Long-Term Stimulation of Neural Progenitor Cell Migration After Cortical Ischemia in Mice

Ahmed M. Osman, MVSc; Michelle J. Porritt, PhD; Michael Nilsson, MD, PhD; H. Georg Kuhn, PhD

Background and Purpose—Cortical ischemia induces neural progenitor cell migration toward the injury site; however, whether these cells are capable of maintaining the migratory response for a longer period after injury remains uncertain.

Methods—We analyzed progenitor migration up to 1 year after induction of photothrombotic stroke to the mouse neocortex. Migrating progenitors identified as doublecortin positive cells (DCX⁺) were assessed using the immunohistochemistry and immunofluorescence. The thymidine analogues chlorodeoxyuridine and iododeoxyuridine were used to birth-date the progenitor cells.

Results—In the striatum, we detected elevated numbers of DCX⁺ cells up to 6 weeks postlesion. In the corpus callosum and the peri-infarct cortex (Ctx), DCX⁺ cell numbers were increased up to 1 year. The orientation of the migrating progenitors was mostly aligned with the corpus callosum fiber tract at all time points; however, in the Ctx, they aligned parallel to the infarct border. The injured cortex continuously receives new progenitors up to 1 year after lesion. Cells born after lesion did not become mature neurons, although a portion of the migrating progenitors showed initial signs of differentiation into neurons.

Conclusions—Neural progenitors might have a role in brain plasticity after cortical stroke, especially considering the prolonged window of migratory responses of up to 1 year after stroke lesion. (Stroke. 2011;42:00-00.)

Key Words: cortical ischemia ■ doublecortin ■ long-term migration ■ neural progenitors ■ neurogenesis

Evidence is accumulating that brain ischemia influences the 2 adult neurogenic niches, the subventricular zone (SVZ) of the lateral ventricle¹ and the subgranular zone of the dentate gyrus² in animal models of the injury and in human patients.³,⁴ In particular, in the SVZ, as a response to the insult, neural stem/progenitor cells proliferate.⁵,⁶ A subset of cells change their migratory path from the natural target in the olfactory bulb⁷ to the striatum, the corpus callosum (CC), and the neocortex.⁸–¹² However, few progenitor cells survive and differentiate into mature functional neurons at the injury site.⁸,¹²,¹³ Previous experimental studies considered the migratory response to ischemic brain injuries as acute and transient, starting within days after induction of lesion, peaking shortly after, and declining within a few weeks.⁵,⁹,¹⁴ In contrast, it has been shown that the striatal migration persists up to 1 year after focal ischemia, when induced by 2 hours of middle cerebral artery occlusion.¹⁵ However, whether smaller and more remote cortical lesions can induce long-lasting effects on SVZ progenitor migration remains open.

In the current study, we assessed migration of the neural progenitor cells up to 1 year after induction of photothrombotic cortical stroke in mice.¹⁶ We analyzed the expression of doublecortin (DCX), a microtubule-associated protein expressed by the neuronal progenitors and early immature neurons¹⁷,¹⁸ in the striatum, the CC, and the Ctx at different time points starting 2 weeks after injury up to 1 year. We determined the orientation of the migrating progenitors in the CC and the Ctx. Furthermore, we birth-dated migrating progenitor cells using the thymidine analogues chlorodeoxyuridine (CldU) and iododeoxyuridine (IdU),¹⁹ and determined the fate of newly generated cells in the neocortex.

Materials and Methods

Animals
Forty-six female C57BL/6 mice (Charles River) were accommodated in equal light/dark cycle (12/12 hours) with free access to food and water. All experimental work was conducted in accordance with European and Swedish animal welfare regulations and approved by the Gothenburg committee of the Swedish Animal Welfare Agency (application no. 164/06 and 290/09).

Induction of the Cortical Ischemia
Animals were initially anesthetized with 5% isoflurane and maintained with 2.5% in a mixture of air and oxygen (1:1), then mounted on the stereotaxic apparatus. Body temperature was maintained with a heating pad. Ischemia was induced on the cortex (left hemisphere)
using the photothrombotic stroke model previously described.\textsuperscript{16} An incision was made over the skull, skin flaps were retracted, and then periosteum was removed to allow proper exposure of the skull. Rose Bengal dye (0.1 mL of 10 mg/mL) was injected intraperitoneally (IP) 5 minutes before laser illumination. The laser source was placed 3 cm above the skull (Cobolt Jive). The skull and underlying tissue were illuminated for 10 minutes with laser beam (power, 50 mW; wave length, 561 nm) at the coordinates relative to bregma: +2.7 mm lateral and +1 mm anterior. The skin was sutured, and animals were kept in a heated recovery box until awake, then returned to their home cage. Lesioned animals were randomly divided into 5 groups with the following termination time points after the injury: 2, 6, 12, 24, and 48 weeks with 8 animals in each group. Six animals were left unlesioned as a control group. Animals that did not display evolved lesions that included cortical layer 6 and those which had lesions involving the CC were excluded from the study.

**Administration of the Thymidine Analogues**

The thymidine analogues CldU and IdU (Sigma Aldrich) were used to birth-date doublecortin positive (DCX\textsuperscript{+}) progenitor cells. All lesioned animals received daily single IP injections of CldU (50 mg/mL) from postlesion day 7 to day 10. Animals euthanized at 6, 12, 24, and 48 weeks had daily, single IP injections of IdU (50 mg/mL) for 4 consecutive days, 2 weeks before perfusion (Supplemental Figure IA; http://stroke.ahajournals.org).

**Detection of the Migrating Progenitors**

Migrating progenitors and newly generated cells were detected by immunohistochemistry and immunofluorescence using the following antibodies: goat anti-DCX (C-18; Santa Cruz Biotechnology); rat anti-BrdU/CldU (clone BU1/75 ICR1; Accurate); mouse anti-BrdU/IdU (clone B4+; BD BioSciences); mouse anti-NeuN (clone A60, Chemicon); biotinylated donkey anti-goat IgG (JacksonImmunoResearch Laboratory); Alexa-488 donkey anti-rat IgG, Alexa-647 donkey anti mouse IgG, Alexa-546 donkey anti-goat IgG, Alexa-488 donkey anti-goat IgG, and Alexa-555 donkey anti-mouse IgG (Molecular Probes/invitrogen). ToPro3 (Molecular Probes/Invitrogen) was used as a nuclear counterstain (for detailed procedure, see Supplemental Methods).

**Histological Quantification**

DCX\textsuperscript{+} cells were analyzed in the ipsilateral and contralateral striatum, the CC, and the Ctx in 6 serial sections per animal spaced 150 \mu m apart and covering the center of the cortical lesion. Using stereology software (Stereo Investigator, MicroBrightField Inc), regions were defined as depicted in (Supplemental Figure IB). For more details, see Supplemental Methods. A confocal laser scanning microscope (TCS SP2; Leica) was used to analyze the percentage of the newly generated progenitors and neurons (Supplemental Methods).

**Determination of the Direction of the Migrating Progenitors**

We assessed the orientation of migrating progenitors in the CC and in the Ctx. The orientation of the leading process for at least 50 randomly chosen DCX\textsuperscript{+} cells per animal was categorized using an 8-directional compass rose (Supplemental Figure IC). Only DCX\textsuperscript{+} cells, which had the bipolar morphology, were analyzed.

**Statistical Analysis**

Two-way ANOVA with Bonferroni posthoc test was used to compare the difference in DCX\textsuperscript{+} cell numbers between the ipsilateral and the contralateral sides in ischemic animals at the studied time points. All other statistical analyses were performed using 1-way ANOVA with Bonferroni posthoc test. Data are expressed as mean±SEM. Significance was considered at probability values <0.05.

**Results**

**Cortical Ischemia Significantly Upregulated the Number of Migrating Progenitors Outside the Known Neurogenic Zones**

Using the photothrombotic stroke model, we induced cortical infarcts that were confined to the cerebral cortex and only touched the dorsal edge of the CC. We quantified the number of DCX\textsuperscript{+} cells in the ipsilateral and contralateral striatum, CC, and Ctx (Supplemental Figure IB) of the lesioned animals at 2 weeks postinjury (n=6) and in age-matched, unlesioned control animals (n=6). After injury, we observed a highly significant upregulation in the number of DCX\textsuperscript{+} cells in all analyzed regions in the ipsilateral hemisphere compared with the contralateral side and with the control group (Figure 1C and Table). No significant difference in the number of DCX\textsuperscript{+} cells was detected when comparing the contralateral side of the lesioned animals with the control group.

**Neural Progenitor Cells Respond to the Cortical Ischemia With Sustained Migration Toward the Injury Site for at Least One Year**

To determine whether the neural progenitor pool of the SVZ is capable of maintaining the migratory response to the cortical lesion over a long period, we counted DCX\textsuperscript{+} cells in the striatum, the CC, and the Ctx (Supplemental Figure IB) in the ipsilateral and contralateral hemispheres up to 1 year after induction of cortical ischemia. In the ipsilateral striatum, we observed significantly increased numbers of DCX\textsuperscript{+} cells up to 6 weeks postlesion (Figure 1). In the ipsilateral CC and cortex, we observed a significant elevation in DCX\textsuperscript{+} cell numbers at all time points up to 1 year (Figure 1).

**Neural Progenitors Migrate in Alignment With Fibers in the Corpus Callosum; However, in the Cortex, They Align With the Infarct Border**

The direction of the leading processes of DCX\textsuperscript{+} cells was analyzed in 2 areas, in the ipsilateral CC and the Ctx, by classifying their orientation in 8 different directions (Supplemental Figure IC). At all time points, significantly more cells in the CC were aligned with the direction of the fiber tract than in any other direction. In the cortex, cells were mostly aligned parallel to the infarct wall at all time points (Figure 2).

**The Injured Cortex Receives a Continuous Supply of New Cells for at Least One Year After the Ischemic Insult**

We had a particular interest in investigating whether DCX\textsuperscript{+} cells in the injured cortex were actually newly generated cells. We injected 2 thymidine analogues, CldU and IdU, at different time points into the same animals to label cohort of cells with different birth-dates. All lesioned animals received daily IP injections of CldU starting on day 7 to day 10 postinjury. Animals euthanized at 6, 12, 24, and 48 weeks also received daily IP injection of IdU for 4 consecutive days beginning 2 weeks before perfusion (Supplemental Figure IA). We determined the percentage of DCX\textsuperscript{+} cells that colabeled with each analog (DCX\textsuperscript{+}/CldU\textsuperscript{+}, early born cells;
DCX⁺/IdU⁺ (late born cells) in the ipsilateral CC and the Ctx. Two weeks after lesion, we found 25.2% of the DCX⁺ cells in the CC were CldU positive (CldU⁺), whereas the proportion declined to 11.5% in the cortex. At 6 weeks after lesion, only 0.5% and 1.9% of DCX⁺ cells were CldU⁺ in the CC and in the cortex, respectively. None of the CldU positive (early-born) cells colabeled with DCX at 12, 24, and 48 weeks postinjury (Figure 3A).

IdU⁺ (late-born) cells that colabeled with DCX were consistently detected at all time points. In the CC, the ratio varied between 0.9% and 0.2%, and in the cortex between 1.8% and 1.3%, respectively. Significant differences in DCX⁺/IdU⁺ ratios were not observed among the studied time points, neither in the CC nor in the cortex (Figure 3B).

Cells Born During the First Ten Days After the Cortical Ischemia Do Not Give Rise to Mature Neurons in the Peri-Infarct Cortex

Last, we examined whether cells born early after lesion (CldU⁺; Supplemental Figure IA) or the migrating progenitors (DCX⁺) are capable of differentiating into mature neurons (NeuN⁺). We analyzed the ratio of CldU⁺/NeuN⁺

### Table. Number of DCX⁺ Cells 2 Weeks Post Injury

<table>
<thead>
<tr>
<th>Area</th>
<th>Control Mean±SEM</th>
<th>Control P (vs. Ipsilateral)</th>
<th>Contralateral Mean±SEM</th>
<th>Contralateral P (vs. Ipsilateral)</th>
<th>Ipsilateral Mean±SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Striatum 1</td>
<td>63.17±10.49</td>
<td>&lt;0.0001</td>
<td>69.33±7.969</td>
<td>&lt;0.0001</td>
<td>231.5±25.11</td>
</tr>
<tr>
<td>Striatum 2</td>
<td>3.167±1.01</td>
<td>&lt;0.0001</td>
<td>2.333±0.33</td>
<td>&lt;0.0001</td>
<td>48.67±7.990</td>
</tr>
<tr>
<td>Corpus callosum</td>
<td>76.17±12.94</td>
<td>&lt;0.0001</td>
<td>104.7±11.50</td>
<td>&lt;0.0001</td>
<td>387.8±42.37</td>
</tr>
<tr>
<td>Cortex</td>
<td>0.33±0.33</td>
<td>&lt;0.0001</td>
<td>3.500±1.310</td>
<td>&lt;0.0001</td>
<td>315.8±44.11</td>
</tr>
</tbody>
</table>

The number of DCX⁺ cells at 2 weeks post injury in the ipsilateral Striatum 1 (adjacent to the SVZ) and Striatum 2 (lateral to Striatum 1), the corpus callosum (CC), and the peri-infarct cortex (Ctx) compared with the corresponding areas in the contralateral hemisphere and to the control group.

See Supplemental Figure IB for more details on anatomic locations of the selected areas.

Data are presented as mean±SEM and were analyzed using one-way ANOVA with Bonferroni post hoc test.

All comparisons between control and contralateral cell counts revealed no significant differences.
and DCX⁺/NeuN⁺, respectively, in the Ctx at 6, 12, 24, and 48 weeks after lesion. We did not detect any CldU⁺ cells expressing NeuN at any time point (Figure 3C); however, the proportion of DCX⁺ cells coexpressing NeuN⁺ was 0.8%, 2.1%, 1.1%, and 0.7% at 6, 12, 24, and 48 weeks, respectively. No significance difference in the proportion of DCX⁺ cells expressing NeuN was detected between the studied time points (Figure 3D).

Discussion

Because the existence of adult neurogenesis has been demonstrated,20 questions arose as to whether these cells have any role in regeneration or replacement of neurons after brain insults.21 In this context, stroke has received much attention, and a large number of studies have been conducted regarding the regenerative capacity of endogenous neural stem cells and progenitors.22 All studies are in agreement that the ischemic environment influences the neural stem/progenitor pools in both adult neurogenic niches, the SVZ of the lateral wall of the lateral ventricle, and the subgranular zone of dentate gyrus of the hippocampus.1,2

In the SVZ, brain ischemia increases proliferation of the neural stem/progenitor cells.5,6 A subset of progenitor cells is redirected toward the injury site8–12 rather than it migrating to distant areas. Figure 2. Orientation of the leading processes of DCX⁺ cells. Using an 8-directional compass rose, DCX⁺ cells were assessed for orientation of the leading processes in the ipsilateral CC A and the peri-infarct cortex B commencing 2 weeks and up to 48 weeks postlesion. For more information on selection of areas, see Supplemental Figure IC. The migrating cells in the CC were mostly oriented parallel to the fiber direction at all time points, whereas in Ctx, cells were mostly aligned parallel to the infarct border. Data presented as mean±SEM, analyzed using 1-way ANOVA with Bonferroni posthoc test. CC indicates corpus callosum; Ctx, neocortex; Stars (*) mark the orientations with significant differences (P<0.05).
via the rostral migratory stream to their normal destination, the olfactory bulb. The migratory response of the neural progenitors has been confirmed using different experimental animal models of brain ischemia. Thus far, studies are in agreement that brain ischemia leads to a transient migratory response of the neural progenitors toward the injury, which peaks within a few weeks after the insult. However, it was demonstrated that neuroblasts are capable of migrating toward a striatal injury site without decline for 4 months, and with sustained migration capacity up to 1 year after middle cerebral artery occlusion. To our knowledge, it is still unknown whether the cerebral cortex receives an influx of SVZ progenitors over a prolonged period of time after ischemia. Our results show a highly upregulated number of DCX-expressing cells in all examined regions in the ipsilateral hemisphere, which is in accordance with previous studies. Surprisingly, we observed a significant elevation in number of migrating progenitors (DCX<sup>+</sup> cells) in the ipsilateral CC and the peri-infarct cortex up to 1 year (48 weeks) postinjury. For the striatum, an influx of progenitor cells was observed only up to 6 weeks after lesion, indicating that the long-term migration is a directed migratory response triggered by the cortical infarct. Several molecular mechanisms have been demonstrated to redirect the SVZ progenitors toward the injury site, including cell death and the inflammation, which seem to be early triggers for migration after injury (for review, see Young et al<sup>23</sup>). However, additional studies are needed to demonstrate whether these early molecular cues are still effective to attract the SVZ progenitors at later time points after injury (1 year), or whether other signals are involved during this late phase.

Under physiological conditions, glial tubes guide neuroblasts during migration in the rostral migratory stream toward the olfactory bulb. In addition, neuroblasts can have intimate contact with blood vessels and use them as scaffolding under normal or injury conditions. The CC is the largest fiber tract in the forebrain and primarily comprises myelinated axonal bundles. It has previously been demonstrated as migratory path for stem cells implanted contralateral to an ischemic injury. Our data indicate that cells from the SVZ

![Figure 3](http://stroke.ahajournals.org/)

Figure 3. Generation of new cells. A, Double-labeling of doublecortin (DCX<sup>+</sup>) and chlorodeoxyuridine (CldU<sup>+</sup>). The confocal image shows DCX<sup>+</sup> (red) and CldU<sup>+</sup> (green) in the peri-infarct cortex at 2 weeks postinjury. Bar graphs show the percentage of DCX<sup>+</sup>/CldU<sup>+</sup> among DCX<sup>+</sup> cells in the ipsilateral corpus callosum (CC) and the peri-infarct cortex (Ctx), respectively, at all studied time points. B, Double-labeling of DCX<sup>+</sup> and iododeoxyuridine (IdU<sup>+</sup>). The confocal image shows DCX<sup>+</sup> (red) and IdU<sup>+</sup> (blue) in the peri-infarct cortex at 24 weeks postinjury. Bar graphs show the percentage of DCX<sup>+</sup>/IdU<sup>+</sup> among DCX<sup>+</sup> cells in the ipsilateral CC and Ctx, respectively. C, Confocal image of CldU<sup>+</sup> (green) and neuronal specific nuclear marker NeuN (NeuN<sup>+</sup>; red) in the peri-infarct cortex (Ctx) at 24 weeks postinjury. No CldU/NeuN double-labeling was seen at studied time points (6, 12, 24, and 48 weeks). D, Double-labeling of DCX<sup>+</sup> and NeuN<sup>+</sup>. The confocal image shows coexpression of DCX<sup>+</sup> (green) and NeuN<sup>+</sup> (red) (DCX<sup>+</sup>/NeuN<sup>+</sup>) in Ctx at 24 weeks postinjury. ToPro (blue) used as nuclear stain. The bar graph shows the percentage of DCX<sup>+</sup>/NeuN<sup>+</sup> of hundred counted DCX<sup>+</sup> cells in Ctx at 6, 12, 24, and 48 weeks postinjury. Data presented as mean ± SEM, analyzed using 1-way ANOVA with Bonferroni post hoc test. ***P<0.0001; *P<0.01; ns indicates not significant. Scale bar=30 μm (A, B), 20 μm (C), and 10 μm (D).
migrate within the CC toward the cortical lesion site. Using the orientation of the leading process to establish the migratory direction, we determined that the majority of migrating cells aligned with the fiber direction of the white matter tract, suggesting a guidance function of the fiber bundles. Once progenitors reach the Ctx, they reorient. Initially, a portion of the cells point toward the infarct center; however, within 6 weeks after lesion, the majority of cells align in the peri-infarct region along the infarct border, indicating that cells are not inclined to enter the infarct region. Whether this alignment is caused by specific support structures, such as neuronal or glia boundaries or blood vessels, is not known and requires additional investigation.

Controversy developed from a recent study reporting that SVZ neural progenitor cells do not migrate toward the cortical lesion, but are rather locally generated from cortical stem/progenitor cells. In addition, ischemia appears to induce neurogenesis of neocortical layer 1 progenitor cells. However, a contrasting report, using transgenic mice in which neuroblasts originating in the SVZ were enhanced green fluorescent protein-labeled, showed that neural progenitors migrate from the SVZ toward the cortical infarct. In our experiment, no labeling tool to identify the origin of DCX-positive cells was used; however, we detected a migratory tract of progenitors, which reached from the SVZ through the CC to the peri-infarct cortex at all time points. This supports the view that neural progenitor cells in cortical stroke areas are, at least, partially SVZ-derived, although we cannot exclude local generation of progenitors in the neocortex.

DCX is widely used as a label for neuronally committed progenitor cells. During adult neurogenesis, DCX is expressed for a transient period of approximately 2 to 3 weeks. However, the detection of DCX cells in the neocortex at up to a year after stroke raises the question whether, under lesion conditions, DCX expression could be prolonged and thus DCX cells detected at late time points had still been generated early after stroke. Using a combined CldU/IdU-labeling paradigm, we found that DCX-positive cells that incorporated CldU during the first 10 days after lesion were detectable in the cortex only up to 6 weeks after lesion. DCX+/IdU- cells, which were generated within the last 2 weeks before sacrifice, were detectable at all time points up to 1 year in the peri-infarct cortex after stroke. We therefore conclude that the lesioned cortex receives continuous supplement of new cells for at least 1 year after the ischemic insult.

Experimental studies have reported varying degrees in the generation of new cortical neurons after brain lesion, and in several studies, cortical neurogenesis was not detected. This may in part be caused by methodological differences in thymidine labeling and microscopy. Moreover, lesion-induced cell responses, such as DNA-repair or aberrant cell cycle entry of injured cells, could lead to thymidine incorporation into cells without cell division. Whether studies have falsely interpreted thymidine-analog signals as cortical neurogenesis remains a controversy.

In the current study, we were not able to detect new mature neurons in the cortex using confocal microscopy with optical slice thickness of 1 μm or less, sequential fluorochrome excitation, and exclusion of autofluorescing cellular elements. Cells labeled within the first 10 days postlesion were not found to colabel with NeuN at later time points. However, this period reflects also the peak of inflammation and could have impaired the differentiation and survival of neuronal progenitor cells. We detected a portion of the DCX+ cells that express NeuN, a sign of beginning neuronal differentiation; however, we have no indication that these cells survive and fully differentiate into functional neurons. DCX+ cells may thus lack differentiation signals and undergo cell death, as previously suggested.

Both survival and proper differentiation seem to be limitations in cortical brain areas, whether transplanted or endogenous stem cells are used. Trophic factors have been successfully used to promote enhanced neural progenitor migration in the ischemic brain and to counteract apoptosis. The demonstrated persistent migratory response up to 1 year after ischemia significantly expands the time window for possible interventions that could target progenitor cell survival and differentiation over longer periods. Prolonged treatment after stroke may thus enhance the potential for cortical neurogenesis and reorganization and lead to more efficient rehabilitation strategies.

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Disclosures

None.

References


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SUPPLEMENTAL MATERIAL

Methods

Induction of the cortical ischemia

Animals were initially anesthetized with 5% isoflurane and maintained with 2.5% in a mixture of air and oxygen (1:1), then mounted on the stereotaxic apparatus, and body temperature was maintained with a heating pad. Ischemia was induced on the cortex (left hemisphere) using the photothrombotic stroke model previously described \(^1\). An incision was made over the skull, the skin flaps were retracted, and the periosteum was removed to allow proper exposure of the skull. Rose Bengal dye (0.1 mL of 10 mg/mL) was injected intraperitoneally (IP) 5 min prior to laser illumination. The laser source was placed 3 cm above the skull (Cobolt Jive\textsuperscript{TM}, Solna, Sweden). The skull and underlying tissue were illuminated for 10 min with laser beam (Power: 50 mW; Wave length: 561nm) at the coordinates relative to bregma: +2.7 mm lateral and +1mm anterior. The skin was sutured, and animals were kept in a heated recovery box until awake, then returned to their home cage.

Tissue preparation

Animals were deeply anesthetized with an IP injection of sodium thiopental and transcardially perfused with 0.9% NaCl, fixed with 4% Paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4). Brains were post-fixed overnight in the same fixative, then transferred to 30% sucrose in 0.1 mol/L phosphate buffer for the cryoprotection and left for a minimum of 3 days. Brains were cyrosectioned coronally using a sliding microtome into 25 \(\mu\)m thick free floating sections and stored as 1:12 series at 4\(^{\circ}\)C in tubes containing cryoprotection solution (25% glycerin, 25% ethylene glycol in 0.1 mol/L phosphate buffer) for the further histological analysis.

Immunohistochemistry and immunofluorescence

For antigen retrieval, free-floating sections were treated for 30 min with sodium citrate (pH 6.0) at 80\(^{\circ}\)C, and allowed to cool for 20 min at room temperature. DCX\(^+\) cells were visualized using the immunoperoxidase method. The endogenous peroxidase was quenched with 0.6% H\(_2\)O\(_2\), followed by blocking the non-specific binding with a solution of 3% donkey serum (JacksonImmunoResearch Lab, West Grove, PA) and 0.1% triton X-100 in Tris buffered saline for 30 min at room temperature. Sections were then incubated over night at 4\(^{\circ}\)C with goat anti-DCX (1:125; C-18; Santa Cruz Biotechnology, Santa Cruz, CA) and for 1 h with a secondary antibody (biotinylated donkey anti-goat IgG; 1:1000; JacksonImmunoResearch Lab), followed by incubation in avidin-biotin solution (1:100; Vectastain ABC Elite kit, Vector Laboratories, Burlingame, CA) for 1 h. The stain was developed with 30% H\(_2\)O\(_2\) and 3-3’ diaminobenzidine tetrahydrochloride (DAB, 1:100; Saveen Werner AB, Malmö, Sweden). Sections were mounted onto glass slides and coverslipped using NeoClear® and NeoMount® (Merck, Whitehouse Station, NJ).

For double-labeling of thymidine analogues (CldU or IdU) with DCX, neuronal specific nuclear marker (NeuN) or DCX/NeuN colabeling, immunofluorescence staining was performed. Since the commercially available antibodies for detection CldU and IdU have been demonstrated to have a degree of cross-reactivity when used for the simultaneous detection of these analogues \(^2,3\), we optimized antibody concentrations for minimal cross-reactivity, and proceeded using the antibody against CldU at dilution (1:1500) that would not show cross-reaction with IdU. For all CldU and IdU staining sections were incubated for 30 min in 2 mol/L of HCl at 37\(^{\circ}\)C followed by 10 min incubation in 0.1 mol/L borate buffer (pH
8.5). After blocking of non-specific binding (as mentioned above), sections were incubated 72 h with the following primary antibodies: to detect CldU (1:1500; rat anti-BrdU clone BU1/75 ICR1; cat. no. OBT0030; Accurate, Westbury, NY); to detect IdU (1:200; mouse anti-BrdU; clone B44; cat. no. 347580; BD BioSciences, San Jose, CA), goat anti DCX (1:200; C-18; Santa Cruz Biotechnology) and mouse anti-NeuN (1:200; clone A60; chemicon, Temecula, CA), followed by 2 h incubation with secondary antibodies: Alexa-488 donkey anti-rat IgG, Alexa-647 donkey anti mouse IgG, Alexa-546 donkey anti goat IgG, Alexa-488 donkey anti-goat IgG, and Alexa-555 donkey anti-mouse IgG (1:1000; Molecular Probes/invitrogen, Carlsbad, CA). ToPro3 (1:10 000; Molecular Probes/Invitrogen) was used as a nuclear counterstain. Sections were mounted onto glass slides and coverslipped with the antifade reagent ProLong Gold (Molecular Probes/ Invitrogen).

Histological quantification
The striatum was divided into two counting regions: striatum 1 (Str1), which covered the area from lateral border of the subventricular zone (SVZ) up to 300 µm laterally, and striatum 2 (Str2) located lateral to Str1. In the corpus callosum (CC), the cell count was performed from the dorsolateral corner of the lateral ventricle to the region beneath the cortical lesion; however, the caudal parts of the rostral migratory stream/ventricle wall, such as the alveus region, were not included. In the cortex (Ctx) cells were counted in a 300 µm-wide peri-infarct region adjacent to the necrotic border of the infarct (see supplemental figure S1B). Counting was performed using the 40x objective and the quantification was based on counting DCX+ cell bodies. The total number of DCX+ cells for each region was the sum of all counted DCX+ cells in six sections per animal.

Confocal microscopy
A confocal laser scanning microscope (TCS SP2; Leica, Wetzlar, Germany) was used to quantify newly generated progenitors, the percentage of DCX+ cells that co-labeled with CldU (DCX+/ CldU+) or IdU (DCX+/ IdU+) out of total DCX+ cells was determined in the ipsilateral corpus callosum (CC) and the peri-infarct cortex (Ctx) on three sections per animal containing cortical lesion and spaced 300 µm apart. Neuronal differentiation was assessed by determining the percentage of NeuN among CldU or DCX positive cells in the peri-infarct cortex. A minimum of 100 cells per animal and staining were analyzed. Confocal analysis was performed using a 63x glycerol immersion objective (numerical aperture 1.3) with optical slices of 1 µm or less (Pinhole setting: airy 1.0), sequential fluorochrome excitation at 488nm, 546nm and 633nm.

References
Supplemental figure S1

Experimental design and methods of analysis. The thymidine analogues chlorodeoxyuridine (CldU) and iododeoxyuridine (IdU) were utilized to birth-date the progenitor cells. All lesioned animals received four injections of CldU on days 7-10 after induction of photothrombotic stroke, whereas four injections of IdU were administered 2 weeks (wks) prior to perfusion at 6, 12, 24 and 48 wks (A). Panel (B) represents areas of histological quantification of the migrating progenitors (Doublecortin positive; DCX+ cells). A diagonal line was drawn from the center of the ventral limit of the infarct passing downward into the striatum. The striatum was divided into two counting regions: striatum 1 (Str1), up to 300 µm lateral to the subventricular zone (SVZ; arrow) and striatum 2 (Str2), lateral to Str1 up to the diagonal line (dashed areas); the corpus callosum (CC); and the peri-infarct cortex (Ctx), from the infarct border up to 300 µm in the surrounding intact cortex (dotted area). In panel (C), the orientation of the leading processes of the DCX+ cells was assessed using an 8-directional compass rose (schematic representation in C) in the corpus callosum (CC) and the peri-infarct cortex (Ctx) (circles). LV= later ventricle.