Enriched Environment Attenuates Cell Genesis in Subventricular Zone After Focal Ischemia in Mice and Decreases Migration of Newborn Cells to the Striatum

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Background and Purpose—Cells proliferate continuously in the adult mammalian brain, and in rodents, cell genesis is affected by housing conditions and brain injury. Increase in neurogenesis after brain ischemia has been postulated to be linked to functional recovery after stroke. Housing rodents in an enriched environment improves motor function after stroke injury. We have investigated whether changes in cell genesis can explain the beneficial effects of an enriched environment.

Methods—Intact mice and mice subjected to transient occlusion of the middle cerebral artery were exposed to an enriched environment for 1 month. Bromodeoxyuridine was injected daily to label proliferating cells during the first postischemic week. Newborn cells were analyzed immunohistochemically after 4 weeks.

Results—The enriched environment increased neurogenesis in the dentate gyrus in both intact and stroke-injured animals. An increased number of newborn cells was found in the subventricular zone of stroke-injured mice, but not in injured mice exposed to an enriched environment. Also, the number of newborn astrocytes (BrdU+/S-100β+ cells), neuroblasts (dcx+ cells), and reactive astrocytes (vimentin mRNA) in the striatum ipsilateral to the ischemic injury was markedly attenuated and new adult neurons (BrdU+/NeuN+) were not found. The enriched environment did not affect infarct size or mortality.

Conclusions—An enriched environment after experimental stroke increased neurogenesis in the hippocampus, whereas there was a decreased cell genesis and migration of neuroblasts and newborn astrocytes in the striatum. (Stroke. 2006; 37:2824-2829.)

Key Words: animal models ■ basic science ■ brain ■ brain ischemia ■ brain recovery ■ cerebral infarct ■ exercise ■ experimental ■ hippocampus ■ inflammation ■ ischemia ■ neuroregeneration ■ stem cells ■ stroke

The adult mammalian brain retains neuronal stem cells, which can develop into neurons or glia.1 They are mainly found in 2 regions: the subgranular zone of the hippocampal dentate gyrus and in the subventricular zone (SVZ) close to the ventricles.2 Housing in an enriched environment and brain injury stimulate cell genesis in these regions. In intact rodents, exposure to an enriched environment promotes the proliferation of cells in the dentate gyrus3,4 and their differentiation into neurons.5 Furthermore, housing in an enriched environment enhances the survival of neuroblasts in the hippocampus of the intact rodent brain.5,6 The effect of an enriched environment on cell genesis after a cortical infarct has been studied in the rat.7 Neurogenesis in the SVZ was depressed in rats subjected to a cortical infarct and then housed in standard cages. In contrast, rats housed in an enriched environment, the level of neurogenesis was unchanged. These results differ from others showing that stroke injury results in an increased cell proliferation in the SVZ,8 migration of the newborn cells to the striatum, and differentiation of the newborn cells into striatal projection neurons.9-11 Likewise, in a recent study in the mouse, transient middle cerebral artery occlusion (tMCAO) was found to induce enhanced cell genesis in the SVZ.12 The aim of the present study was to study the effects of exposure to an enriched environment on the genesis, migration, differentiation, and survival of cells born in the SVZ in mice previously subjected to tMCAO.

Materials and Methods

Focal Cerebral Ischemia

Male C57BL/6 mice (Taconic M&B; Copenhagen, Denmark), 9 to 10 weeks old and weighing between 25 and 30 g at the start of the experiment, were housed under diurnal light conditions with free access to food and water. The ethical committee at Lund University approved the experiments.

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Middle cerebral artery occlusion\textsuperscript{13} in which the artery was occluded for 50 minutes by an intraluminal filament was applied. Occlusion and established reperfusion was confirmed by a laser-Doppler (Periflux System 5000; Perimed).

**Bromodeoxuridine Injections**

5-Bromo-2'-deoxyuridine (BrdU; Sigma-Aldrich) was dissolved in phosphate-buffered saline. Twenty-four hours after ischemia, mice were injected with BrdU (50 mg/kg) subcutaneously and subsequently once daily for 7 days before the daily period of enriched environment.

**Housing Conditions**

Mice were housed in standard cages together with 2 or 3 cage mates and were part of the same experimental group. Forty-eight hours after focal ischemia, enriched animals were transferred to an enriched environment\textsuperscript{13} consisting of a multilevel cage containing toys, ramps, and platforms for 3 hours a day. Animals in standard cages were handled once a day and treated in the same way as the enriched group.

**Behavioral Test**

Gross sensorimotor function was evaluated in a separate group of mice 2 weeks after tMCAO by the mice’s ability to transverse a rotating pole as described previously.\textsuperscript{13}

**Immunohistochemistry**

Mice were killed and their brains analyzed morphologically 4 weeks after tMCAO. Brains were fixed with 4% paraformaldehyde and sectioned coronally at 40-µm thickness on dry ice. The sections were double-stained for BrdU and various markers with standard immunohistochemistry procedures. Primary antibodies used were rat anti-BrdU (1:100; Oxford Biotechnology), mouse anti-NeuN (1:100; Chemicon; Malmö, Sweden), mouse anti-S-100β (1:1000; Sigma; Stockholm, Sweden), goat anti-doublecortin (1:200; Santa Cruz; SDS Bioscience; Falkenberg, Sweden). Secondary antibodies: Cy3 donkey anti-rat IgG, biotin horse anti-mouse (both 1:200; Molecular Probes; Invitrogen; Stockholm, Sweden) and biotin horse anti-goat (1:200; Vector; Invitrogen; Stockholm, Sweden). Finally, the sections were incubated with Alexa 488 (1:200; Molecular Probes; Invitrogen; Stockholm, Sweden).

**Measurement of Infarct Volume**

Sections separated by 50 µm were stained by immunohistochemistry for the neuronal-specific antigen NeuN (1:500) and visualized by peroxidase-DAB. Infarct area was evaluated using a light microscope and software (Olympus Bx51, Cast2) at x10 magnification. The border between infarct and viable brain tissue was outlined so that the infarct area contained no NeuN-positive neurons.

**Areas Chosen for Analysis of Newborn Cells**

The numbers of BrdU- and doublecortin-positive cells were counted in all mice (control standard n = 10, control-enriched n = 10, tMCAO standard n = 14, and tMCAO-enriched n = 13) in the SVZ in 2 brain sections, bregma +0.86 mm and +0.76 mm according to the mouse brain atlas.\textsuperscript{14} The number of cells immunostained for doublecortin (dcx), which labels neuroblasts, was counted in the entire striatum from corpus callosum to the anterior commissure. Analysis of double-labeled BruU and S-100β-positive cells was performed in the following groups: control standard n = 5, control-enriched n = 5, tMCAO standard n = 7, and tMCAO-enriched n = 8. The number of BrdU+/S100β+ cells were counted in one section (bregma +0.86) and within 200 µm from the SVZ so predominantly newborn cells, which had migrated from the SVZ were counted. The number of BrdU-labeled cells in the dentate gyrus was counted in all animals (3 enriched and one standard brain in the tMCAO group were excluded resulting from extensive hippocampal damage) in the ipsilateral side in 2 rostrocaudal sections at Bregma −1.7 and −1.8. Colabeled BrdU– and NeuN-positive cells in the hippocampus was calculated as a percentage of BrdU + cells with the help of a confocal microscope.

At least 15 BrdU+ cells were counted in 7 brains from each experimental group.

**Microscopical Analysis**

Microscopic analyses were made on a fluorescence microscope (Leica BX60) with a FITC/CY3 filter at x40× magnification by a blinded investigator. Confocal imaging was conducted on a confocal laser scanning microscope (Leica DM IRE3, Leica Confocal Software Version 2.77) at steps of 1-µm optical thickness.

**In Situ Hybridization of Vimentin mRNA**

After tMCAO, mice were placed in an enriched environment (n = 3) for 2 weeks or housed in standard cages (n = 3). The mice were decapitated and brains were removed and frozen at −80°C. In a cryostat, 14-µm thick coronal sections of striatum were cut and thaw-mounted onto SuperFrost plus glass (Menzel-Gläser). The vimentin oligonucleotide (Invitrogen) was used for in situ hybridization.\textsuperscript{15} The level of vimentin mRNA was assessed by measuring the optical density in the infarcted area (Image J 1.32j; National Institutes of Health).

**Statistics**

Analysis of variance with Bonferroni post hoc test/ correction was used to compare differences in cell identity between groups. Motor function was analyzed with Mann–Whitney U test. Unpaired Student t test was used to compare the infarct volume and vimentin mRNA levels between groups. The χ² test was used to assess differences in mortality between groups. Data were presented as mean±SD. P<0.05 was considered statistically significant.

**Results**

**Infarct Volume, Mortality, and Neurologic Function**

Analysis of infarct volume did not reveal any differences between enriched and standard housed animals (20.3±11.1 mm\textsuperscript{3} and 21.2±6.9 mm\textsuperscript{3}, respectively). Three animals were excluded as a result of absence of infarct (2 from the enriched and one from the standard group). There was no difference in mortality between enriched (one of 16) and standard housed animals (2 of 17) nor in body temperature 1 and 2 hours after reperfusion. In a separate group of mice, motor function tested on the rotating pole test was improved in mice exposed to an enriched environment for 2 weeks after tMCAO (4.7±0.9, n=10) compared with standard housed animals (3.7±0.5, n=6) (P<0.05 Mann–Whitney U test).

**Neurogenesis in the Dentate Gyrus**

The number of BrdU+ cells in the ipsilateral hippocampal dentate gyrus was increased in control and stroke-injured mice when housed in an enriched environment compared with mice kept in standard cages (Figure 1B). Double labeling of BrdU and NeuN (Figure 1A) showed an increased neurogenesis in both control and stroke-injured mice after exposure to an enriched environment (Figure 1C).

**Cell Genesis and Number of Neuroblasts in the Subventricular Zone**

In control animals, the housing conditions did not alter the number of BrdU+ cells in the SVZ. In stroke-injured mice, there was an increase in the number of BrdU+ cells in standard housed animals compared with either control standard housed or control enriched mice (81% and 55% more cells, respectively). The number of BrdU+ cells in mice...
exposed to tMCAO followed by daily housing in an enriched environment was similar to that of control animals (Figure 2). There was no difference between groups or controls in number of BrdU+ cells on the contralateral side. To assess the number of neuronal precursor cells, sections were stained with an antibody against dcx, a marker for migrating neuroblasts. In control mice, the number of neuroblasts in the SVZ was increased by 80% when housed in an enriched environment compared with standard environment ($P < 0.01$, t test, Bonferroni correction). In contrast, there was no difference in the number of neuroblasts in the SVZ of stroke-injured animals housed in standard or enriched conditions on the ipsilateral side (Figure 3) nor on the contralateral side. We did not find any cells double labeled for BrdU and NeuN in the SVZ or its vicinity regardless of whether the brains were intact or injured or whether the mice had been housed under standard or enriched conditions. This suggests that none of the newborn cells developed into mature neurons. Importantly, in the dentate gyrus of the same mice, we found numerous BrdU and NeuN double labeled neurons, indicating successful immunostaining procedures (Figure 1A).

**Neuroblasts and Newborn Astrocytes in the Striatum**

In the striatum, dcx+ neuroblasts were typically found close to the SVZ or just beneath corpus callosum. There was no difference in the number of dcx+ neuroblasts in the striatum...
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In situ Hybridization of Vimentin mRNA in the Striatum

Two weeks after tMCAO, there was a marked upregulation of mRNA for vimentin, an intermediate filament upregulated in reactive astrocytes ipsilateral to the lesion. Mice housed in an enriched environment exhibited significantly lower levels of vimentin mRNA in the infarcted striatum (240±65 nCi/g) compared with mice kept under standard housing conditions (418±84 nCi/g) (P<0.05, Student t test, Figure 6).

Discussion

The adult brain can regenerate neurons lost after brain ischemia.8 Increasing neurogenesis after stroke could be of importance for future rehabilitation strategies.16 Physical therapy is an effective clinical treatment of neurologic deficits after an established stroke. This can partially be mimicked in animal studies by housing the animals in an enriched environment, which effectively improves recovery of both motor and cognitive functions.13,17,18 Assuming that postischemic neurogenesis contributes to functional recovery after stroke, it is important to characterize the effect of enriched environment on cell genesis after experimental stroke. We demonstrate that the number of newborn cells in the striatum of mice exposed to an enriched environment for 4 weeks after experimental stroke is reduced and that the stroke-induced increase in number of BrdU-positive cells in the subventricular zone is absent. Moreover, intact mice exposed to an enriched environment displayed an increased number of neuroblasts in the SVZ compared with mice housed under standard conditions, whereas no difference was seen between stroke-injured mice housed in either of the 2 housing conditions. Importantly, the enriched environment paradigm that we used in our study enhanced hippocampal neurogenesis as previously reported.3,19 Thus, the enriched housing conditions increased neurogenesis in the dentate gyrus in both control and stroke-injured mice, showing that hippocampal neurogenesis indeed is enhanced in our experimental model. Increased hippocampal neurogenesis by enriched environment has been suggested to enhance cognitive function.20 Still, because an enriched environment improves sensorimotor function of stroke-injured mice, we focused our

between control mice housed under standard or enriched conditions. After 2 weeks of recovery after stroke injury, no differences in the number of dcx+ cells in the ipsilateral striatum were found between standard (84±33 dcx+ cells, n=5) and enriched housed mice (76±34 dcx+ cells, n=8). Interestingly, after 4 weeks of recovery after stroke injury, the number of dcx+ cells in the striatum of standard housed mice was 388±166 (n=14), whereas in the enriched housed animals, the number was 178±111 (n=13; P<0.001, ANOVA). Hence, stroke-injured mice housed in an enriched environment exhibited 54% fewer dcx+ neuroblasts in the striatum ipsilateral to the lesion than animals kept in a standard environment (Figure 4). On the contralateral side, there was no difference between housing conditions (data not shown).

The number of newborn astrocytes, labeled by antibodies against both BrdU and S100β, was quantified close to the ventricular wall in the striatum ipsilateral to the lesion (Figure 5A). In control mice, the housing condition did not affect the number of newborn astrocytes. In stroke-injured mice, however, the number of newborn astrocytes was reduced by 49% in the mice housed in an enriched environment compared with standard (Figure 5B). There was no difference between groups on the contralateral side.

Figure 4. Stroke-injured mice kept in an enriched environment (MCAO EE) had a 54% reduction in number of neuroblasts in the injured striatum compared with ischemic mice kept in standard condition (MCAO ST) 4 weeks after 50 minutes tMCAO. Control mice from both housing conditions (control ST and EE) had only a few dcx+ cells in the striatum. Mean±SD (**P<0.001, ANOVA, Bonferroni post hoc test, df=3, f-value=31.6).

Figure 5. Newborn astrocytes counted in a 200-μm wide area lateral to the SVZ to include migrating cells from the SVZ. A. Confocal imaging of newborn astrocytes (S-100β [green] and BrdU [red] double-labeled cells) in striatum. Scale bar is 20 μm. B. Stroke-injured mice kept in an enriched environment (MCAO EE) had a reduction in number of newborn astrocytes (S-100β+ cells and BrdU+ cells) in the injured striatum next to the SVZ compared with ischemic mice kept in standard condition (MCAO ST) 4 weeks after 50 minutes tMCAO. The housing condition did not affect the number of newborn astrocytes in control mice (control ST and EE), in which only few newborn astrocytes were found. Mean±SD (*P<0.05, ANOVA, Bonferroni post hoc test, df=3, f-value=13.3).
attention on cell genesis in the subventricular zone and the striatum.

The enriched environment had no significant effect on net cell genesis in the SVZ in intact animals as measured by BrdU incorporation in agreement with previous reports. On the other hand, we found an increase in the number of neuroblasts in the SVZ in intact mice exposed to an enriched environment, suggesting that mitosis is enhanced, cell death depressed, or the cellular fate changed toward neuronal differentiation by the environmental enrichment.

In agreement with a recent report, we found that experimental stroke enhances cell genesis in the SVZ. However, in contrast to the stimulating effect of an enriched environment on cell genesis in the dentate gyrus of our injured mice, no such effect was seen in the SVZ. Evidently, housing mice in a stimulating environment attenuates the activation of cell genesis in the SVZ. The influence of tissue damage per se on cell genesis in mice housed in the 2 different conditions should be the same, because there was no difference in brain infarct size between the groups.

Neuroblasts migrate from the SVZ as well as from the corpus callosum into the striatum and are located close to the ventricular wall or the white matter tracts of the corpus callosum. The mitigated cell genesis in the SVZ of stroke-injured mice housed in enriched conditions was further substantiated when we analyzed the specific phenotypes of the newborn cells in the striatum. Injured mice housed in an enriched environment have both fewer newborn astrocytes and immature neurons compared with mice housed in standard cages. Our results indicate that the improvement in motor function after exposure to enriched environment in mice subjected to tMCAO is not associated with an increase in the number of neuronal precursor cells in the striatum. On the contrary, there is a clear decrease in the number of both astrocytes and neuronal precursors. We have not studied or revealed the mechanisms behind our findings; however, one interpretation is that exposure to enriched environment after tMCAO enhances the cell death of newly generated cells in the striatum, at least within the first month of recovery. The mechanisms leading to a reduced number of neuroblasts in the striatum and a reduction of newborn astrocytes in the medial striatum after an enriched environment are currently unknown.

A decrease in astrocytogenesis is supported by the vimentin in situ hybridization data, which show a decreased level of mRNA in animals exposed to an enriched environment. This suggests that the enriched environment mitigates the reactive astrogliosis in the injured hemisphere. This could be beneficial because deletion of the vimentin and GFAP genes have a positive effect on functional recovery after central nervous system injury possibly by enhancing neurite outgrowth of surviving motor neurons. Some caution is warranted when drawing these conclusions because the sample size (n=3 in each group) for the vimentin data are relatively small.

In conclusion, experimental stroke in mice causes an increase in cell genesis in the subventricular zone, which is attenuated by housing stroke-injured animals in an enriched environment. This latter effect is associated with a decrease in migration/survival of the newborn astrocytes and neuroblasts into the adjacent striatum and with a lack of development of the newborn cells into mature neurons. Furthermore, an enriched environment increases neurogenesis in the dentate gyrus in both intact and stroke-injured mice, and the exposure to an enriched environment after experimental stroke results in an improved motor function.

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Disclosures
None.

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