of the contralateral SVZ was 1.8-fold (fig. 3).

In animals injured on P21, BrdU+ cells were sparse compared to younger animals (fig. 2). In the medial aspect of the SVZ on the ipsilateral side, the BrdU labeling index was 7.5% compared to 5% in the sham-operated animals. There was no significant difference in the percentage of BrdU-labeled cells in the medial aspect of the SVZ in the ipsilateral SVZ compared to sham-operated animals. The only area where there was a statistically significant increase was in the mediolateral aspect of the ipsilateral SVZ. There was a 1.6-fold increase in BrdU-labeled cells compared to sham-operated controls. As in the medial aspect, only 8.5% of the cells were BrdU labeled (fig. 3).

Neocortical Neurogenesis after Cryoinjury

To determine whether new neocortical neuroblasts were produced in response to the cryoinjury, P6 and P10 animals were subjected to the cryoinjury and BrdU was injected beginning at 24 h of recovery for 4 days (fig. 4). The animals were sacrificed and sections stained for BrdU and DCX after an additional 7 days, or for DCX and NeuN 14 days later. DCX+ cells were extremely rare in the neocortex in the hemisphere contralateral to the cryoinjury and were rarely observed in the sham-operated animals; however, in regions both lateral to and ventral to the injury site, bipolar, DCX+ cells were readily observed in both P6 and P10 animals (fig. 4a–e). The processes of those neuroblasts located along the medial border were predominantly oriented perpendicular to the pial surface

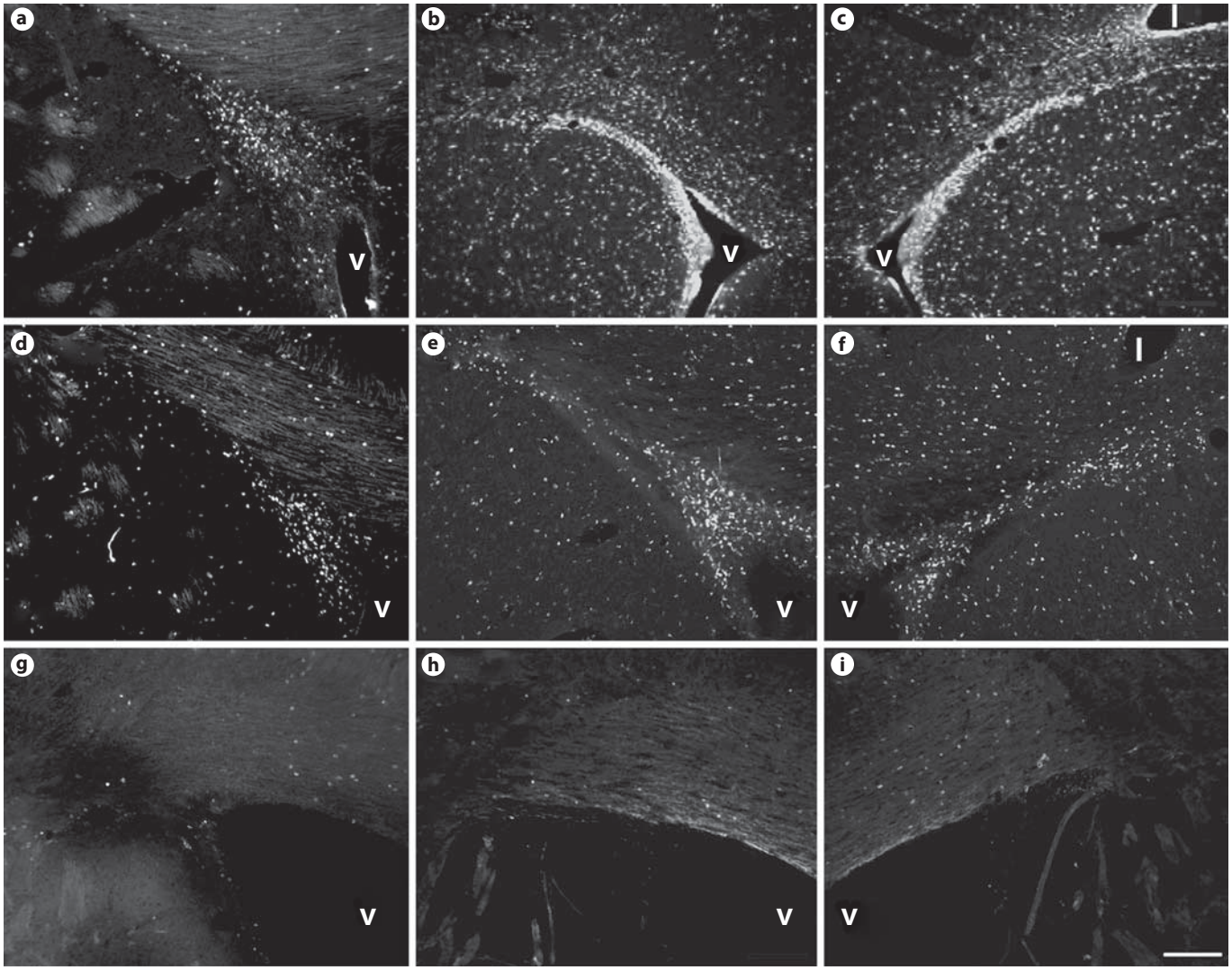


Fig. 2. A 60-second cryoinjury increases proliferation bilaterally in the SVZ after injury. The animals received once daily injections of 50 mg/kg BrdU (i.p.) on days 1–4 of recovery and were sacrificed 7 days later and stained for BrdU incorporation. I = Cryoinjury; V = ventricle. Scale bar = 250 μm. **a–c** BrdU staining in the SVZ of ipsilateral (**c**) and contralateral hemispheres (**b**) after 60 s of cryoinjury in the P6 rats. **d–f** BrdU staining in the SVZ of ipsilateral (**f**) and contralateral hemispheres (**e**) after 60 s of cryoinjury in the P10 rats. **g–i** BrdU staining in the SVZ of ipsilateral (**i**) and contralateral hemispheres (**h**) after 60 s of cryoinjury in the P21 rats. **a**, **d**, **g** Sham.

(i.e. parallel to the lesion) (fig. 4a, b, f). At 18 days of recovery, BrdU+/DCX+ cells persisted and some DCX+ cells had acquired NeuN, indicating that these neuroblasts were differentiating into mature neurons (fig. 4e, f). Quantifying the relative numbers of BrdU+, DCX+ and DCX+/BrdU+ cells in the ventral, medial and lateral regions of the cryoinjury lesion of P6 and P10 animals revealed significantly more DCX+ cells in both the neocortex medial and ventral to the injury in the P6 animals compared to P10 animals (fig. 4g, h). There were virtually no BrdU+/DCX+ cells observed outside the SVZ in animals injured at P21. Quantifying BrdU+ cells adjacent to the cryoinjury confirmed an increase in the overall proliferative response in the P6 animals versus the P10 animals. Additionally, there were more DCX+/BrdU+ cells in the P6 animals than in the P10 animals. The proportion of double-positive cells was approximately 10% of the total number of DCX+ cells.

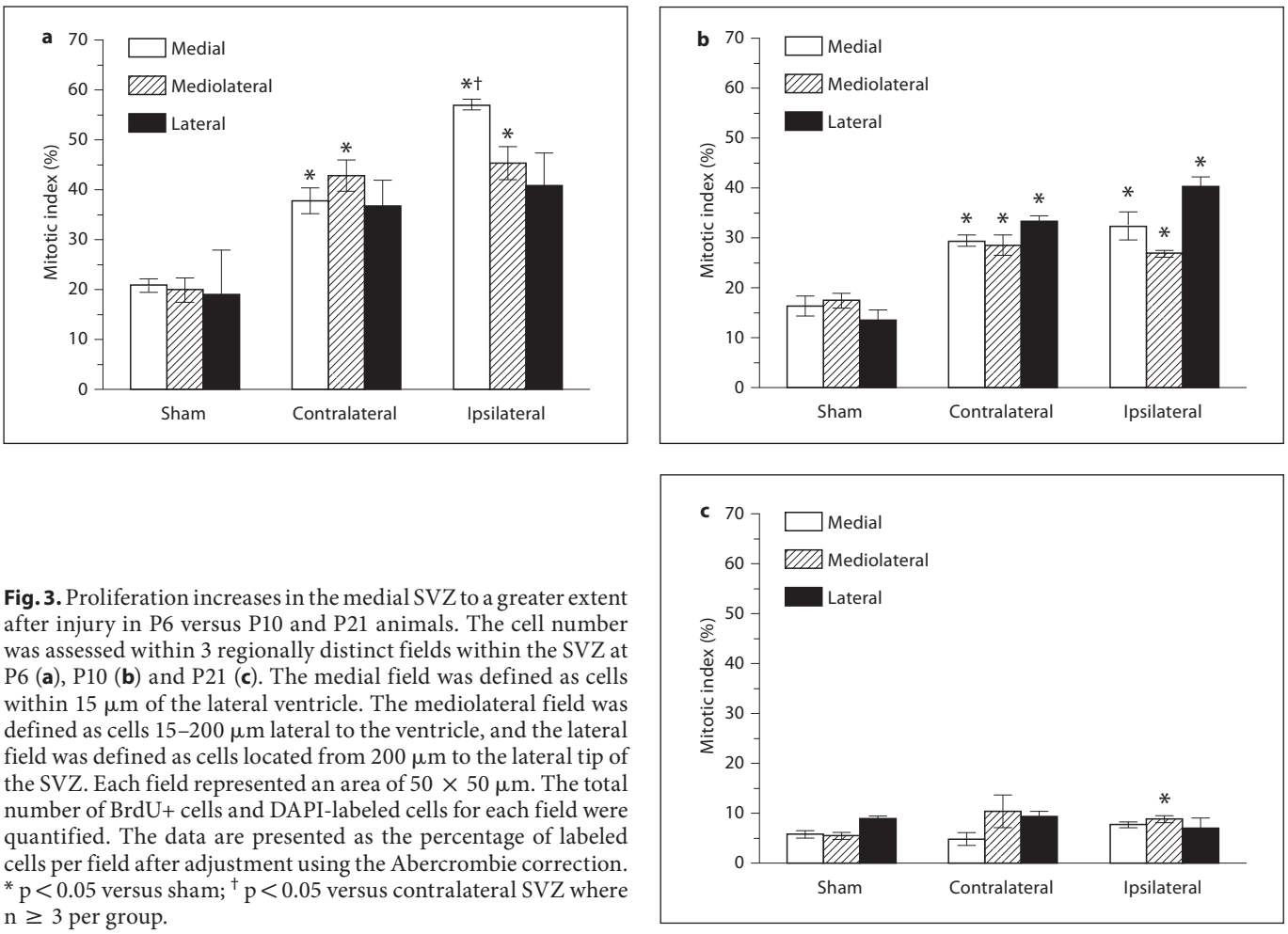


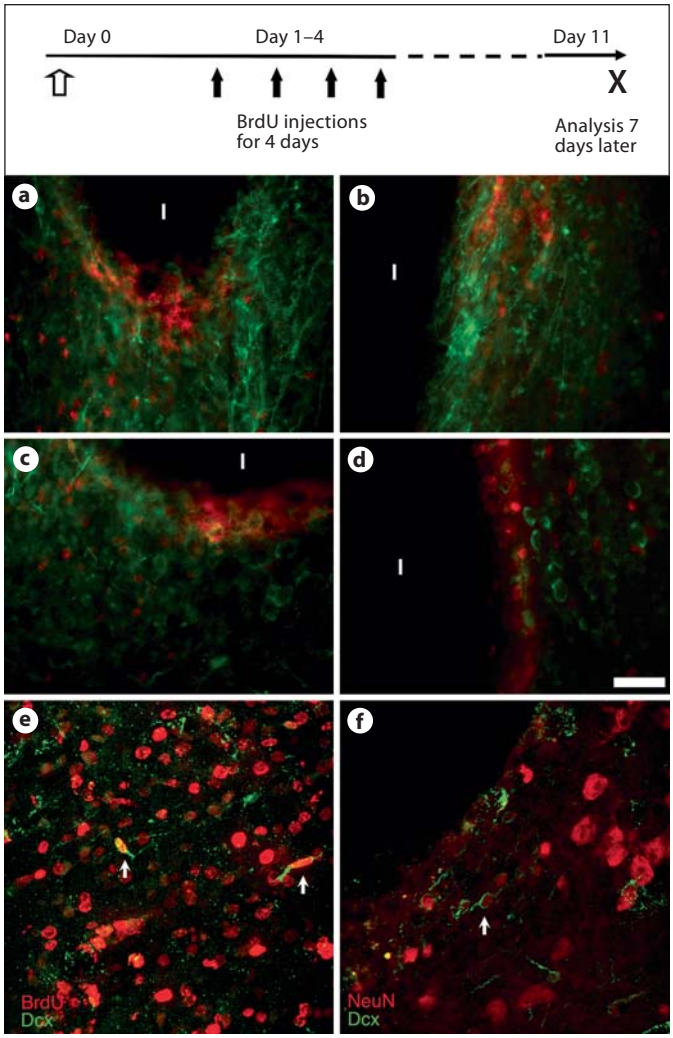
Fig. 3. Proliferation increases in the medial SVZ to a greater extent after injury in P6 versus P10 and P21 animals. The cell number was assessed within 3 regionally distinct fields within the SVZ at P6 (**a**), P10 (**b**) and P21 (**c**). The medial field was defined as cells within 15 μm of the lateral ventricle. The mediolateral field was defined as cells 15–200 μm lateral to the ventricle, and the lateral field was defined as cells located from 200 μm to the lateral tip of the SVZ. Each field represented an area of 50 × 50 μm. The total number of BrdU+ cells and DAPI-labeled cells for each field were quantified. The data are presented as the percentage of labeled cells per field after adjustment using the Abercrombie correction. * $p < 0.05$ versus sham; † $p < 0.05$ versus contralateral SVZ where $n \geq 3$ per group.

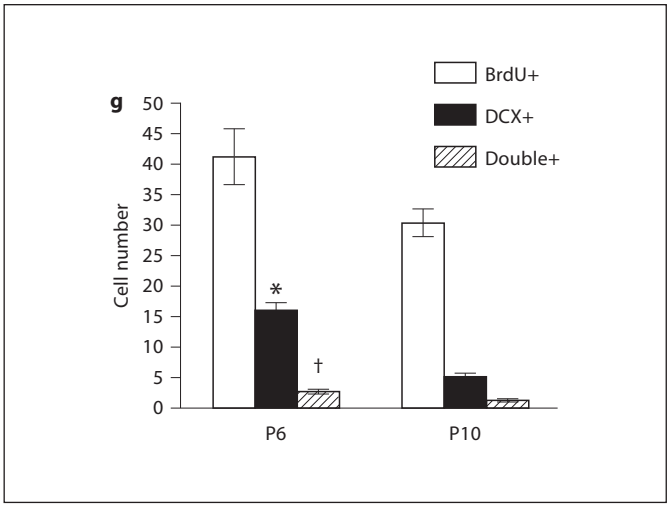
Time Course of Neocortical Neurogenesis after Cryoinjury

To determine when the greatest number of new neocortical neurons is produced during recovery from the cryoinjury in P6 animals, the animals received BrdU injections once daily for 3 days at 4 time points of recovery. BrdU was injected daily beginning at either day 1, 4, 7 or 21, and the animals were sacrificed 6 days later. DCX-expressing cells were produced at all time points evaluated and could be found in the neocortex immediately below the lesion (fig. 5a–d) as well as in the medial and lateral regions of the lesion penumbra (fig. 5e–h) when compared to controls. The total numbers of BrdU+, DCX+ and DCX+/BrdU+ cells in the ventral, medial and lateral regions of the cryoinjury penumbra of P6 animals were quantified. This analysis revealed significantly more BrdU+ cells in ventral and medial regions in groups 1 and 2, where labeling began at days 1 and 4 of recovery, compared to groups 3 and 4, where BrdU labeling was initiated on days 7 and 21 (fig. 5i, j). When the numbers of DCX-labeled and DCX+/BrdU+ cells were quantified, the increase in DCX+ and BrdU+/DCX+ cells was most significant in the ventral and medial regions for group 2, where cells were labeled between days 4 and 7 of recovery, compared to groups where cells were labeled beginning on days 1, 7 or 21 of recovery (fig. 5i, j).

Discussion

Whereas many studies have evaluated the mechanisms of cell death after TBI, accompanied by significant interest in applying neuroprotective strategies, fewer studies have evaluated the extent to which regeneration is occurring. That said, there have been studies performed over the last several decades that have suggested that the





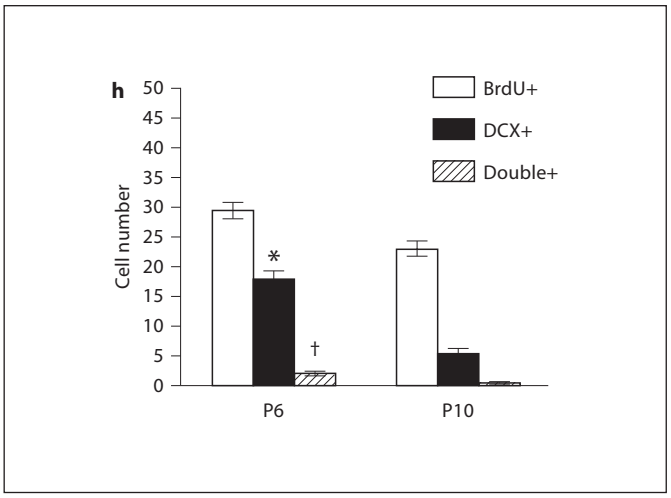


Fig. 4. Neocortical neurogenesis is greater in P6 versus P10 animals after cryoinjury. P6 or P10 animals were injected with 50 mg/kg BrdU once daily (i.p.) for 4 days, beginning 1 day after the injury, and were sacrificed either 7 (**a–d**) or 14 days later (**e**, **f**). * $p < 0.05$ versus P10 animals; † $p < 0.05$ versus P10 animals. Arrows: double-labeled cells. Scale bar = 30 μm (**a–d**) and 15 μm (**e**, **f**). **a** Ventral surface of the cryoinjury lesion (I) from P6 animals stained for DCX (green) and BrdU (red). **b** Lateral surface of the lesion from P6 animals stained for DCX. **c** Ventral surface of the cryoinjury lesion from P10 animals stained for DCX. **d** Lateral surface of the lesion from P10 animals stained for DCX. **e** Higher-power view of cells labeled for BrdU and DXC. **f** Cells double-labeled for BrdU and NeuN at 2 weeks of recovery. **g** Number of BrdU+, DCX+ and BrdU+/DCX+ cells at the ventral surface of the cryoinjury lesion of P6 and 10 animals. **h** Number of BrdU+, DCX+ and BrdU+/DCX+ cells at the lateral surface of the cryoinjury lesion of P6 and 10 animals.

Fig. 5. Neurogenesis is greatest early during recovery from cryoinjury. After cryoinjury at P6, animals were split into 4 groups and injected with BrdU once daily for 3 days beginning either at 1, 4, 7 or 21 days of recovery. The animals were sacrificed 7 days later. Arrows: double-positive cells. Scale bar = 25 μm (**a–h**). **a–d** Ventral surface of the cryoinjury lesion from the 4 groups of P6 animals stained for DCX. **a** Group 1. **b** Group 2. **c** Group 3. **d** Group 4. **e–h** Medial surface of the cryoinjury lesion from the 4 groups of P6 animals stained for DCX. **e** Group 1. **f** Group 2. **g** Group 3. **h** Group 4. **i** Number of BrdU+, DCX+ and BrdU+/DCX+ cells at the ventral surface of the cryoinjury lesion of P6 animals for each group. **j** Number of BrdU+, DCX+ and BrdU+/DCX+ cells at the medial surface of the cryoinjury lesion of P6 animals for each group. **k** Number of BrdU+, DCX+ and BrdU+/DCX+ cells at the lateral surface of the cryoinjury lesion of P6 animals for each group. Statistical analyses: **i** [a] vs. group 2; [b] vs. group 3; [c] vs. group 4; [d] vs. group 2; [e] vs. group 4; [f] vs. group 4; [g] vs. group 3; [h] vs. group 4; [i] vs. group 4. **j** [a] vs. group 2; [b] vs. group 3; [c] vs. group 4; [d] vs. groups 3; [e] vs. group 4; [f] vs. group 1; [g] vs. group 3; [h] vs. group 4. **k** [a] vs. group 4; [b] vs. group 3; [c] vs. group 4; [d] vs. group 3; [e] vs. group 4.
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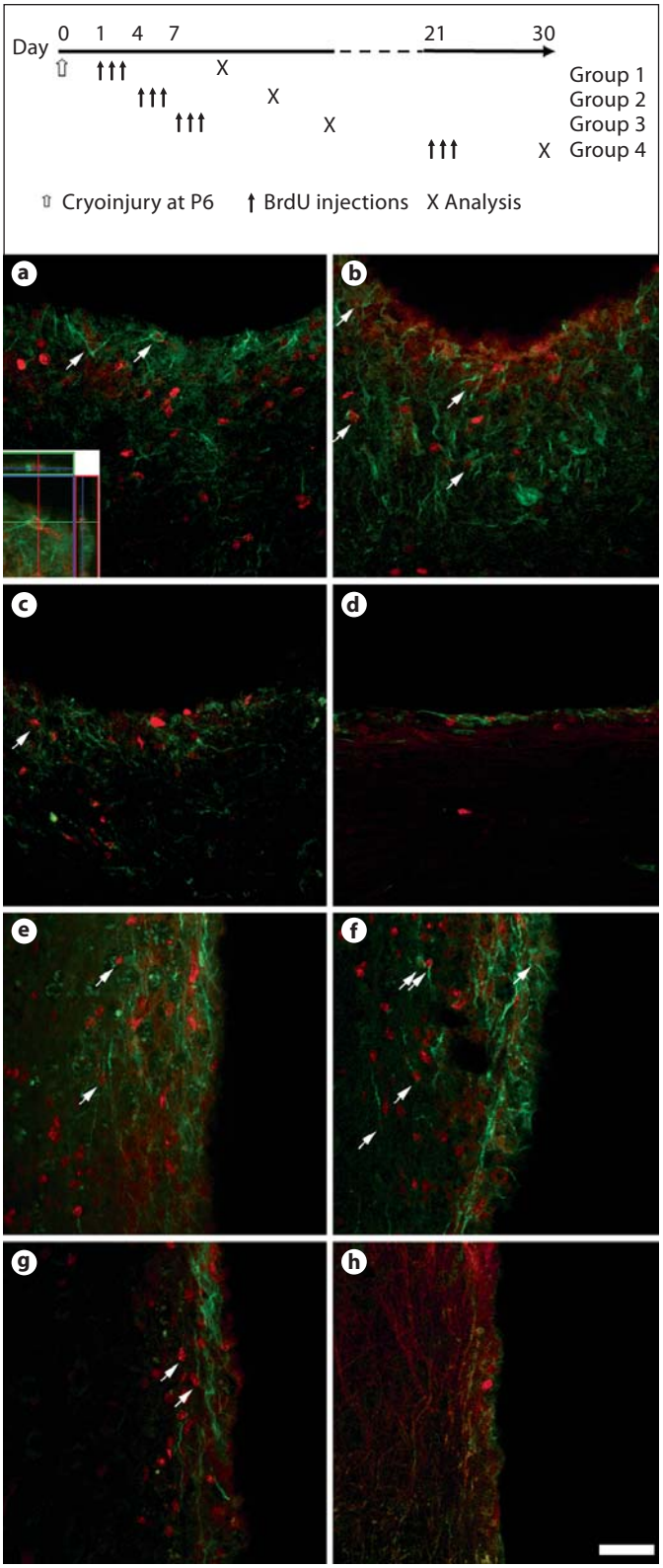
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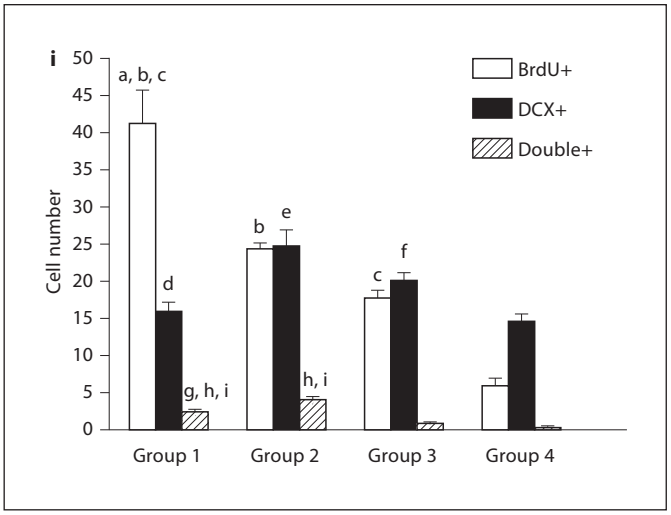
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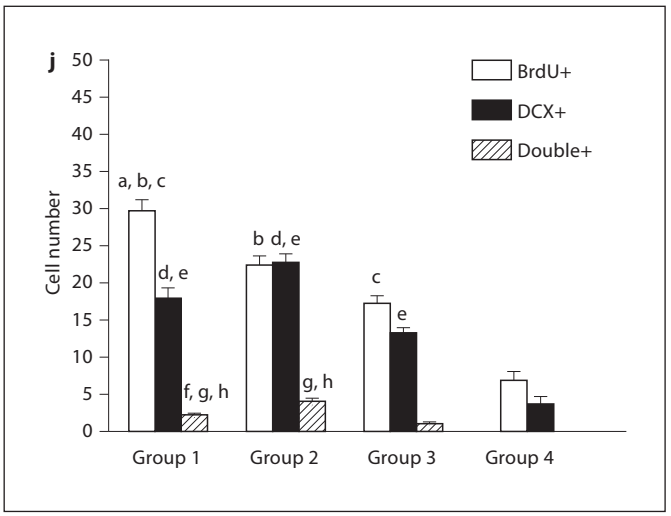
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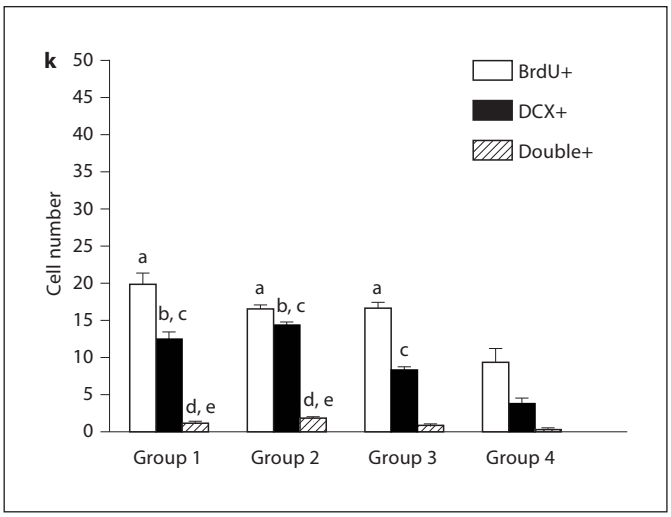
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5

brain is capable of mounting an endogenous repair response, thus raising hope that regeneration after brain damage might be stimulated [3, 19, 20]. Of particular relevance, studies have documented injury-induced neurogenesis in the hippocampus after TBI [21, 22]. The aim of this study was to characterize the timing of the neuroregenerative response of the SVZ in neonates and juveniles after applying a cold probe to the skull for 60 s, which produced a neocortical lesion as a consequence of secondary necrosis.

In previous studies where injuries have been inflicted upon the adult brain, cell proliferation in the SVZ has been found to increase with subsequent migration of newly formed cells into non-OB regions. In a study in adult mice using this same injury paradigm, the vast majority of proliferating cells after injury were astrocytes and NG2 cells [17]. The Szele laboratory injected a library of retroviral vectors into the lateral ventricles of mice and examined migration to the OB and the penumbra of a neocortical aspiration injury [23]. Four days after the lesion, fewer cells had migrated to the OB, and a greater number of cells had instead migrated into the corpus callosum adjacent to the injury. However, relatively few cells migrated through the corpus callosum into the cortex. Since approximately twice as many retrovirally labeled cells had survived in the lesioned brain compared to brains of control mice, the authors concluded that the lesion promoted the survival of the labeled population as well as migration.

In a more recent study, the Szele group used DCX and BrdU labeling to assess the migration of newly born neurons after cortical aspiration lesions and confirmed that newly born DCX+ cells migrate towards the lesion, but they seem inhibited in the adult brain from entering the neocortex. They did not find any decrease in migration into the OB. These results are consistent with a scenario in which those cells that migrate from the SVZ to the area of injury are newly born cells, as opposed to cells that fail to follow their normal migratory routes [24]. In these studies, those SVZ cells that emigrated from the SVZ into the neocortex differentiated into astrocytes and oligodendrocytes, but not neurons [23]. Of those cells that adopt a neuronal fate, the vast majority of these new neurons migrated into subcortical regions such as the striatum, or they appeared to get trapped in the white matter [24]. Subsequent to knife cuts through the rostral migratory stream in mice, an enormous number of polysialic acid-neural cell adhesion molecule (PSA-NCAM)-positive cells oriented in chains migrate caudal to the lesion to the striatum [25]. A subset of these cells express the neuronal markers β-tubulin, tyrosine hydroxylase and γ-aminobutyric acid up to a month and a half after the surgery [25]. In our studies, we attempted to stain the migrating neuroblasts for PSA-NCAM, but even using confocal microscopy could not obtain convincing images of DCX+/PSA-NCAM+ cells as PSA-NCAM is highly expressed in the immature brain.

When we compared the proliferation of cells in the SVZ after cryoinjury at P6, P10 and P21, we found that there was increased proliferation as measured by BrdU incorporation in P6 and P10 animals, with a much diminished response in P21 animals. Although the increase in proliferating cells was similar in P6 and P10 animals, the absolute number of proliferating cells was much greater in P6 animals than in P10 animals as a result of a higher mitotic index. The cells in the medial SVZ were stimulated in both P6 and P10 animals, which suggests that this injury increases the proliferation of neural stem cells and/or multipotential progenitors which are known to reside within this domain of the SVZ, and which we have previously shown are activated after hypoxic/ischemic injuries to newborn rats [26, 27]. Clearly, additional studies are necessary to test the validity of this hypothesis.

To determine whether the increase in SVZ cell proliferation leads to emigration of neuroblasts, we examined the number of DCX+ cells present after cryoinjury in P6 and P10 animals. We found that neuroblast production was more robust in the injured P6 than in P10 animals. These data are reminiscent of those generated in experiments performed by Bryan Kolb et al. [28, 29], who found that with aspiration lesions of the prefrontal cortex there was a dramatic regenerative response that was most robust in P10 animals. That we see a more robust response in slightly younger animals in the somatosensory cortex is consistent with the concept that there is a caudal-to-rostral gradient in neocortical maturation.

To establish when the peak of neuroblast production occurs after injury to the P6 rat somatosensory neocortex, we examined 4 different time points of recovery to determine when the most DCX+ neurons were born. We found that more DCX+ cells were generated early in the recovery process, on days 1 and 4, indicating a strong neurogenic response that was pronounced in regions close to the SVZ. As recovery continued through days 7 and 21, there was a decrease in the number of DCX+ cells observed. This observation was unexpected since in ischemic injury models both in adults as well as in neonatal animals, the injury stimulates a persistent neurogenic response [9, 30]. Studies have shown that a number of

growth factors and their receptors are induced after brain injury, which may regulate the production of new neurons. It is possible that the levels of these factors are differentially regulated in ischemic versus traumatic injury models, but at the present time one can only speculate as to which factors are critically important but not sustained after the cryoinjury.

Whereas DCX+ cells were common in the medial aspect of the neocortex, DCX+ cells were far less frequently observed on the lateral side of the injury, furthest from the SVZ. This observation raises the issue of mechanisms that are affecting SVZ cell migration. The developing brain is permissive of long-range cell migration; furthermore, a number of studies have shown that chemoattractant signals are produced subsequent to brain injury that promote neuroblast migration. For instance, there are studies supporting a role for the SDF-1α/CXCR4 complex in the migration of neuroblasts toward lesions [31]. Other studies support a role for MCP-1 [8, 30, 32]. Assuming that the reduced numbers of neuroblasts observed on the lateral aspect of the lesion is due to reduced migration, the lack of neuroblasts may be due to either a lack of attractant signals or the production of molecules in the reactive neuropil that are nonpermissive of neuroblast migration. It is also conceivable that these DCX+ cells arose from progenitors residing within the neocortex rather than from the SVZ; however, there are studies to directly support the hypothesis that these cells arise from dividing precursors in the SVZ, whereas there are no conclusive studies showing that, in vivo, neocortical precursors can generate neuroblasts [8, 9, 33].

In this study, we have shown that the proliferation of neural precursors in the SVZ increases after a TBI and, importantly, that the age at which the injury occurs significantly affects the magnitude of the repair response. Our data also demonstrate that the greatest number of new neurons is produced during the first few days of recovery. This observation suggests that factors that promote this neurogenic response either become less available as the lesion resolves, or that factors that inhibit neurogenesis are produced which quell the repair response. Having established that there is a window of time during which endogenous recovery is robust, it will be important to understand which factors are involved in this process and why the neurogenic response diminishes in this injury, whereas it does not after moderate ischemic injuries that occur at the same age.

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