Cortical Neurogenesis in Adult Rats After Transient Middle Cerebral Artery Occlusion

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Background and Purpose—This study explored the possible occurrence of newly generated nerve cells in the ischemic cortex of adult rats after middle cerebral artery occlusion and reperfusion.

Methods—Nine- to 10-week-old male Wistar rats were subjected to 2 hours of middle cerebral artery occlusion by the monofilament method. Rats received repeated intraperitoneal injections of the cell proliferation–specific marker 5-bromodeoxyuridine (BrdU) after stroke induction. Brain sections were processed for immunohistochemistry with an avidin-biotin complex–alkaline phosphatase and/or –peroxidase method. Brain sections processed with double-immunofluorescent staining were further scanned by confocal microscopy.

Results—Interspersed among the predominantly newly formed glial cells, some cells were double labeled by BrdU and 1 of the neuron-specific markers, Map-2, β-tubulin III, and Neu N, at 30 and 60 days after stroke onset. These cells were randomly distributed throughout cortical layers II through VI, occurring with highest density in the ischemic boundary zone. Three-dimensional confocal analyses of BrdU and the neuron-specific marker Neu N confirmed their colocalization within the same cortical cells.

Conclusions—This study suggests that new neurons can be generated in the cerebral cortex of adult rats after transient focal cerebral ischemia. Cortical neurogenesis may be a potential pathway for brain repair after stroke. (Stroke. 2001;32:1201-1207.)

Key Words: bromodeoxyuridine ■ cerebral ischemia ■ middle cerebral artery occlusion ■ neurons ■ rats

In most parts of normal adult mammalian brains, neurons are not replaced after cell death. However, self-renewing stem cells do exist in discrete brain regions, including the dentate gyrus of the hippocampus and the subependymal layer close to the subventricular zone of lateral ventricles.1–3 In vitro and in vivo, neural stem cells from adult animals and humans can proliferate and then generate either neurons or glial cells,2,4–8 depending on the externally applied growth factors. Under physiological conditions, the stereotyped pathway taken by the newly generated daughter cells in the subventricular zone when they migrate toward their final destination, the olfactory bulb,1 strongly prevents their random dispersion into the adjacent cortical tissues. An exception to this was observed in adult macaques, in which migration of the proliferating cells from the subventricular zone toward the cortex was considered a source of newly generated neurons.9 However, in adult mammalian brains of most species, neurogenesis in the cerebral cortex cannot be observed under physiological conditions. However, a different situation occurs after pathological brain injury. In our recent study of adult rats subjected to photothermotic stroke with spontaneous reperfusion, newborn cortical neurons were identified in the morphologically restored cortical region at risk,10 a part of the somatosensory cortex that underwent successive critical hypoperfusion and severe morphological damage followed by spontaneous reperfusion and histological recovery. These newly generated cortical neurons were randomly scattered among the widespread proliferating cortical cells that appeared in the cortical region at risk after reperfusion. The neurogenesis accounted for 3% to 6% of the newly generated cortical cells as counted by conservative estimation at 7 and 100 days after stroke. To further study the generality of poststroke cortical neurogenesis, the present study explored the possible occurrence of cortical neurogenesis in adult rats subjected to transient unilateral middle cerebral artery (MCA) suture occlusion, which is a focal cerebral ischemic model commonly used in experimental stroke studies.

Materials and Methods

The animal care and all experimental procedures were performed in accordance with European Communities Council Directive 86/609/EEC, and the experimental protocol was approved by the Ethics Committee for Animal Research at Umeå University.

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Animal Preparation
Nine- to 10-week-old nonfasted male Wistar rats (B&K Universal Laboratory, Sol lentuna, Sweden) were first anesthetized with 3.0% halothane in an induction chamber (Stoelting) and then maintained by 2% halothane and a mixture of 70% nitrous oxide and 30% oxygen delivered through a closely fitting face mask. Head temperature was kept at 37.0°C to 37.5°C with a needle thermistor probe in the temporal muscle and a thermostatically controlled heating lamp system (Omega 6000). Rectal temperature was maintained at 37.5°C with a rectal thermistor probe and a thermostatically regulated heating pad (CMA/150, CMA/Microdialysis). The right common carotid artery was exposed through a midline incision and separation of the omohyoid muscle. The external carotid artery (ECA) was dissected from the surrounding fascia and nerves, and the occipital artery and superior thyroid artery branches of the ECA were ligated with 4-0 silk sutures. Polylysine, the suture was heated in 60°C for 60 minutes.13 The ECA was incised, and a vessel loop was placed around the vessel to prevent bleeding. The microvascular clamp was removed, and the filament was advanced 19 to 20 mm into the internal carotid artery. The neck incision was then closed. After 2 hours of ischemia, the neck incision was reopened and the intraluminal filament was withdrawn into the ECA. Sham-operated rats underwent the same surgical procedures as ischemic rats but without the suture insertion.

5-Bromodeoxyuridine Delivery
To detect many proliferating cells while minimizing any potential cellular toxicity, we administered the cell proliferation-specific marker 5-bromodeoxyuridine (BrdU; Sigma) repeatedly in small doses intraperitoneally (10 mg/kg BrdU dissolved in saline for each injection).14 In the 30-day group (n = 5), BrdU injections were started at 24 hours after stroke onset and were continued twice daily during the first and second weeks and once a day during the third and fourth weeks, ending on day 28. In the 60-day group (n = 5), BrdU was injected as described above, but delivery was reduced to 2 times per week during the fifth to eighth weeks and ended on day 56. In a separate experiment, a shorter delivery protocol was used to study whether the newborn cortical cells could survive for a longer interval after BrdU had been incorporated. In those rats, BrdU injection was injected as described above, but delivery was ended on day 14; the rats were killed on day 30 (n = 4) or day 60 (n = 4) after stroke induction. In sham-operated rats (n = 3 in each group), BrdU was injected in the same way as the corresponding rats submitted to ischemia. Normal rats without BrdU delivery were used as BrdU-negative controls (n = 3). All rats were killed by transcardiac perfusion with 37°C Histochoice tissue fixative. Brains were either immediately frozen and stored at −80°C or immersed in the same tissue fixative at 4°C for at least 24 hours before being processed.

Immunohistochemistry
Paraffin-embedded coronal brain sections (10 μm thick) through the ischemic lesion were immunostained according to the protocol recommended for the Vector staining kit (Vector Laboratories). Briefly, brain sections were deparaffinized in xylene and dehydrated through graded ethanol series. Endogenous peroxidase activity was quenched by 30-minute incubation in freshly prepared 3% H2O2–methanol solution. Antigen retrieval was performed by boiling the brain sections in 0.1 mol/L citrate buffer (pH 6.0) in a microwave oven for 3×8 minutes with 5-minute cooling intervals in ambient temperature. This handling also completely deactivates endogenous alkaline phosphatase activity. After 2×5-minute wash in 0.01 mol/L PBS buffer (pH 7.4), the sections were incubated with 20% normal horse serum (Vector)–PBS for 30 minutes. After excessive normal serum was blotted from the sections, mouse anti-BrdU (Becton Dickinson) diluted at 1:30 in PBS buffer was added, and the slides were incubated at 4°C overnight. After 2×5-minute wash in 0.01 mol/L PBS buffer, the sections were incubated with 1:200 biotinylated horse anti-mouse IgG (rat absorbed, Vector)–PBS containing 1.5% normal horse serum at ambient temperature for 30 minutes. After 2×5-minute wash in 0.01 mol/L PBS buffer, the sections were incubated with an Elite Vectastain avidin-biotin complex (ABC)–peroxidase kit (Vector) and stained with diaminobenzidine (DAB; Sigma). For double-immunohistochemical labeling of 2 antigens in the same cell, anti-BrdU was first detected by Vectastain ABC–alkaline phosphatase and then stained with Vector-red or Vector-blue as chromagen. The same staining procedure was then repeated by incubating brain sections with one of the neuron-specific markers mouse anti–Map-2ab (1:400, Boehringer Mannheim), mouse anti–β-tubulin III (1:1800, Promega), and mouse anti–Neu N (1:800, Chemicon) or the glial cell marker rabbit anti–glial fibrillary acidic protein (GFAP) (1:2000, DAKO, A/S). Immunolabeling was detected with 1:200 biotinylated horse anti-mouse IgG (rat absorbed, Vector)/goat anti–rabbit IgG (Vector) followed by the Elite Vectastain ABC-peroxidase kit (Vector) and stained with DAB or Vector-VIP (Vector) as chromagen. To verify the specificity of the immunolabeling, different primary antibodies were omitted from the staining procedure in a set of control sections. Naive animals without BrdU injections were used as anti-BrdU-negative controls.

Immunofluorescence and Confocal Microscopy
Cryostat (10 μm thick) coronal brain sections were processed for immunofluorescence staining according to the protocol recommended for Jackson immunofluorescent kits (Jackson ImmunoResearch). Brain sections were first pretreated for antigen retrieval as described above. After 2×3-minute wash in 0.01 mol/L PBS, the slides were immersed in 1% Triton X-100/PBS for 20 minutes, washed 2×3 minutes in 0.01 mol/L PBS, blocked with 5% normal donkey serum for 20 minutes, and incubated with 1:800 mouse anti–Neu N–PBS overnight at 4°C. After 2×3-minute wash in 0.01 mol/L PBS, the specimen was blocked with 5% normal donkey serum for 20 minutes and then incubated with Cy3-donkey anti-mouse F(ab)2 (Jackson) for 2 hours. To double label brain sections, the staining procedure described for the first staining was repeated, 1:50 rat anti-BrdU (Accurate Chemical & Scientific Corporation) was added, and the sections were incubated overnight at 4°C. The anti-BrdU antibody was then detected by 2-hour incubation with SP-biotin-donkey-anti-rat Fab2 (Jackson) and 30-minute incubation with 5-(4,6-dichlorotriazin-2-yl)aminofluorescein (DTAF)–streptavidin (Jackson). After 3×3-minute wash in 0.1 mol/L PBS, the sections were mounted in Vectashield medium and scanned with a laser scanning confocal microscope equipped with an argon–krypton laser (Multiprobe 2001, Molecular Dynamics). Sets of fluorescent images were acquired sequentially for the red and green channels to prevent crossover of signals from green to red or red to green channels.

Cell Count
To estimate the density of newborn cortical cells, rats that received long-term BrdU delivery in the 30-day and 60-day (n = 3 in each group, randomly selected from paraffin-embedded brains) ischemic groups and sham-operated rats (n = 3) were studied. Three randomly selected coronal brain sections through the ischemic lesion from each rat were double labeled with BrdU and Map-2 by immunohistochemistry. Cell counting was performed in the ischemic boundary cortex under a CAST-Grid system (Olympus). This region was delineated from the edge of the panneurotrophic cytic cavity approximated 1 mm into the adjacent cortex and then to the folds of the corpus callosum. Within these boundaries, optical dissectors sized at 80×45×10 μm were systematically randomly sampled,14 and the number of BrdU-immunopositive cells (neurons, nonneurons) in each dissector was counted. The density of BrdU-immunopositive cells in the investigated region was calculated by dividing the total number of BrdU-positive cells counted by the total volume of the optical dissectors. The data are expressed as the number of BrdU-positive cells per cubic millimeter and are presented as mean±SE within the investigated region. The newborn neurons were identified through their double labeling by Map-2 and BrdU.
Nonneuronal newborn cells were identified through their single labeling by BrdU.

Results

BrdU Single Labeling

Widespread BrdU single-immunopositive cells were observed in the postischemic cerebral cortex, corpus callosum, and dentate gyrus of the hippocampus ipsilateral to the ischemic infarct at 30 and 60 days after stroke induction (Figure 1A). In the cerebral cortex, these cells were distributed randomly through cortical layers II through VI, at higher density in the peri-infarct regions (Figure 1B) than in remote cortical regions. The frequency of BrdU-positive cells in the ipsilateral dentate gyrus of hippocampus was also increased compared with sham-operated controls (Figures 1A, 1C, and 1D). Some BrdU-positive cells were also observed in the striatum close to the ischemic lesion. In the cortex contralateral to the ischemic lesion, a few BrdU-immunopositive cells were also observed. Very few BrdU-immunopositive cells were seen in the sham-operated rats that received the same doses of systemic BrdU administration as the ischemic animals (Figure 1D). In the BrdU-negative control animals, ie, rats that did not receive any BrdU injection, no BrdU immunolabeling was detected in the brain sections examined. In the 30- and 60-day ischemic groups, no major difference in the cell density or the spatial distribution of the BrdU-positive cells was observed between the rats that received long-term BrdU delivery (28 and 56 days) and the rats that received short-term BrdU (for 14 days) and then were immunofluorescently labeled.

Immunohistochemical Double Labeling

Numerous GFAP-immunopositive glial cells stained in purple (Vector-VIP) were observed in the ipsilateral cortex at 30 and 60 days after stroke induction. Many of these cells had blue-stained (Vector-blue) BrdU-immunopositive cell nuclei representing newly generated glial cells (Figure 2A). Interspersed among these GFAP and BrdU double-labeled newborn glial cells in the cortex were BrdU single-immunopositive cells representing endothelial cells and macrophages. In addition, some of the BrdU single-immunopositive cells demonstrated a morphological appearance reminiscent of neurons, ie, cells with a single large, round cell nucleus surrounded by pyramidal cell outlines. To determine whether those cortical cells actually had a neuronal lineage, 3 different neuron-specific markers, Map-2,
β-tubulin III, and Neu N, were used in conjunction with BrdU to perform double-labeling immunohistochemistry. In general, a similar morphological appearance was observed in the BrdU and Map-2 as well as in the BrdU and β-tubulin III double-labeled cortical cells (Figure 2B and 2D). In those cells, the BrdU-immunopositive cell nuclei were stained either in blue (Vector-blue; Figure 2B) or red (Vector-red; Figure 2D) and surrounded by the Map-2–immunopositive cytoplasm stained in purple (Vector-VIP; Figure 2B) or by the β-tubulin III–immunopositive cytoplasm stained in brown (DAB; Figure 2D). These cells varied in shapes and sizes and often had 1 or more recognizable Map-2– or β-tubulin III–immunopositive dendrites extending from the cell bodies. In contrast, a different cellular appearance was revealed by Neu N compared with the Map-2 or β-tubulin III immunostaining. In sham-operated and ischemic rats, Neu N stained the cell nuclei and perinuclear cytoplasm in most cortical neurons, whereas in some cells, only the nuclei or the perinuclear cytoplasm was immunolabeled. Proximal parts of the dendrites were frequently stained in cortical neurons (Figure 2E, arrowhead). The Neu N immunostaining patterns in the present study were thus in agreement with previous reports. In rats subjected to MCAO, many Neu N and BrdU double-labeled cells were found at 30 or 60 days in sham-operated animals. In specificity tests, no specific labeling was observed after omission of the anti-BrdU, anti–GFAP, anti–Map-2, anti–β-tubulin III, and Neu N antibodies. Bars=20 μm (A through E).
randomly through cortical layers II through VI, more densely in the peri-infarcted regions than in distant cortical regions. Furthermore, some BrdU-immunopositive cells, double labeled by Map-2, β-tubulin III, or Neu N, were also found in the striatum close to the ischemic lesion. In sham-operated rats, no cells that were immunopositive to Map-2 (Figure 1C), β-tubulin III, or Neu N showed immunoreactivity to BrdU.

Cell Counting
In the 30-day group, an average of 58 dissectors and 285±95 BrdU-positive cells per brain was counted. The density of BrdU-immunopositive cells was 77.100±8500 (mean±SE) cells per cubic millimeter. Of the BrdU-positive cells counted, a density of 4900±800 neurons and 72.200±8.400 nonneurons per cubic millimeter was found. In the 60-day group, an average of 42 dissectors and 151±59 BrdU-positive cells per brain was counted. The density of BrdU-positive cells was 49.700±7.800 nonneuronal cells per cubic millimeter were observed. In sham-operated control brains, similar to what we observed in the previous study, only a few BrdU-positive cells in the whole cortex were seen. None of these cells was Map-2 and BrdU double positive.

Confocal Microscopy
Z-series consecutive scans of the double immunofluorescence facilitate 3-dimensional analyses of the colocalization of 2 different signals within the same cells. Colocalization of BrdU and Neu N immunofluorescence signals was detected in the cortical cells at 30 days (Figure 3A) and 60 days (Figure 3B) after stroke onset. In these cells, the intense BrdU immunofluorescent signal in the cell nuclei was completely merged with the nuclear Neu N immunofluorescent signal (Figure 3A and 3B). In some BrdU-immunopositive cell nuclei, a brighter immunofluorescent signal was detected in the peripheral than in the central part of the nuclei (Figure 3A). When analyzed in Z-series direction, the colocalization of BrdU and Neu N immunofluorescence extended for several consecutive z-axis planes. Confocal Microscopy

Discussion
This study shows a persistent appearance of neurogenesis in the frontal, temporal, and parietal cortex and the striatum in...
adult rats after transient monofilament MCAO followed by reperfusion. This revelation is to the best of our knowledge unprecedented. Neurogenesis, ie, appearance of cells with nuclear incorporation of the cell proliferation–specific marker BrdU and double labeled by 1 of the neuron-specific markers Map-2, β-tubulin III, or Neu N was observed at 30 and 60 days after stroke onset. As a thymidine analogue, BrdU is incorporated into the proliferating cell nuclei during the S-phase of a cell cycle for DNA duplication.17 BrdU has been widely used to explore neural stem cell proliferation in the central nervous system.4,18 The procedure of double immunolabeling the cells with BrdU and 1 of the neuron-specific makers by sequential ABC–alkaline phosphatase and -peroxidase methods, as presently used, is one of the most common ways to explore neurogenesis in vivo and in vitro.4,5,19–21 To further confirm the presence of cortical neurogenesis, we performed 3-dimensional confocal analyses of BrdU and Neu N immunofluorescence. When confocal microscopy is used, the strict spatial limitation of signal detection of colocalization of 2 signals helps to exclude possible signal overlays from cell juxtaposition.16 The complete merge of the BrdU and Neu N signals in multiple consecutive Z-series planes observed at 30 and 60 days after MCAO verifies a real colocalization of BrdU and Neu N in the same cells. The visualization of proximal Neu N–positive dendrites in these BrdU and Neu N double-labeled cells further authenticates morphologically the neuronal lineage of these newborn cells. Therefore, our data strongly indicate that cortical neurogenesis occurred in the cerebral cortex of the adult rats after the temporal MCAO.

Under physiological conditions, neurogenesis occurs exclusively within the dentate gyrus of the hippocampus and olfactory bulb in normal adult mammalian brains. Altered environments, including active running,2,22 learning,23 seizure,24 aging,19 and global cerebral ischemia,21 are among the factors that influence dentate gyrus neurogenesis. Increased cell proliferation in the dentate gyrus of hippocampus was also observed in the present study. However, except in normal adult macaques, in which constant addition of new neurons in the neocortical associated area has recently been observed,2 3-dimensional confocal analyses do not support the colocalization of BrdU with neuronal markers within the same cortical cells in adult mammalian brains of other species under physiological conditions.10,16,25 This is in agreement with the present observation that no BrdU and neuron marker colocalization was detected in the cortical cells of the sham-operated rats. Therefore, neurons in the cerebral cortex of adult mammalian brains are generally considered to be terminally differentiated. However, our original observation, first presented in June 1999,26 showed that newborn neurons were generated in the reperfused cortical region at risk in response to a reversible photothrombotic cortical ring stroke.19 Judged with immunohistochemical and morphological criteria similar to those in the present study, the BrdU+Map-2 and BrdU+Neu N double-labeled neurons were distributed randomly through cortical layers II through VI, with a more dense distribution in the region at risk close to the cortical ring lesion. A similar spatial distribution of the newborn neurons in the cortex was observed in the present study. However, neurogenesis was also observed in the striatum close to the ischemic lesion. The dispersed distribution of newborn cells in the distal temporal cortex and the striatum in the present study is likely explained by the fact that MCAO of the rats resulted in larger ischemic cortical lesions also involving the striatum compared with the cortical ring stroke model.10 In addition, induction of synchronous apoptosis in a subset of corticothalamic neurons in cortical layer VI also resulted in neurogenesis in this particular cortical region in adult mice.25 Approximately 1% to 2% of the BrdU-positive cells in the damaged cortical layer were shown to express Neu N, and these cells survived up to 28 weeks after the induced injury.25 In the present study 6% to 10% of the BrdU-positive cells were of neuronal origin. This percentage is higher than that reported by Magavi et al25 and also somewhat more than we observed in our previous report, in which 3% to 6% of BrdU-positive cells were immunopositive for Map-2 after reversible photothrombotic stroke.10 The reason for this difference is unclear; possible factors that may partly explain this include the following. First, different species and ages of animals were used. In the present study 9- to 10-week-old male Wistar rats weighing 260 to 280 g were used compared with 12-week-old Wistar rats in our previous report.10 The goal of using the somewhat younger, albeit adult, rats in the present study was to achieve an ischemic lesion of appropriate size with the monofilament used to occlude the MCA. Second, various types of cortical lesions were performed. Finally, the spatial difference from the lesion to the part of the cortex where cell counts were performed varied. In the present study the density of the total population of BrdU-positive cells decreased from 30 to 60 days, whereas the density of cells double immunopositive for BrdU and Map-2 was unchanged. This indicates a persistent appearance of newborn cortical neurons after stroke and also indicates that no significant cell death of these cells took place between those time intervals. Disappearance of macrophages and a turnover of endothelial and glial cells may explain the decreased density of newborn nonneuronal cells.

The origin of the newborn cortical neurons after MCAO is unknown. Multipotent neural stem cells have been isolated from adult brains.4 When exposed to media containing epidermal growth factor, these cells can proliferate and differentiate into neurons as well as glial cells in vitro. The ependymal cells of the lateral ventricle have been further traced as the possible origin of the stem cells residing in the subventricular zone.3 In normal adult macaques,9 a temporal migration of the proliferating cells from the subventricular zone toward the cortex was observed and considered as the possible origin of the newborn cortical neurons. However, the early appearance of the newborn neurons in cortical layer II of the postischemic cortex after a reversible photothrombotic ring stroke in our recent study prompted us to assume that the newborn neurons might originate from the proliferation of the neural stem cells inside the cortex after stroke.10 This assumption is further supported by a recent report that mature neurons can be cultured in vitro from the stem cells isolated from the cerebral cortex of adult rats.7

In the present study the newborn neurons survived for at least 45 days after the last BrdU injection in the 60-day
ischemic group. The normal morphological appearance of these neurons excludes the possibility that these cells were apoptotic. However, little is known about the functional status of these newly generated cortical neurons. Further study should address this issue. Moreover, the possibility that nuclear BrdU incorporation occurs as a sign of DNA repair is under debate. Indeed, there is clear-cut evidence of DNA damage and repair in brain cells after cerebral ischemia. In vitro, neural progenitor cells originally isolated from Sprague-Dawley rat fetuses aged up to 4 years in epidermal growth factor–containing media revealed a subpopulation of APE/ref-1 immunopositive cells indicating DNA repair. To the best of our knowledge, there have been no reports on in vitro or in vivo experiments demonstrating BrdU incorporation in ischemically injured neurons. The signal intensity data on BrdU incorporation in the confocal image in the present study were strong and as intense as nearby proliferating nonneuronal newborn cells. In the case of DNA repair, considerably less BrdU incorporation would be anticipated than that observed. The present findings therefore strongly support our previous suggestion that the BrdU-immunoreactive cortical cells represent neurogenesis rather than DNA repair.

The MCA suture occlusion in rats resembles, in the context of stroke initiation, the brain infarct caused by MCAO in humans. The finding of cortical neurogenesis in this stroke model may present a potential pathway of brain repair through the generation of new neurons in the postischemic adult brain.

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