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*Biol Res Nurs* 2005 6: 167
DOI: 10.1177/1099800404271328

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What is This?
Dentate Gyrus Neurogenesis after Cerebral Ischemia and Behavioral Training

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Neurogenesis in the mammalian brain continues throughout adulthood. Several factors have been shown to influence neurogenesis, including experience in a complex environment (EC), exercise (EX), and ischemic insult. The authors investigated the effects of behavioral rehabilitation training following transient global cerebral ischemia on the number of new cells in the dentate gyrus that incorporated bromodeoxyuridine (BrdU), a thymidine analog that labels cells undergoing DNA replication. Seventy-two animals were included in the study, and 4-vessel occlusion was used to induce cerebral ischemia while control animals were subjected to anesthesia and sham surgery alone. Within 3 days of surgery, rats were randomly assigned to either EC, EX, or control (paired housing in standard laboratory conditions) groups. All animals were sacrificed 2 weeks after behavioral training. Immunohistochemistry results showed an increased number of BrdU-labeled cells in the subgranular zone of the dentate gyrus in all ischemic groups and in the EC and EX sham groups, although no significant group differences were seen. Examination of cell phenotype showed that almost all BrdU-positive cells colabeled with TuJ1, an immature neuron marker, in all animals whereas only a few BrdU-positive cells colabeled with NeuN, a mature neuron marker. BrdU/NeuN-labeled cells were seen only in the sham and ischemia EC groups. No new cells showed glial fibrillary acidic protein, astrocyte marker, colabeling. These results suggest that the adult brain has an inherent regenerative capacity after insult and that behavioral training following injury does not have an additive effect on neurogenesis. Finally, the enhanced maturation of BrdU-positive cells seen in the EC rats is probably modulated by environmental cues.

Key words: neurogenesis, cerebral ischemia, exercise, complex environment, immunohistochemistry, stereology, neural stem cells

The adult central nervous system (CNS) was previously thought to be limited in its regenerative potential. However, recent identification of neural stem cells in discrete brain regions such as the subgranular zone of the dentate gyrus of the hippocampus (Altman and Das 1964; Eriksson and others 1998; Kornack and Rakic 1999; Dayer and others 2003) and the subventricular zone of the lateral ventricles (Doetsch and others 1999; Johansson and others 1999) raises the possibility that the adult mammalian brain has the potential to repair itself after injury. Factors shown...
to influence hippocampal neurogenesis are adrenal steroids and excitatory neurotransmitters. For example, adrenalectomy, glutamatergic deafferentation, and administration of N-methyl-D-aspartate (NMDA) receptor antagonists resulted in increased neurogenesis, whereas administration of adrenal steroids resulted in decreased neurogenesis (Cameron and others 1998; Gould and Tanapat 1999).

Environmental experience can also influence neurogenesis in the adult brain. Housing adult intact mice and rats in a complex environment (EC) not only produces a host of structural and functional changes in the brain but also results in a significant increase in hippocampal neurogenesis (Kempermann and others 1997; Kempermann and Gage 1999; Nilsson and others 1999). Moreover, voluntary physical activity using a running wheel enhances cell proliferation in the dentate gyrus of adult intact mice (van Praag, Christie, and others 1999; van Praag, Kempermann, and others 1999; Ra and others 2002). Even though the mechanism(s) through which production of new neurons is influenced by environmental experience and physical activity is poorly understood, these studies demonstrate the possibility that behavioral experience may be a potential therapy to repair neurons damaged by disease or injury.

Pathological insults such as mechanical lesions, artificially induced seizures, and cerebral ischemia have also been shown to increase neurogenesis. Mechanical injury to the dentate gyrus granule cell layer in the rat increased proliferation of granule cell neuron precursors in the adjacent subgranular zone (SGZ), evidenced by enhanced cell labeling of $[^{3}H]$thymidine and 5-bromo-2'-deoxyuridine-5' monophosphate (DNA precursors that incorporate only in dividing cells) and coexpression of mature neuronal markers in $[^{3}H]$thymidine-labeled cells (Gould and Tanapat 1997). Seizures also triggered neurogenesis in the SGZ, as demonstrated by the increased number of 5-bromo-2'-deoxyuridine-5' monophosphate (BrdU)-labeled cells expressing the neuronal markers MAP-2, TuJ1, and TOAD-64 (Parent and others 1997). In addition, global and focal cerebral ischemia in adult rats, mice, and gerbils stimulated neurogenesis, as shown by enhanced proliferation of BrdU-labeled cells coexpressing the neuronal markers MAP-2 and neuronal nuclear antigen (NeuN) in the SGZ (Liu and others 1998; Takagi and others 1999; Jiang and others 2001; Kee and others 2001; Yagita and others 2001; Nakatomi and others 2002). From these studies, it is evident that both physiologic and pathologic events have an influence on adult neurogenesis. What is not clear, however, is the effect of rehabilitation strategies such as EC housing and exercise (EX) after cerebral ischemia on adult neurogenesis. Both EC housing and EX have been well documented as strategies that can facilitate neuronal growth and restructuring following CNS damage (reviewed in Jones and others 1998; Jones and others 1999; Briones and others 2004); thus, it is possible that these behavioral paradigms may also have an influence on neurogenesis after cerebral ischemia. Since the role of EC housing and EX in adaptive plasticity after CNS damage is now beginning to be understood, it is important to examine whether these behavioral strategies can influence ischemia-induced cell proliferation, differentiation, and maturation. Recently, it has been reported that housing rats in a complex environment postischemia did not have an additive effect on neurogenesis but did increase astrocyte production (Komitova and others 2002). However, the literature addressing the issue of rehabilitation effects on ischemia-induced neurogenesis is still limited; thus, the present study was conducted to determine whether EX or EC can modulate ischemia-induced cell differentiation and maturation in the dentate gyrus. Our hypothesis was that using EX and EC as rehabilitation training strategies after ischemic injury would facilitate the differentiation and enhance the maturation of the newly produced neurons.

**Materials and Methods**

**Cerebral Ischemia**

Adult male Wistar rats 3 to 4 months of age (with mean body weight of 350–375 g) were used in the study. Transient global cerebral ischemia was induced by the 4-vessel occlusion method (Pulsinelli and Brierly 1979). On the 1st day, rats were anesthetized with an isoflurane/oxygen (2.5% isoflurane and 30% oxygen) mixture using a specially designed mask. Then, an incision was made to isolate both common carotid arteries. A silastic ligature was placed loosely around each artery without interrupting blood flow, and the incision was closed using absorbable sutures.
Immediately after this procedure, the vertebral arteries were electrocauterized in the alar foramina at the level of the 1st cervical vertebra. Inhalation anesthesia was maintained continuously throughout the surgical procedure, and body temperature was kept at 37 °C to 37.5 °C using a heating pad until the animals recovered from surgery. The next day, both common carotid arteries were occluded for 12 min while the animals were awake. This period of carotid occlusion was used because it confines resulting damage to the hippocampal area (Nunn and others 1994; Briones and Therrien 2000; Briones and others 2004). The criterion used to determine transient global cerebral ischemia was the bilateral loss of righting reflex. Animals that did not lose their righting reflex within 2 min of occlusion \((n = 3)\) or did not experience return of the righting reflex within 30 min of removal of the silastic ligatures \((n = 1)\) and those that developed complications at any time after surgery or during the recovery period were excluded from the study. Postoperative complications were excessive weight loss \((>20\%\) of preoperative body weight, \(n = 4)\), bleeding \((n = 3)\), and seizures \((n = 1)\) during the recovery period. Pain level was assessed by observing animals for sluggishness, extreme aversion to being touched, or weight loss. Animals were not given any postoperative analgesia but were euthanized immediately when persistent pain was observed \((n = 1)\). A total of 72 animals were included in the study. Sham-operated animals were subjected to the same anesthesia and surgery that consisted of a neck incision without carotid manipulation and an incision behind the occipital bone without vertebral artery electrocauterization. All efforts were made to minimize animal distress and to reduce the number of animals used. Experimental protocols were approved by the Institutional Animal Care and Use Committee and were in accordance with the National Institutes of Health guidelines.

**Animal Housing**

Within 3 days of recovery, rats were randomly assigned for 14 days to 1 of 3 environmental conditions: EC housing, EX, or social condition (SC; paired housing). All animals were housed in the same room under a 12-h light/12-h dark cycle and had free access to food and water. Room temperature was maintained at 22 ± 2 °C, and noise level was kept to a minimum. Animals in the EC group \((n = 14\) ischemia and \(n = 10\) shams) were housed in a sensory-rich living condition, with 8 to 10 animals in a wire cage measuring 2 m × 1 m × 1.65 m that contained a variety of objects such as toys, wooden blocks, running wheels, Plexiglas tunnels, ladders, plastic castles, a swing, and so on. In addition, EC rats were placed daily in an open field measuring 1.2 m × 1.2 m where they were allowed to explore a novel arrangement of objects for 30 min while their home cage was being changed. Objects in both EC housing and the open field were changed daily to maintain novelty.

Animals in the EX group \((n = 14\) ischemia and \(n = 10\) sham) were housed in pairs in standard laboratory cages and taken out daily and placed in the treadmill for 5 min. Five minutes was chosen as the cutoff time to prevent fatigue. On the 1st day, rats were placed in the treadmill at 0 speed and allowed to walk for the first 2 min, then the treadmill was turned on at a very low speed for the next 3 min. Treadmill speed was gradually increased daily until a maximum speed of 10 m/min was reached. Since this was a forced exercise paradigm, the experimenter held the animals at the base of the tail throughout the 5-min period and tapped the tail gently to get the animals moving continuously.

Animals assigned to the SC group were housed in pairs in standard laboratory cages \((16.5\) cm × 22.5 cm × 13.5 cm). Although rats in this group \((n = 14\) ischemia and \(n = 10\) shams) were able to observe the ongoing activity in the room, they did not receive any stimulation, and contact was limited to daily handling and routine cage changing. The sham SC rats also served as controls for the social interaction effects in animals housed in EC.

**BrdU Administration**

The thymidine analog BrdU (Chemicon, Temecula, CA) was used to label proliferating cells. BrdU is incorporated within 2 h after injection into the genetic materials on mitotic division, after which it can be detected immunohistochemically in the daughter cells (Kuhn and others 1996). BrdU was dissolved in 0.9% sterile NaCl and filtered at 22 µm. The resulting solution was injected at 50 mg/kg (concentration of 10 mg/mL) intraperitoneally in all rat groups. Injections were given 7 days after ischemic insult—a time that is documented to be within the peak of cell proliferation after...
ischemia (Liu and others 1998)—and given daily for 7 consecutive days to maximize labeling of cell proliferation (Fig. 1). This repeated-injections protocol allowed us to examine the number of cells that incorporated BrdU and to investigate the phenotype and survival pattern of these newborn cells, and it also allowed us to reduce the effects of dilution of the BrdU labeling. That is, using a single-injection protocol would show not just the originally labeled cells but also the progeny of labeled cells that would have divided once BrdU was no longer available, thus increasing the effects of dilution of BrdU labeling.

Tissue Preparation

On the day after rehabilitation training, all rats were anesthetized with pentobarbital (100 mg/kg) and perfused according to the guidelines of the Panel on Euthanasia of the Veterinary Medicine Association. Perfusion consisted of transcardial infusion with heparinized phosphate-buffered saline (pH 7.3) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3). The brains were then removed and placed in fixative for 24 hours at 4 °C. Postfixation, the brains were cryoprotected with 30% sucrose in 0.1 M phosphate-buffered saline containing 25% ethylene glycol and were stored at −20 °C until they were sectioned and processed for immunohistochemistry or immunofluorescence. Coronal sections were obtained throughout the entire hippocampal formation using a cryostat at 30 µm thickness.

Immunohistochemistry

For immunocytochemical detection of BrdU-labeled nuclei, DNA was denatured to expose the antigen before incubation in anti-BrdU primary antibody. Briefly, free-floating sections were pretreated in 50% formamide/50% 2× saline-sodium citrate buffer (SSC) at 65 °C for 2 h, rinsed in 2× SSC, and then incubated in 2 N HCl at 37 °C for 30 min. Tissues were then rinsed in borate buffer (pH 8.5) for 15 min and placed in 0.6% H2O2 in Tris-buffered saline (TBS) for 30 min to block endogenous peroxidase, followed by several rinses in TBS (pH 7.5). Tissues were then placed in TBS/0.1% Triton X-100/3% donkey serum (TBS-TS) for 1 h followed by incubation with anti-BrdU primary antibodies at a concentration of 1:400 (monoclonal mouse; Boehringer Mannheim; Indianapolis, IN) in TBS-TS overnight at 4 °C. The following day, the primary antibodies were detected using biotinylated immunoglobulin G (IgG) donkey antimouse secondary antibodies (Vector Laboratories; Burlingame, CA) at a concentration of 1:200 for 2 h. Tissues were then rinsed in TBS and incubated in avidin-biotin complex (ABC kit; Vector Laboratories) for 1 h at room temperature. Immunoreactions were visualized by treatment of section with hydrogen peroxide and 3,3′-diaminobenzidine tetrahydrochloride in Tris buffer.
(pH 7.3) enhanced with nickel. After thorough rinsing, the tissue sections were mounted on gelatin-coated slides and dried, and coverslips were applied. To minimize intergroup and interbrain staining variability and to ensure reproducibility of results, tissues from all experimental groups were run simultaneously and under identical conditions.

**Immunofluorescence**

To determine cell differentiation and maturation, double labeling was done using fluorescent-tagged secondary antibodies. For double labeling of BrdU and the different cell markers, sections were incubated with an anti-BrdU antibody and antibodies for each cell marker at 4 °C overnight after DNA denaturation. Immunostaining was performed as described above. The combination of antibodies used in each experiment was 1) rat anti-BrdU (1:400; Serotec) and mouse anti-NeuN (1:100; Chemicon), a cell marker for mature neurons, as primary antibodies, and Cy3-conjugated antirat IgG (1:200) and Cy5-conjugated antimouse IgG (1:400) as secondary antibodies; 2) mouse anti-BrdU (1:400; Boehringer Mannheim) and rabbit anti–glial fibrillary acidic protein (1:1000; Chemicon), a cell marker for astrocytes, as primary antibodies, and fluorescein isothiocyanate–conjugated antimouse IgG (1:200) and Texas red antirabbit IgG (1:200) as secondary antibodies; 3) rat anti-BrdU and mouse anti-β tubulin (TuJ1, 1:500; Covance), a cell marker for immature neurons, and Cy3-conjugated antirat IgG and Cy5-conjugated antimouse IgG as secondary antibodies. All secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA). Fluorescent signals were imaged with a confocal laser microscope equipped with a krypton/argon mixed-gas laser (Zeiss) using individual collections of wavelengths to minimize artifactual detection of nonspecific fluorescent emission. To assess the percentage of newborn cells that acquired the neuronal phenotype, 6 tissue sections (240 µm apart) from each rat were used for every antibody combination. The percentage of double-labeled cells (BrdU/NeuN–positive or BrdU/TuJ1–positive cells) was obtained for each rat, and the group means were calculated. For all immunohistochemical procedures, the free-floating-section method was used because previous studies have demonstrated that it produces good antibody penetration on relatively thick sections (30 µm) with high specificity and low background (Hawrylak and Greenough 1995; Jones and others 1996). In addition, optimal dilution of Triton X-100 in buffers and antibody diluent was determined to enhance antibody penetration.

**Assessment of Cell Loss**

Tissue sections adjacent to those used for immunohistochemistry were stained to investigate histological damage caused by cerebral ischemia. Sections were mounted on gelatin-coated slides and air dried, rehydrated in descending grades of alcohol, and then dipped in 1% methylene blue azure II for 7 to 10 min. Tissue sections were then differentiated in 70% acetic alcohol, dehydrated in ascending grades of alcohol, and cleared in Histoclear, and coverslips were applied using Permount.

**Quantification of BrdU Labeling**

The total number of BrdU-positive cells in the granule cell layer and its corresponding sample volume were determined in 8 coronal sections (covering the entire hippocampal region in its rostro-caudal extension), 240 µm apart, using the optical dissector method. Using the StereoInvestigator (MicroBrightfield; Colchester, VT) computerized analysis system, BrdU-labeled cells were counted using the optical dissector method. Briefly, each section was examined at a magnification of 40×, and an unbiased counting frame was positioned randomly across the dentate gyrus area. The 1st focal plane (i.e., the top of the tissue section where cells came into focus) was identified, and cells in this field of view were disregarded. A focal plane (approximately 3 µm apart) was then gradually passed through each section by adjusting the focus of the microscope slowly, and the labeled cells encountered while focusing through the section were counted. The number of labeled cells was related to the number of sections counted and was multiplied by the reference volume to provide an unbiased estimation of the total number of BrdU-positive cells. Reference volume in the dentate gyrus was obtained using the Cavalieri principle (Gundersen and others 1988), wherein the granule cells were counted at random systematic sam-
pling points superimposed onto the image projected on the computer. The reference volume was the product of the sum of the number of points that fell within the boundaries of the granular layer and the mean post-processing thickness of Nissl-stained sections. The section thickness of 30 µm (micrometer setting) was used because it was assumed that the net error by using the whole-section thickness for the volume was smaller than the error introduced by measuring the postprocessing section thickness on each slide and counting in a fixed fraction of it. For the present study, only the granule cell layer of the dentate gyrus was analyzed because very few BrdU-labeled cells were seen in the hilar region; thus, the hilar region was not analyzed in the present report.

Quantification of Neurodegeneration

A modification of the optical disector technique (as described above) was used to determine the percentage of degenerated cells in the Nissl-stained tissue sections. The total number of cells within the unbiased counting frame encountered while focusing on the sections was counted, as was the number of degenerated cells. Degenerated cells were identified as having smaller and darker nuclei, either swollen or shrunken cell bodies, and absence of cytoplasmic material. The percentage of degenerated cells was calculated as % degeneration = number of degenerated cells/total number of cells × 100.

Statistical Analysis

The SAS general linear model (SAS Institute; Cary, NC) procedures for 2-way analysis of variance (ANOVA) was used to examine effects of injury, rehabilitation training, and injury and rehabilitation training interaction for all quantitative measures. When appropriate, the SAS CONTRAST statement was used for planned comparisons of the effects of injury (ischemia groups versus sham groups), rehabilitation training (EC versus EX versus SC), and the combination of injury and rehabilitation training (ischemia and rehabilitation training versus sham and rehabilitation training) on cell proliferation and differentiation. Planned comparisons are considered to be more correct statistically than the post hoc t-test comparisons because they use all the data to estimate the error variance. All slides used for analysis were coded to preclude experimenter bias.

Results

Cell Loss after Ischemia

Analysis of Nissl-stained tissue sections revealed no cell loss in the sham-operated animals and dramatic neuronal loss in ischemic rats. Ischemia-induced neuronal loss largely involved the CA1 region (Fig. 2), and all quantifiable cell loss was found only in the dorsal area of the hippocampus. No cell loss was seen in the ventral area of the hippocampus or in the dentate gyrus. Qualitative examination of ischemic tissues demonstrated a significant disruption in the neuropil and tissue vacuolization. These results underscore the fact that the CA1 region is selectively vulnerable to the effects of ischemia. No significant difference was seen in the degree of cell loss between ischemic rats in the complex environment (31% ± 2.44%) and physical activity (33% ± 1.86%) groups and those assigned to the social condition (33% ± 1.46%) group, suggesting the reproducibility of the 4-vessel occlusion procedure.

Cell Proliferation

Proliferating cells were detected in the SGZ of the dentate gyrus in the hippocampal region in all groups of rats. A significant main effect of ischemia was that the number of BrdU-positive cells per hippocampal volume increased by approximately 40% in all rats subjected to cerebral ischemia (F(1/70) = 20.01, P < 0.005) compared to the sham SC group (Fig. 3). Interestingly, no differences were seen in the number of BrdU-labeled cells in ischemic rats assigned to EC and EX groups compared to those in the SC group, suggesting that the cell proliferation seen in these animals is most likely due to ischemic insult alone and not the rehabilitation training.

A significant main effect of environment (F(2/69) = 6.22, P < 0.05) was that sham animals assigned to EC and EX showed increased BrdU-labeled cells compared to the sham SC rats. Comparison of BrdU labeling in the sham animal groups showed that behavioral experience in a sensory-rich environment resulted in a
42% increase and physical activity in a 45% increase in BrdU labeling compared to the sham SC group. Furthermore, the presence of BrdU-positive cells in sham SC rats suggests that there is a basal level of proliferative activity present in the mature nervous system. It is unlikely that the BrdU immunoreactivity seen in these sham-operated SC rats was due to the stress of surgery since previous studies have demonstrated that sham operation does not result in increased cell proliferation (Kee and others 2001; Yagita and others 2001).

**Cell Differentiation and Maturation**

Phenotype of the BrdU-labeled cells was examined by immunofluorescent labeling with TuJ1 as a marker for immature neurons, NeuN as a marker for mature neurons, and glial fibrillary acidic protein (GFAP) as a marker for astrocytes. NeuN immunofluorescence stained predominantly cell nuclei whereas TuJ1 staining was confined to the cytoplasm (Fig. 4). Overall, a significantly higher proportion of BrdU-labeled cells expressed TuJ1 compared to NeuN ($F_{1,36} = 11.71$, $P < 0.005$), whereas no new cells expressed GFAP in the granule cell layer of the dentate gyrus. However, a small number of BrdU-positive cells were found to express GFAP in the hilar region of the dentate gyrus. BrdU-positive cells expressing TuJ1 were seen in all animal groups. These results suggest that ischemia and rehabilitation training enhanced not only cell proliferation but also differentiation into neuronal phenotype. Planned comparisons did not show significant differences between the ischemic groups and the sham EC and EX animals in the number of BrdU-positive cells that differentiated into neuronal phenotype. Given that

Figure 2. No cell loss was seen in sham-operated animals (A and B), whereas in ischemic animals (C and D), neuronal cell loss was confined to the CA1 region of the hippocampus proper (B and D). Ischemia did not produce any cell loss in the dentate gyrus. SO = stratum oriens; SP = stratum pyramidale; SR = stratum radiatum. Scale bar for A and C is 100 µm and for B and D is 40 µm.
the intrinsic signals activated in the injured brain may be different from those that are activated in the intact brain in association with the EC and EX paradigms, these results suggest that physiologic as well pathologic factors can independently enhance adult neurogenesis.

We next examined colabeling of BrdU and NeuN, a neuron-specific developmentally regulated nuclear protein associated with withdrawal from the cell cycle and terminal differentiation. A very limited number of BrdU-positive cells that express NeuN were seen (Table 1). Furthermore, the BrdU/NeuN–labeled cells were seen only in animals housed in EC, suggesting that although ischemia and exercise enhanced cell proliferation and differentiation into neurons, exercise did not lead to enhanced neuronal maturation. The BrdU-positive cells that colabeled NeuN were found only at the lower edge of the granule cell layer (GCL) of the dentate, indicating that this neuronal marker was probably acquired after cells migrated. Previous studies have shown that cell migration from the SGZ into the GCL correlates with neuronal maturation (Liu and others 1998; Kee and others 2001).

Discussion

In the present study, we demonstrated the selective vulnerability of the CA1 region to dramatic changes in blood flow and the relative resistance of the dentate gyrus to ischemic insult. These were evidenced by the confined pyramidal cell loss in CA1 and preservation of the integrity of dentate granule cells following 12 min of cerebral ischemia. These findings are in line with previous reports on the histopathological effects
Figure 4. Confocal images of double-labeled cells. (A) TuJ1/bromodeoxyuridine (BrdU) labeling, (B) TuJ1, and (C) BrdU; (D) neuronal nuclear antigen (NeuN)/BrdU labeling, (E) NeuN, and (F) BrdU. Small arrows show BrdU labeling surrounded by TuJ1-labeled cells (A) Large arrows show TuJ1 labeling, and open arrows show BrdU labeling. Asterisks show NeuN labeling, whereas arrowheads show double labeling of BrdU and NeuN. Scale bars are 60 µm for images on the left panel and 40 µm for images on the right panel.
of ischemic damage (Schmidt-Kastner and Freund 1991; Briones and Therrien 2000; Briones and others 2004). The reason for the selective sparing of the dentate gyrus in ischemic insult is still not fully understood, but it is possible that the enhanced cell proliferation seen in this brain region may be a contributing factor in regulating the mechanisms that promote its tolerance to ischemic injury.

Cell proliferation in the dentate gyrus has been shown to be dynamically regulated (Kempermann and others 2000). In this study, we addressed whether rehabilitation training contributes to enhanced cell proliferation following ischemic insult. Our results showed that the number of BrdU-positive cells does increase after ischemic damage, but the lack of significant differences between the ischemia EC, ischemia EX, and ischemia SC rats indicates that behavioral training after cerebral ischemia does not have an additive effect on cell proliferation. Our results on ischemia-induced cell proliferation are similar to those of others who reported an increased number of BrdU-labeled cells in the dentate gyrus between 1 to 2 weeks after BrdU labeling (Cameron and others 1998; Gould and Tanapat 1999). The combination of ischemia and rehabilitation training in the present study did not result in a further increase in cell proliferation in the dentate gyrus, suggesting that EC housing and EX did not have an additive effect on ischemia-induced cell proliferation. It may be that there is some maximal level of cell proliferation that occurs in the adult brain and that the activation of additional signals that trigger cell proliferation have no added effects. It is also possible that there are differences in responsiveness to rehabilitation training–induced cell proliferation in ischemia versus sham animals. This possibility is supported by studies that reported neurogenesis in the dentate gyrus but no effect on the generation of astrocytes in intact rats housed in the complex environment (Kempermann and others 1997; Nilsson and others 1999). On the

<table>
<thead>
<tr>
<th>Group</th>
<th>BrdU/TuJ1 %</th>
<th>BrdU/NeuN %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ischemia EC</td>
<td>74.6 ± 9.2**</td>
<td>1.8 ± 0.9*</td>
</tr>
<tr>
<td>Ischemia EX</td>
<td>71.4 ± 8.8**</td>
<td>None</td>
</tr>
<tr>
<td>Ischemia SC</td>
<td>73.3 ± 8.1**</td>
<td>None</td>
</tr>
<tr>
<td>Sham EC</td>
<td>78.9 ± 10.7**</td>
<td>2.5 ± 0.6*</td>
</tr>
<tr>
<td>Sham EX</td>
<td>74.2 ± 9.3**</td>
<td>None</td>
</tr>
<tr>
<td>Sham SC</td>
<td>59.3 ± 7.2</td>
<td>None</td>
</tr>
</tbody>
</table>

NOTE: BrdU = bromodeoxyuridine; NeuN = neuronal nuclear antigen; EX = exercise; EC = complex environment; SC = social condition.

*P < 0.05 significantly different from the EX and SC groups. **P < 0.05 significantly different from the sham SC group.
other hand, cortical ischemic injury increased the generation of astrocytes in the ipsilateral brain region in animals housed in EC postischemia, but no effect on neurogenesis was seen (Komitova and others 2002). The reason for this differing response to EC and EX (factors known to independently influence cell proliferation in the dentate gyrus) between the intact and ischemic brains is not clear, although it may be part of an important compensatory response since astrocytes are essential to neuronal function. Because ischemia results in neurogenesis, the ratio between neurons and astrocytes may be altered after ischemic injury, and it is possible that EC housing and exercise may play a role in correcting this disturbance.

Our results on enhanced neurogenesis in intact animals assigned to the EC and EX groups parallel those of others (Kempermann and others 1997, 1998; Kempermann and Gage 1999; Nilsson and others 1999), although in the present study, we tried to separate the effects of the different components of EC such as diet, increased activity, socialization, and novelty of surroundings. In our EC paradigm, we excluded the confounding effects of diet in that no caloric restrictions were imposed and animals did not receive any treats such as apples or cheese, which were given as part of the enrichment protocol in other studies (Kempermann and others 1997, 1998). Socialization was also controlled in our study wherein both the EX and SC groups were housed in pairs; however, it is possible that interaction with a large social group such as that seen in EC housing may have a different effect on neurogenesis. Furthermore, by including an exercise group in our study, we were able to separate the effects of increased activity on neurogenesis.

Increased cell proliferation cannot be considered beneficial after CNS insult without consideration of cell maturation. Our data indicate that most of the proliferating cells seen in all ischemic rats and in the sham EC and sham EX groups differentiated into neuronal phenotype, and we infer that these new neurons were generated from progenitor cells in the dentate gyrus based on their localization at the SGZ, the only region of the hippocampal formation known to produce neurons during adulthood. Almost all of the new neurons colabeled with TuJ1, a marker for immature neurons, and the new neurons that colabeled with NeuN, a marker for developed neurons, were seen only in the EC animal groups. Our results showing an increased number of new immature neurons in all ischemic animals and in the sham EX and sham EC groups are consistent with the results of others who reported increased TuJ1 at the early time points of cell proliferation and differentiation (1 to 4 weeks; Cameron and McKay 2001; Dayer and others 2003). However, contrary to previous findings by others (Komitova and others 2002), we demonstrated a small number of new mature neurons in the granule cell layer of the dentate gyrus in the sham EC and ischemia EC animals, as evidenced by BrdU/NeuN colabeling. This discrepancy in findings may be explained by the different model of ischemia used. The 4-vessel occlusion method that results in direct damage to the hippocampus was used in the present study, whereas a neocortical infarct model that results in loss of cortical connectivity to the hippocampus was used in the Komitova study. It may be that effects of behavioral training on ischemia-induced neurogenesis are specific to the brain region carrying out the task. That is, since the hippocampus is involved in memory processing, it may be influenced by rehabilitation therapy that includes some form of learning such as EC housing, whereas damage to brain regions, such as the cortex, that participate in motor activity may be influenced by physical activity. This line of reasoning is supported by studies that demonstrated behavioral tasks that activate and depend on hippocampal functioning, such as are found in EC housing, promote the survival of immature neurons in the granule cell layer and enhance its maturation (Gould and others 1999; Ambrogini and others 2000). Therefore, it is possible that tasks that activate the hippocampus but do not require hippocampal functioning (such as exercise) may influence cell proliferation but may have a minimal effect on cell differentiation and maturation.

In summary, our data showed that both ischemia and rehabilitation training independently induced cell proliferation and differentiation in the dentate gyrus, which demonstrates the brain’s endogenous repair mechanism. However, enhanced maturation of newly formed cells was seen only with environmental enrichment, raising the possibility that behavioral experience in a complex environment may be used as a rehabilitation strategy following ischemic insult.
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