

Enriched Environment Increases Neural Stem/Progenitor Cell Proliferation and Neurogenesis in the Subventricular Zone of Stroke-Lesioned Adult Rats

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Background and Purpose—The subventricular zone in the adult brain is identified as an endogenous resource of neuronal precursors that can be recruited to adjacent lesioned areas. The hypothesis was tested that postischemic environmental enrichment might enhance subventricular zone cell genesis.

Methods—A cortical infarct was induced in adult spontaneously hypertensive rats by ligating the middle cerebral artery distal to the striatal branches, after which animals were housed in either standard or enriched environment and allowed to survive for 5 weeks. The thymidine analogue bromodeoxyuridine was administered during the first postischemic week. The generation of neural stem/progenitor cells and neuronal precursors in the subventricular zone were studied with cell specific markers such as Ki67 and phosphorylated histone H3 (cell proliferation), Sox-2 (neural stem/progenitor cells), bromodeoxyuridine (slowly cycling, nonmigratory putative neural stem cells), and doublecortin (newborn immature neurons).

Results—Proliferating cells in the subventricular zone were identified as chiefly neural progenitors but also putative neural stem cells and neuronal precursors. Five weeks after stroke, proliferation in the subventricular zone was lower in stroke-lesioned rats housed in standard environment compared with nonlesioned rats. Postischemic environmental enrichment normalized cell proliferation levels, increased the numbers of putative neural stem cells as assessed with bromodeoxyuridine, and increased doublecortin-positive neuroblasts, which extended in migratory chains toward the infarct.

Conclusions—Enriched environment increased the neural stem/progenitor cell pool and neurogenesis in the adult subventricular zone 5 weeks after a cortical stroke. This might be of potential importance for tissue regeneration. (*Stroke*. 2005;36:1278-1282.)

Key Words: cerebral ischemia, focal ■ progenitor cells ■ recovery ■ rehabilitation ■ stem cells

Experimental stroke leads to increased generation of new neurons from dividing endogenous neural stem and progenitor cells in the 2 germinal niches in the adult mammalian brain: the hippocampal dentate gyrus and the fore-brain subventricular zone (SVZ) along the lateral wall of the lateral ventricles. Newly generated neuronal precursors are recruited from the SVZ to nearby infarcted areas and some express region-appropriate phenotypes.^{1,2} Identifying ways to enhance this endogenous regenerative capacity is therefore particularly pertinent. Environmental enrichment (social interactions, voluntary and varied physical activity, introduction of novel objects) exerts pronounced effects on the intact and lesioned brain, from the molecular to the structural level, ultimately influencing beneficially brain function and recovery after injury.³⁻⁶ However, data on the effects of postischemic environmental enrichment on lesion-induced cell genesis in the adult germinal zones are currently scarce. Previously it

was shown by us that postischemic environmental enrichment can affect cell differentiation in the hippocampus.⁷ The aim of the present study was to investigate the effects on the SVZ in the same material.

Materials and Methods

Animals, Housing Conditions, and Surgery

As described previously,⁷ 6-month-old spontaneously hypertensive male rats (Møllegaard Breeding Centre, Ejby, Denmark) were anesthetized with methohexital sodium (Brietal, 50 mg/kg, intraperitoneal injection) and the right middle cerebral artery was ligated distal to the origin of the striatal branches, resulting in a neocortical infarct. Lesioned and sham-operated rats were postoperatively housed in individual cages for 24 hours. The thymidine analogue 5-bromodeoxyuridine (BrdUrd; Boehringer Mannheim, Scandinavia AB; 50 mg/kg body weight, intraperitoneal), which incorporates into the DNA of dividing cells and their progeny, was administered as a daily injection starting 24 hours after surgery for 7 days. The experimental protocol was approved by the Ethics Committee for

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Animal Research at Lund University. The groups of animals were the following: standard stroke group (n=7) housed in standard cages (550×350×200 mm, 3 to 4 rats in each cage); early enriched group (n=9) introduced in enriched environment 24 hours after surgery; delayed enriched group (n=10) introduced in enriched environment 7 days after surgery, ie, after completed BrdUrd administration; and a sham-operated group (n=4) housed in standard environment. The enriched environmental conditions consisted of housing 9 to 10 animals per 815×610×1280 mm cage, equipped with horizontal and vertical boards, chains, swings, wooden blocks, and objects of different sizes and materials. The distance between the boards and the objects was changed twice per week.

Behavioral Tests

Sensorimotor function was tested with the limb placement and the rotating pole test⁸ preoperatively and 4 weeks postoperatively.

Tissue Preparation

Five weeks after surgery, the rats were deeply anesthetized with an overdose of Brietal and then perfused transcardially with saline solution, followed by 4% paraformaldehyde in 0.1 mol/L phosphate buffer. The brains were removed, postfixed in 4% paraformaldehyde in 0.1 mol/L phosphate buffer, transferred to 30% sucrose, and thereafter sectioned coronally (40 μ m).

Measurement of Total Tissue Volume Loss

Total tissue volume loss was measured on series of cresylviolet-stained sections and expressed as percentage of the contralateral hemisphere volume as previously described.⁷

Immunohistochemistry

Briefly, sections were incubated in blocking solution (3% donkey serum and 0.1% Triton X-100 in Tris-buffered saline, pH 7.5) for 30 minutes and then incubated with primary antibody in blocking solution for 16 hours at 4°C. Subsequently, sections were incubated for 2 hours at room temperature with fluorophore-conjugated secondary antibody or biotinylated antibody; the latter reacted with avidin-peroxidase for 1 hour (ABC-kit; Vectastain Elite, Vector Laboratories), followed by detection solution (0.25 mg/mL diaminobenzidine, 0.01% H₂O₂, 0.04% NiCl). Pretreatment for BrdUrd detection was as follows: 2 hours at 65°C in sodium citrate buffer containing 50% formamide, followed by incubation in 2 mol/L HCl for 30 minutes at 37°C. For immunoperoxidase staining, endogenous peroxidase was blocked with 0.6% H₂O₂ in TBS for 30 minutes.

Antibodies

Primary antibodies were: rabbit anti-Ki67 IgG (1:100; Novocastra Laboratories, Newcastle, UK), which labels cells in all active parts of the cell cycle; rabbit anti-phosphorylated histone H3 (1:400; Upstate, Charlottesville, VA), which labels the mitotic fraction of actively cycling cells; rat anti-BrdUrd IgG (1:300; Harlan, Loughborough, England); mouse anti-BrdUrd IgG (1:400; Boehringer Mannheim); goat anti-Sox-2 IgG (1:200; Santa Cruz Biotechnology, Santa Cruz, Calif), a marker for neural stem and progenitor cells; rabbit anti-NG2 (1:100; Chemicon, Temecula, Calif), a marker for oligodendrocyte precursors; goat anti-doublecortin IgG (1:400; Santa Cruz Biotechnology) a marker for newborn, immature, and migratory neurons. Mature neuronal markers were: mouse anti-NeuN IgG (1:50; Chemicon); mouse anti-GAD-67 IgG (1:500; Chemicon); mouse anti-MAP-2ab IgG (1:200; Sigma, St Louis, Mo); mouse anti-NF-200 IgG (1:400; Sigma); mouse anti-gial fibrillary acidic protein (GFAP) IgG (1:300; Chemicon), an astrocytic marker; and mouse anti-CD11b clone Ox-42 (1:100; Chemicon), a marker for macrophages and microglia.

Secondary antibodies were: fluorothioisocyanate-conjugated donkey anti-rat and anti-mouse IgG, Texas Red-conjugated anti-goat IgG, Cy5-conjugated donkey anti-goat and anti-rabbit IgG (1:150; Jackson ImmunoResearch, West Grove, Pa); and biotinylated rat-adsorbed horse anti-mouse and anti-rabbit IgG (1:125; Vector

Laboratories). No nonspecific labeling was observed when omitting incubation with primary antibody.

Quantification of Immunopositive Cells

Immunopositivity for Ki67, phosphorylated histone H3, doublecortin, and BrdUrd was determined in the SVZ, defined as a band of \approx 50 μ m along the entire length of the lateral wall of the lateral ventricle in 5 anatomically matched forebrain sections, 240 μ m apart each. Coronal forebrain sections between the crossing of the corpus callosum and the anterior commissure were analyzed.

The numbers of proliferating and doublecortin-positive cells in the SVZ were very high, which precluded counting under a microscope. Instead, immunoreactivity was semi-quantitatively estimated based on an image analysis approach. Confocal microscopy was used to scan the entire thickness of the stained section with step 2 μ m. The images were then superimposed using LCS Lite software. The resulting projection image was converted to grayscale in Adobe Photoshop. A similar threshold was set for all images and the area of specific immunoreactivity was measured using Image J National Institutes of Health software. Immunoreactivity was then expressed as the total area of specific immunoreactivity per total SVZ area. Area measurements of the SVZ were performed using the same software.

Cells positive for phosphorylated histone H3 and BrdUrd were counted in peroxidase stained sections under a Nikon Eclipse E 600 light microscope (Nikon). Area measurements of the length of the lateral ventricle wall were performed on micrograph images using digital image processing software (Nikon). Cell density was calculated by dividing the number of positive cells by the sample volume (SVZ area multiplied by 40- μ m sample thickness).

Determination of Cell Phenotype

Colocalization of cell-specific markers was determined with confocal laser scanning microscopy on multi-labeled tissue sections (Leica TCS SP2, Leica Microsystems, Heidelberg, Germany).

Slowly proliferating neural stem cells in the adult SVZ germinal zone give, via fast amplifying neural progenitor cells, rise to neuroblasts that continue to divide and migrate to the olfactory bulb to become interneurons.⁹ GFAP immunoreactive cells are considered to be neural stem cells in the neurogenic areas of the adult mammalian brain.⁹ Therefore, the combination of GFAP and the neural stem/progenitor cell marker Sox-2, which in the adult brain is essentially absent in immature neurons but is expressed by GFAP-negative neural progenitors and GFAP-positive cells,¹⁰ was used to distinguish between proliferating putative neural stem cells and neural progenitor cells. Approximately 50 Ki67-labeled cells were evaluated in the SVZ of each animal with respect to colabeling with Sox-2 and GFAP. Newborn immature and migrating neuronal precursors were identified with doublecortin.

Moreover, by administering BrdUrd repeatedly and allowing the animals to survive for an extended period of time, thus allowing the label to be diluted in fast amplifying neural progenitor cells and allowing newly generated neurons to migrate away from the SVZ, postischemically generated cells were identified that were likely slowly cycling and nonmigratory, characteristics of SVZ neural stem cells.¹¹ By placing rats in enriched environment at the initiation or after the completion of BrdUrd administration, ie, having an early enriched and a delayed enriched group with respect to BrdUrd administration, it could be distinguished between effects on cell proliferation during BrdUrd incorporation and possible subsequent effects on asymmetric cell divisions of putative neural stem cells and/or cell survival when the numbers of BrdUrd-positive cells between the 2 enriched groups were compared. The phenotype of strongly BrdUrd immunopositive cells was evaluated in 3 stroke early enriched animals with respect to Sox-2, GFAP, doublecortin, NG2, and CD11b. Approximately 100 BrdUrd-positive cells were phenotypically evaluated per animal and marker in the SVZ.

In the peri-infarct cortex, at least 200 BrdUrd-positive cells were examined with respect to mature neuronal (NeuN, MAP-2ab, NF-200, GAD-67) and astrocytic (GFAP) markers in at least 3 stroke

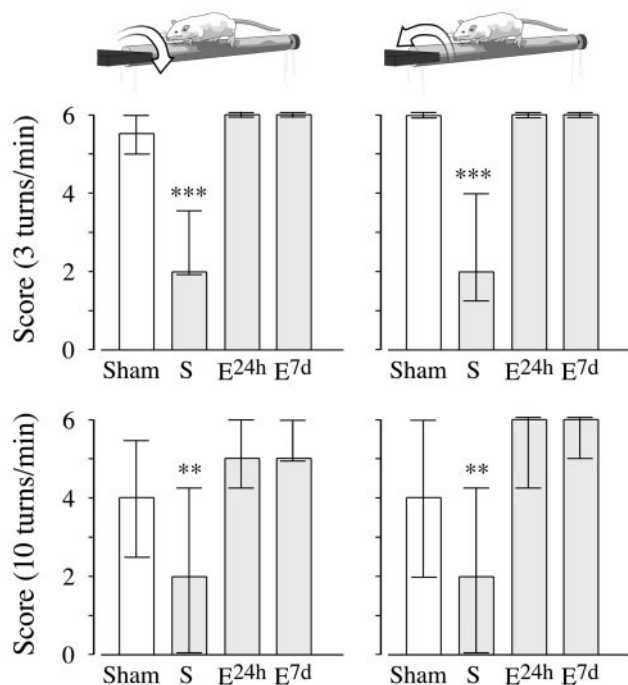


Figure 1. Performance on a horizontal pole, rotating 3 and 10 turns per minute, in rats postoperatively housed in standard cages (S), or transferred to enriched environment 24 hours (E24h) or 7 days (E7d) after a permanent middle cerebral artery occlusion distal to the striatal branches, resulting in a neocortical infarct. The pole rotated either to the left or to the right as indicated in the cartoon above the columns. Results are presented as median values and 25% upper and lower percentiles. The top score, 6, indicates that the rats managed the tests without any problems. *** $P < 0.001$ for difference between stroke standard and all other groups; ** $P < 0.01$ for difference to the enriched groups.

standard and stroke-enriched animals, respectively. Colabeling of BrdUrd with CD11b was also visualized.

Statistical Analysis

The presented values are mean values \pm SEM. Data were statistically processed with 1-way ANOVA, followed by Tukey/Kramer or Dunnett post-hoc test for comparisons between the lesioned groups or between lesioned groups and control, respectively (Statview 4.01 for Macintosh). $P < 0.05$ was considered statistically significant. For the behavioral tests, the Kruskal–Wallis nonparametric ANOVA with a multiple comparison post-hoc test at the 95% significance level was used.

Results

Behavioral Tests

In the limb placement test with maximum scores of 16, the median scores (upper and lower 25% percentiles in brackets) were 14.5 (16 and 10), 2 (4.25 and 1.25), 6 (8.25 and 3), and 6 (7 and 4) for sham, stroke standard, stroke early, and delayed enriched, respectively. Lesioned rats had significantly lower scores than sham-operated controls and the stroke early and delayed enriched groups performed significantly better than the stroke standard in the limb placement test. In the rotating pole test with full score of 6, the enriched groups performed significantly better than standard rats (Figure 1).

Measurement of Total Tissue Volume Loss

The total tissue volume loss did not differ significantly between the groups ($20.4 \pm 1.3\%$ in stroke standard, $16.1 \pm 1.2\%$ in stroke early enriched, and $17.1 \pm 1.8\%$ in stroke delayed enriched rats).

Cell Proliferation in the SVZ

At 5 weeks after stroke, Ki67-positive cells were below basal levels in the stroke standard group but did not differ from controls in stroke enriched rats, which actually had significantly more phosphorylated histone H3-positive cells in the contralateral SVZ compared with controls (Figure 2A to 2C). Stroke-enriched animals exhibited significantly higher numbers of Ki67 and phosphorylated histone H3-positive cells bilaterally compared with stroke standard animals.

Phenotype of Proliferating SVZ Cells

The majority of Ki67-positive cells at the 5-week time-point after surgery were identified as Sox-2-positive and GFAP-negative neural progenitors ($\approx 90\%$) (Figure 2D). There was also a small fraction of Ki67, Sox-2, and GFAP triple-labeled putative neural stem cells ($\approx 2\%$). A fraction of the Ki67-positive cells were not found to express Sox-2 or GFAP ($\approx 8\%$) and likely included proliferating neuroblasts, an example of which can be seen in Figure 2E as a phosphorylated histone H3 and doublecortin double-positive cell. The fractions of the different phenotypes of Ki67-positive cells did not differ significantly between the groups, suggesting that the effects on proliferating cells were reflected on the 3 main proliferating cell types in the SVZ.

Slowly Cycling Nonmigratory Putative Neural Stem Cells

At 5 weeks after surgery and thus 4 weeks after completion of BrdUrd administration, which was performed during the first week after surgery, the numbers of strongly BrdUrd-immunopositive cells, likely representing slowly cycling nonmigratory cells that are putative SVZ neural stem cells, were higher bilaterally in the early enriched stroke animals compared with the stroke standard group and ipsilaterally in the early stroke-enriched compared with controls (Figure 2F). In stroke animals transferred to enriched environment after completed BrdUrd administration (delayed enriched), the numbers of BrdUrd-positive cells in the SVZ were similarly increased as in the early enriched (data not shown), suggesting modulating effects on subsequent symmetric divisions of putative neural stem cells and/or cell survival rather than initial effects on proliferation of this cell population during tracer administration.

As we indicated, the phenotype of strongly BrdUrd immunopositive cells in the SVZ was evaluated in stroke early enriched animals. The fraction of BrdUrd-positive cells that co-labeled with GFAP was $8 \pm 3\%$. Most of these cells were also strongly Sox-2-positive (Figure 2G). A considerable fraction of the BrdUrd-positive cells were strongly Sox-2-positive but GFAP-negative ($29 \pm 8\%$) (Figure 2H). Some strongly BrdUrd-labeled cells were also found to be positive for NG2 ($5 \pm 1\%$) (Figure 2I), an oligodendrocyte precursor marker that has also been described in multipotent central

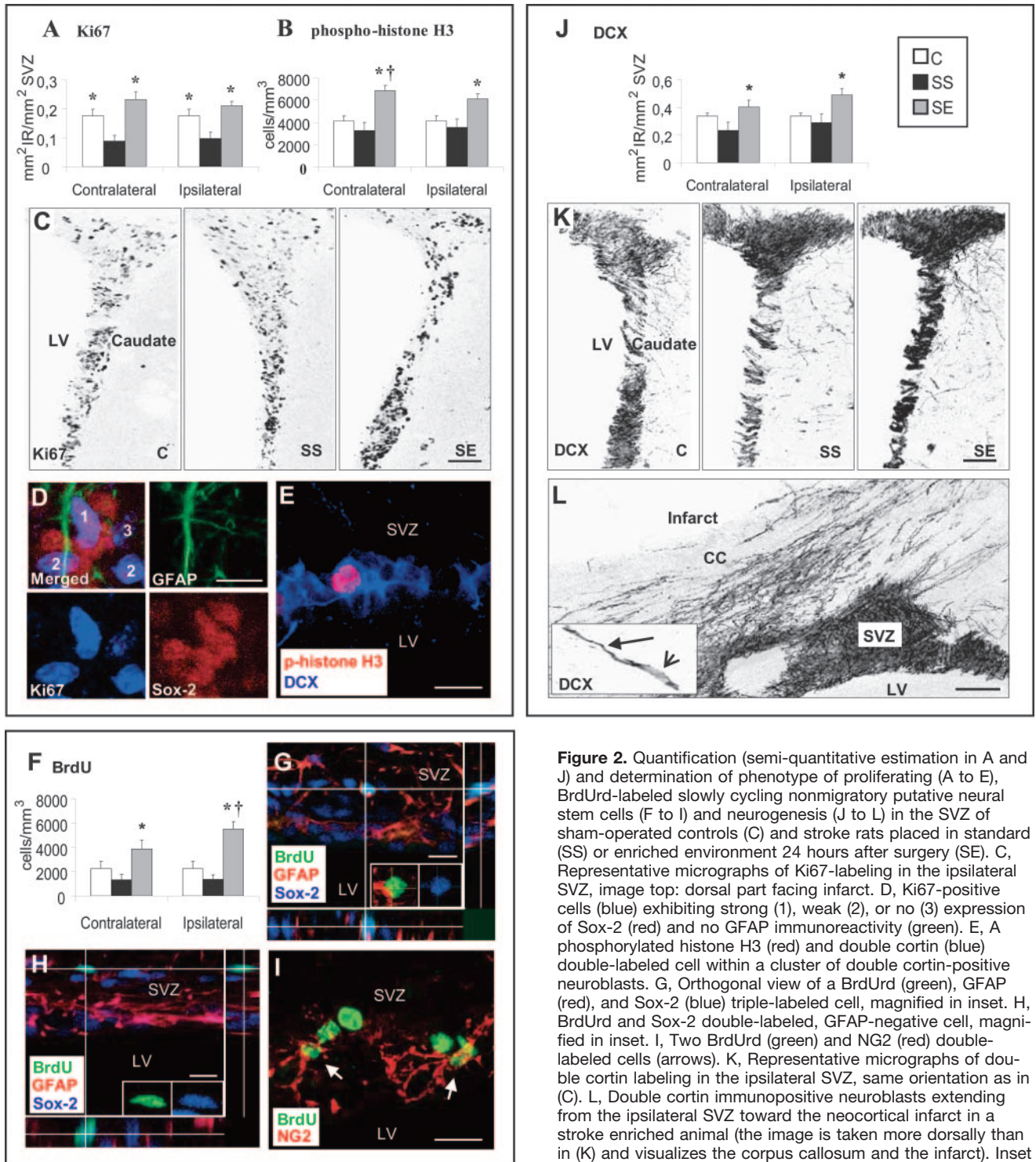


Figure 2. Quantification (semi-quantitative estimation in A and J) and determination of phenotype of proliferating (A to E), BrdUrd-labeled slowly cycling nonmigratory putative neural stem cells (F to I) and neurogenesis (J to L) in the SVZ of sham-operated controls (C) and stroke rats placed in standard (SS) or enriched environment 24 hours after surgery (SE). C, Representative micrographs of Ki67-labeling in the ipsilateral SVZ, image top: dorsal part facing infarct. D, Ki67-positive cells (blue) exhibiting strong (1), weak (2), or no (3) expression of Sox-2 (red) and no GFAP immunoreactivity (green). E, A phosphorylated histone H3 (red) and double cortin (blue) double-labeled cell within a cluster of double cortin-positive neuroblasts. G, Orthogonal view of a BrdUrd (green), GFAP (red), and Sox-2 (blue) triple-labeled cell, magnified in inset. H, BrdUrd and Sox-2 double-labeled, GFAP-negative cell, magnified in inset. I, Two BrdUrd (green) and NG2 (red) double-labeled cells (arrows). K, Representative micrographs of double cortin labeling in the ipsilateral SVZ, same orientation as in (C). L, Double cortin immunopositive neuroblasts extending from the ipsilateral SVZ toward the neocortical infarct in a stroke enriched animal (the image is taken more dorsally than in (K) and visualizes the corpus callosum and the infarct). Inset L, Magnification of a doublecortin-positive neuroblast with migratory morphology with elongated cell soma (small arrow) and a leading process (large arrow). Scale bars: C, K, L, 50 μ m. D to E, G to I, 10 μ m. $P < 0.05$. *Compared with stroke standard. †Compared with controls. BrdUrd indicates bromodeoxyuridine; CC, corpus callosum; DCX, double cortin; GFAP, glial fibrillary acidic protein; IR, immunoreactivity; LV, lateral ventricle.

and a leading process (large arrow). Scale bars: C, K, L, 50 μ m. D to E, G to I, 10 μ m. $P < 0.05$. *Compared with stroke standard. †Compared with controls. BrdUrd indicates bromodeoxyuridine; CC, corpus callosum; DCX, double cortin; GFAP, glial fibrillary acidic protein; IR, immunoreactivity; LV, lateral ventricle.

nervous system cells,¹² whereas 3 \pm 1% expressed double-cortin, which probably indicated continuous cell division and neurogenesis from BrdUrd-labeled putative neural stem cells. Only one BrdUrd-positive cell out of a total of 442 phenotyped cells was identified as a CD11b-positive and thus likely microglial cell. Subsequently, at least more than half of the

BrdUrd-labeled cells remaining in the SVZ 4 weeks after tracer administration were not found to express any of the aforementioned markers. Analysis of the BrdUrd-labeled putative neural stem cells with several relevant markers revealed phenotypical heterogeneity, a characteristic of neural stem cells that has been discussed.¹³

Neurogenesis in the SVZ

Doublecortin immunoreactivity was significantly higher bilaterally in the stroke-enriched animals compared with the stroke-lesioned animals housed in standard environment at 5 weeks after stroke (Figure 2 J and 2K). In lesioned animals doublecortin-positive cells often exhibiting typical migratory morphology with an elongated cell soma and a single leading process extended in chain-like structures from the ipsilateral SVZ toward the infarct (Figure 2L). Moreover, doublecortin-positive cells with migratory morphology could also be found in the contralateral corpus callosum, likely emanating from the contralateral SVZ and with a leading process directed toward the ipsilateral hemisphere.

Phenotype of BrdUrd-Labeled Cells Perilesionally

At 5 weeks after surgery and thus 4 weeks after completion of BrdUrd administration, which was performed during the first week after surgery, BrdUrd-positive cells were numerous perilesionally. No colocalization of BrdUrd with mature neuronal markers was observed. A small fraction of the BrdUrd-positive cells colabeled for GFAP with no significant differences between the lesioned groups (stroke standard: $4.7 \pm 0.6\%$; early enriched: $5.5 \pm 1.6\%$; delayed enriched: $10.8 \pm 2.2\%$). Numerous BrdUrd-positive cells colabeled for the macrophage/microglia marker CD11b.

Discussion

This study suggests that posts ischemic environmental enrichment: (1) normalized perturbed SVZ cell renewal; (2) increased the SVZ neural stem/progenitor cell pool; and (3) increased the generation of neuronal precursors that can be recruited to the stroke injury at 5 weeks after a cortical stroke. Analysis of the SVZ in intact spontaneously hypertensive rats from a previous unpublished study showed that the same enriched environment paradigm did not alter significantly cell proliferation, assessed as the number of phosphorylated histone H3-positive cells (data not shown).

Despite evidence of robust recruitment of immature neuroblasts to the infarct, posts ischemically born BrdUrd-labeled cells were not found to colocalize with mature neuronal markers in the peri-infarct region. Strongly limited survival of recruited newborn neuronal precursors has been identified previously after proximal middle cerebral artery occlusion, an experimental model with a combined cortical and striatal infarct, and only newborn cells recruited to the ischemic striatum but not those in the peri-infarct cortex expressed neuronal markers after several weeks.^{14,15} In our study, functional improvement in enriched rats was present despite an apparent lack of neuronal replacement and is likely caused by other types of plasticity and re-organization.⁵ It cannot, however, be ruled out that immature neuronal cells could nevertheless exert some effects on recovery, considering the fact that neural stem cells and neuronal precursors have been reported to synthesize and secrete trophic factors that might promote neural repair.^{16,17}

The exact mechanisms of lesion-induced activation of the SVZ are not established but it is known that various brain injuries affect the SVZ differentially and with a different time course as for instance after brain trauma, in which increased cell proliferation can persist in the SVZ for a year.^{2,18} A decreased supply of neuronal precursors to the lesion area during the chronic phase after a cortical stroke might be prevented with posts ischemic environmental enrich-

ment. Environmental enrichment in combination with exogenous administration of trophic factors is likely necessary to create beneficial conditions for regeneration after stroke.

In conclusion, this study shows that posts ischemic environmental enrichment has enhancing effects on SVZ cell genesis in the chronic phase after a cortical stroke. Future efforts need to be focused on identifying mechanisms to stimulate functional neuronal replacement.

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