Transient focal cerebral ischemia–induced neurogenesis in the dentate gyrus of the adult mouse

KUDRET TÜREYEN, M.D., RAGHU VEMUGANTI, PH.D., KURT A. SAILOR, B.S., KELLIE K. BOWEN, AND ROBERT J. DEMPSEY, M.D.

Department of Neurological Surgery and Cardiovascular Research Center, University of Wisconsin, Madison, Wisconsin; and Department of Neurosurgery, University of Süleyman Demirel, Isparta, Turkey

Object. Throughout the life of a mammal, new neurons are produced each day from resident progenitor cells located in the hippocampal dentate gyrus (DG). The availability of transgenic and knockout mice enables the evaluation of specific molecular mediators of this phenomenon. To facilitate such studies the authors characterized the proliferation, survival, and maturation of progenitor cells in the DG of adult mice following transient focal cerebral ischemia.

Methods. Anesthesia was induced in adult C57BL/6 mice by administering halothane. The middle cerebral artery (MCA) was then occluded for 120 minutes by applying an endovascular suture. The marker used to detect the presence of proliferating cells, 5-bromodeoxyuridine (BrdU; 50 mg/kg) was administered intraperitoneally twice daily on Days 2 through 6 after the MCA occlusion. Cohorts of mice were killed on Days 7 and 21, after which their brains were sectioned and BrdU-positive cells were detected using immunohistochemical analysis. The phenotype of the BrdU-positive cells was identified by fluorescent triple labeling by using antibodies specific for neuronal and astroglial markers together with anti-BrdU antibodies. The infarction was confirmed by applying cresyl violet staining.

Compared with sham-operated control animals, there was a 4.6-fold (p < 0.05) increase in BrdU-positive cells in the ipsilateral DG at Day 7 postischemia. Twenty percent of the newly proliferated cells survived to Day 21 postischemia. At this time, the newly proliferated cells expressed the immature and mature neuron markers doublecortin and NeuN, respectively, but none expressed the astroglial marker glial fibrillary acidic protein.

Conclusions. Focal ischemia induces neurogenesis in the DG of the mouse brain; this may be critical for postischemic brain repair.

KEY WORDS • middle cerebral artery occlusion • stroke • progenitor cell proliferation • dentate gyrus • 5-bromodeoxyuridine • mouse

Transient Focal Cerebral Ischemia

Adult male C57BL/6 mice (24 ± 2 g body weight; Jackson Laboratories, Bar Harbor, ME) were used in this study and were
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cared for in accordance with the Guide for the Care and Use of Laboratory Animals (US Department of Health and Human Services Publication No. 86-23 [revised 1986]). The Research Animal Resources and Care Committee of the University of Wisconsin–Madison approved all the surgical procedures.

Anesthesia was induced in the mice by administration of 5% halothane in an N,O,O,mixture (70:30%) and was maintained using a mixture containing 1.5% halothane. Animals were placed in a lateral position, after which the left temporal muscle was dissected and an LDF probe (Vasamedic, St. Paul, MN) was attached to the temporal bone by using cyanoacrylate to measure rCBF. The MCA occlusion model was modified from the one we used in our recent study with rats.8 The mice were turned supine and a midline neck incision was made to expose the left CCA, ECA, and ICA. One microclip was placed on the CCA and another one on the ICA. The ECA was ligated using a 7-0 silk suture placed 2 mm distal from the ECA–CCA branch and was cut distal from the ligated point. Another 7-0 silk suture was loosely tied around the ECA at the ECA–CCA branch for future placement of the occlusion suture. A small V-shaped incision was made in the ECA 1.5 mm distal from the ECA–CCA branch, and an 8-0 occlusion suture with its tip coated with dental resin (Ethicon, Sommerville, NJ; 180–200 μm diameter) was inserted 9 ± 1 mm into the ECA from the bifurcation. When a sharp drop in CBF was observed, the occlusion suture was secured to the loosely tied proximal suture. Ischemia was confirmed by a decrease of rCBF greater than 85% from the baseline value. After 20 minutes, the LDF probe was disconnected, the wound closed, and the animal allowed to awaken. At 100 minutes of occlusion, the animal was anesthetized, the LDF probe reconnected, and the rCBF measured. At 2 hours of occlusion, the suture was withdrawn from the artery to restore blood flow, which was confirmed by LDF. The rCBF recovered to 90 ± 4% of the baseline value within 20 minutes. The wound was closed and the LDF probe detached, after which the mouse was returned to its cage and allowed free access to food and water. Sham-operated mice were subjected to the same surgical procedure except for the MCA occlusion, including placement of ligation clips on the CCA and ICA, and ligation of the ECA by using a 7-0 silk suture. With the aid of a heating blanket, the rectal temperature was maintained at 37 ± 0.5°C until the animals recovered from anesthesia. One hour after reperfusion, the mice were examined for neurological deficits, as described previously.9 Animals without any deficits were excluded from the study.

Administration of BrdU

Cohorts of mice subjected to MCA occlusion and sham operation (14 each) were given injections of BrdU (Sigma Chemical Co., St. Louis, MO; 50 mg/kg, administered intraperitoneally twice daily) for 5 days starting 1 day after reperfusion commenced. To evaluate the rates of progenitor cell proliferation and survival after ischemia, seven mice from each group—the MCA occlusion group and the sham-operated group—were killed on Day 7 (1-week groups) and the remaining animals on Day 21 (3-week groups). Deep halothane anesthesia was used to kill the mice and their brains were quickly removed and sectioned coronally (20-μm-thick slices) on a cryostat. Sections of tissue were mounted on slides and stored at −80°C.

Measurement of Infarction Volume

From each brain serial coronal sections were obtained from sites 1.34 mm anterior and 0.7, 2.7, and 4.72 mm posterior to the breg- ma. The sections were stained with 0.1% cresyl violet and scanned on a flat-bed scanner. Infarction volumes were measured using ImageJ software (version 1.29, written by Wayne Rasband; National Institutes of Health, Bethesda, MD; downloaded from http://rsb.info.nih.gov/ij) as described previously.7,8 The BrdU Immunohistochemical Analysis and Counting of BrdU + Cells

The tissue sections were incubated in 0.3% H,O in 100% metha- nol for 10 minutes, washed in 50 mM TBS (three 5-minute washes), placed in double-distilled water, and heated for 2 minutes in a microwave oven two times. The tissue sections were blocked with 10% NHS in TBS for 30 minutes, incubated with mouse anti–BrdU antibody (dilution 1:200; Oncogene Research Products, San Diego, CA) in 3% NHS for 1 hour, and incubated with rabbit preadsorbed, biotinylated horse anti–mouse–immunoglobulin–G (dilution 1:200; Vector Laboratories, Burlingame, CA) in 3% NHS for 1 hour. The slides were washed in TBS (three 5-minute washes), conjugated with avidin–biotin complex (dilution 1:100; Vector Laboratories), and developed using a 3,3’-diaminobenzidine DAB substrate kit (Vector Laboratories). The tissue sections were again washed, de- hydrated, and mounted in Permount. Sections from mice injected with BrdU and incubated without the anti–BrdU antibody served as negative controls. Sections were analyzed microscopically and im- ages were acquired using a charge-coupled device spot camera (Di- agnostic Instruments, Inc., Sterling Heights, MI). The BrdU-posi- tive cells that reacted to the 3,3’-diaminobenzidine were quantified by examining four sections from each mouse. The cell-counting strategy has been described in our recent paper.9 In each case, the combined area of the GL and SGZ in the DG was traced and mea- sured in square millimeters by using ImageJ software. The total number of BrdU-positive cells in this area were counted and divided by the area to obtain a measurement of cells per square millime- ter. For each case, the cells for each section were obtained and added with PBS (three 5-minute washes), and washed with PBS (three 5-minute washes), and incubated in 100% methanol for 30 minutes. To detect BrdU-labeled nuclei, the sections were denatured by microwave irradiation and blocked for 1 hour in 10% normal don- key serum in TBS at 37°C. The sections were then incubated for 1 hour at 37°C with sheep anti-BrdU antibodies (dilution 1:100; BioDesign International, Saco, ME) in 3% normal donkey serum in TBS, washed in PBS (three 5-minute washes), and incubated for 1 hour at 37°C with Alexa Fluor 488 donkey anti–sheep secondary antibodies (dilution 1:200; Molecular Probes, Eugene, OR). Following these steps, the sections were diluted in casein-PBS-0.05% Tween-20; washed in PBS (three 5-minute washes), and incubated for 1 hour in the M.O.M. kit blocker (Vector Laboratories) followed by the M.O.M. kit diluent (one 5-minute washes; and incubated for 1 hour at 37°C with mouse anti–NeuN antibodies (dilution 1:50; Chemicon International, Temecula, CA) and rabbit anti–cowl GFAP antibody (1:1000; Dako Corp., Carpinteria, CA) in the M.O.M. kit diluent. The sections were washed with PBS for 5 minutes and incubated with Alexa Fluor donkey anti–mouse 594 and donkey anti–rat 647 secondary antibodies (dilution 1:200 each in PBS) for 1 hour at 37°C. The sections were washed, placed on slides, and covered with Vectashield (Vector Laboratories) mounting medium. For BrdU–DCX double labeling, sections of tissue were incubated overnight at 4°C with goat anti–DCX antibodies (dilution 1:100; Santa Cruz

Fluorescent Immunostaining and Confocal Microscopy

The phenotype of the newly proliferated BrdU-positive cells was estimated using triple and double immunolabeling with antibodies to BrdU, NeuN, DCX, and GFAP on parallel sections from each brain. Of these immunohistochemical labels, BrdU is specific for all proliferating cells and NeuN, DCX, and GFAP are specific for mature neurons, immature neurons, and astrocytes, respectively. For these studies, mice subjected to transient MCA occlusion or sham operation were injected with BrdU (50 mg/kg administered intraperitoneally twice daily) for 5 days starting on postoperative Day 1. The mice were killed on Day 7 or Day 21 after MCA occlusion by a transcardiac perfusion of ice-cold 4% paraformaldehyde in 0.01 M PBS. The brains were removed, postfixed overnight in paraformaldehyde, and cryoprotected in a solution of 20% sucrose and 5% glycerol in PBS. The brains were sectioned coronally (20-μm-thick slices) covering the area of the DG. The sections were collected on glass slides, air dried, and used for an immunohisto- chemical analysis. The tissue sections were incubated in 0.03% H,O in 100% methanol for 30 minutes, washed in PBS (three 5-minute washes), and incubated in 100% methanol for 30 minutes. To detect BrdU-labeled nuclei, the sections were denatured by microwave irradiation and blocked for 1 hour in 10% normal donkey serum in TBS at 37°C. The sections were then incubated for 1 hour at 37°C with sheep anti-BrdU antibodies (dilution 1:100; Molecular Probes, Eugene, OR). Following these steps, the sections were diluted in casein-PBS-0.05% Tween-20; washed in PBS (three 5-minute washes), and incubated for 1 hour in the M.O.M. kit blocker (Vector Laboratories) followed by the M.O.M. kit diluent (one 5-minute washes; and incubated for 1 hour at 37°C with mouse anti–NeuN antibodies (dilution 1:50; Chemicon International, Temecula, CA) and rabbit anti–cowl GFAP antibody (1:1000; Dako Corp., Carpinteria, CA) in the M.O.M. kit diluent. The sections were washed with PBS for 5 minutes and incubated with Alexa Fluor donkey anti–mouse 594 and donkey anti–rat 647 secondary antibodies (dilution 1:200 each in PBS) for 1 hour at 37°C. The sections were washed, placed on slides, and covered with Vectashield (Vector Laboratories) mounting medium. For BrdU–DCX double labeling, sections of tissue were incubated overnight at 4°C with goat anti–DCX antibodies (dilution 1:100; Santa Cruz

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Results

The completeness of the MCA occlusion and reperfusion were confirmed by measuring rCBF before and during the occlusion and reperfusion periods by using LDF. In all animals within 30 seconds after insertion of the suture, the rCBF dropped to 85% of the baseline (preocclusion) value (Fig. 1). This decreased flow continued until the time of suture withdrawal (following 2 hours of occlusion). Within 20 minutes after suture withdrawal, the rCBF returned to the control level (Fig. 1).

In mice subjected to transient MCA occlusion, the volume of the infarction was 33.6 ± 6.3 mm³ (mean ± SD) at 7 days of reperfusion, which was not significantly different from the volume observed at 21 days of reperfusion. The survival rate was 80% in the 1-week group and 55% in the 3-week group. Ninety percent of the animals displayed right paw flexion and/or rotation to the paralyzed side 1 hour after reperfusion, indicating the presence of postischemic neurological deficits.

In the sham-operated mice, the number of BrdU-positive cells was not significantly different between the contralateral and the ipsilateral DG either at 1 week or 3 weeks after surgery (Fig. 2). Compared with mice subjected to transient MCA occlusion, by 1 week the number of BrdU-positive cells increased significantly in both the ipsilateral (by 364%; p < 0.05) and contralateral (by 67%; p < 0.05) DGs compared with the respective DGs in the 1-week sham-operated group (Fig. 2). Twenty-one percent of the progenitor cells that proliferated during the 1st week after ischemia survived to 3 weeks. At this time point the number of BrdU-positive cells in the ipsilateral DG of ischemic mice was 124% higher (p < 0.05) than that in the DG of 3-week sham-operated animals (Fig. 2). Figure 3 shows representative photomicrographs of the ipsilateral DG and the contralateral DG in animals in the 1-week sham-operated group (Fig. 3A and Fig. 3B, respectively); the 1-week MCA occlusion group (Fig. 3C and Fig. 3D, respectively); the 3-week sham-operated group (Fig. 3E and Fig. 3F, respectively); and the 3-week MCA occlusion group (Fig. 3G and Fig. 3H, respectively). Confocal microscopic analysis of the immunofluorescence in triple- and double-labeled brain sections revealed that some of the BrdU-positive cells that appeared in Week 1 after ischemia developed into neurons that expressed either the mature neuronal marker NeuN (Fig. 4A and B) or the immature neuronal marker DCX (Fig. 4C and D). At 3 weeks after ischemia, BrdU–DCX double-positive cells were observed within the SGZ, indicating that some newly proliferated cells remained at their site of formation and

**Fig. 1.** Graph showing CBF measurements for the 10-minute preocclusion, 120-minute occlusion, and 20-minute reperfusion periods. The CBF dropped 30 seconds after the MCA occlusion (MCAo) and remained low (<10% of baseline) throughout the 120 minutes of occlusion. The rCBF returned to the baseline level 20 minutes after withdrawal of the suture.

**Fig. 2.** Bar graph showing the numbers of BrdU-positive cells in the ipsilateral and contralateral DGs of sham-operated and MCA-occluded mice killed 7 or 21 days after ischemia. a: p < 0.05 compared with the DG in the respective sham-operated group; b: p < 0.05 compared with the respective contralateral DG.
developed into immature neurons (Fig. 4C and D). At the end of the 3rd week of reperfusion following transient MCA occlusion, approximately 55% of the BrdU-positive cells in the ipsilateral DG (B, D, F, and H) in animals in the 1-week sham-operated (A and B), 1-week MCA-occlusion (C and D), 3-week sham-operated (E and F), and 3-week MCA-occlusion (G and H) groups. The brain sections were immunostained with BrdU antibodies using the DAB method. Tissue from animals in the 1-week MCA occlusion group demonstrated a significant increase in BrdU-positive cells in the SGZ (C). Twenty-one percent of the cells survived 3 weeks (G). Original magnification × 40.

**Discussion**

The findings of the present study show that transient focal cerebral ischemia in an adult mouse induces the proliferation of progenitor cells in the SGZ of the hippocampal DG. Some progenitor cells that form during the 1st week of reperfusion after ischemia survive to 3 weeks, none of them displayed positive BrdU staining (Fig. 4A and B). This suggests that progenitor cells proliferating in the mouse DG after an ischemic injury do not mature into astroglial cells.
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migrate into the GL from the SGZ, and mature into neurons expressing the neuron-specific marker NeuN.

It had been generally assumed that few new neurons are formed in adult mammals; however, recent studies have demonstrated that specific regions of the mammalian brain continue to produce neurons throughout the life of the animal. In these species, adult neurogenesis has been observed in the rostral subependymal zone of the lateral ventricles, the olfactory bulb, and the SGZ of the hippocampal DG.11,19

There is a substantial increase in the number of granule cells in the DG as rodents develop from newborn to adult.2 In the hippocampus of an adult rodent, despite the daily death of neurons, the number of neurons is maintained almost constantly due to the proliferation of new resident progenitor cells that mature into neurons.

Recent studies have also shown that acute neurological injuries, including ischemia and traumatic injury to the brain, increase the rate of progenitor cell proliferation in the DG.12,14,15,21,29 A recent study performed in our laboratory characterized postischemic neurogenesis in adult spontaneously hypertensive rats that had been subjected to transient MCA occlusion. This study showed that in the SGZ of the DG, several new cells will be formed in the 1st week after ischemia and some of them will survive, migrate to the GL, and mature into adult neurons.6 Mice living in an enriched environment have also been found to have a greater amount of granule neuron formation in the DG, which correlates with improved learning.17 These studies raise the possibility that increased neurogenesis following ischemia and trauma may compensate for neuronal loss to facilitate the repair and regeneration process in the damaged brain. Although cerebral ischemia can stimulate the proliferation of progenitor cells, the endogenous response is low in magnitude and may not be sufficient to replace the lost neurons.

We observed that some of the BrdU-positive cells in the 3-week MCA occlusion group also stained positively for DCX. In a previous study the authors suggested that the DCX-positive phenotype is transient and disappears with-

**Fig. 4.** Representative confocal photomicrographs of triple-immunostained (BrdU [green], GFAP [blue], and NeuN [red]) tissue sections showing the ipsilateral DG of a mouse from the 3-week MCA occlusion group. One of the double-stained cells is seen in the center of the GL of the DG (arrow in A). This cell has a BrdU–NeuN double-positive stain indicating a mature neuron but does not stain for GFAP, which would indicate an astrocyte (B). Some of the BrdU-positive (green) cells in the ipsilateral DG also displayed positive staining for DCX (red), indicating an immature neuron (C and D). Cytosolic and fibrillar staining is shown with DCX (red) and nuclear staining is shown with BrdU (green). The fibers display extension into the GL (arrowheads in C and D). Bars = 60 μm in A, 20 μm in B and D, and 120 μm in C.
in 1 or 2 weeks after a new neuron is generated. It is possible that the primary effect of ischemia is to maintain new neurons in an immature state. In this case, it is also possible that there are fewer new neurons that are functionally integrated into the DG circuitry, even though more neurons appear to survive. Similar to previous studies, approximately 30% of the BrdU-positive cells were also found to stain positively for NeuN 21 days after MCA occlusion. Also, proliferating BrdU-positive progenitor cells have been demonstrated to generate neurons and astrocytes in the mouse SGZ under normal conditions (after administration of 45 mg/kg BrdU every 2 hours for 48 hours). Sequential administration of BrdU was observed to result in 53% NeuN-positive and 14% GFAP-positive cells in 3 weeks. This remarkable difference in phenotype differentiation after focal ischemia may be due to the relatively intense ischemia (120 minutes of transient occlusion) or to differences in the BrdU administration protocol.

Recent progress in developmental neurobiology as well as in neural stem cell/progenitor cell research has demonstrated that GFAP-positive astrocytes in the SGZ can divide (are BrdU positive) and generate new neurons under normal conditions or after the chemical removal of actively dividing cells in mice. In our study we could not find any convincing BrdU–GFAP double-positive cells.

The transcriptional regulators Pax6, Emx2, and Mash1 are expressed in the developing hippocampus, and each of these factors distinguishes a specific subpopulation of neural progenitor cells during embryogenesis. A recent study demonstrated that dividing BrdU-positive cells in the SGZ of the DG expressed these molecular markers in adult rodents. The use of these transcriptional markers may help elucidate the molecular mechanism by which ischemia induces neurogenesis.

Several studies show that treatment with a growth factor may increase ischemia-induced proliferation of progenitor cells and, given the correct growth factor cocktail, this effect can be maximized to aid the postischemic repair process. Yoshimura and colleagues demonstrated that endogenous fibroblast growth factor–2 is essential for the proliferation and differentiation of progenitor cells in the DG by studying mice deficient in this growth factor. In this case, it is also possible that the death of the glutamatergic neurons that project to progenitor cells induces neurogenesis. Changes in NMDA receptors also mediate ischemia-induced neurogenesis. It is possible that the death of neurons within the hippocampus provides a stimulus for increased neurogenesis after ischemia. In support of this hypothesis, limbic seizures that cause the apoptosis of granule cells increase DG neurogenesis.

At present, the precise molecular mechanisms responsible for stroke-induced neurogenesis are not known. Recent studies in which GeneChip microarrays were used have demonstrated upregulation of several putative endogenous mediators of neurogenesis such as cytokines, growth factors, and transcription factors following MCA occlusion. Some of these factors that can diffuse from the site of production (ischemic cortex) to the site of neurogenesis (hippocampus) may promote postischemic neurogenesis, and studies of the interplay of these factors will lead to the development of novel pharmacological agents to induce the endogenous repair mechanism following cerebral ischemia.

Conclusions

The results of the present study indicate that focal ischemia in the adult mouse induces the proliferation of progenitor cells in the hippocampal DG, which may be part of the endogenous response of the central nervous system to repair structures after an acute injury. Some of the progenitor cells that newly proliferate after ischemia survive and differentiate into neurons.

References

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Address reprint requests to: Robert J. Dempsey, M.D., Department of Neurological Surgery, University of Wisconsin, K4/822 CSC, 600 Highland Avenue, Madison, Wisconsin 53792. email: dempsey@neurosurg.wisc.edu.