Induced Spreading Depression Activates Persistent Neurogenesis in the Subventricular Zone, Generating Cells With Markers for Divided and Early Committed Neurons in the Caudate Putamen and Cortex

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Abstract—Status epilepticus and cerebral ischemia stimulate persistent neurogenesis in the adult brain, but both conditions cause neuronal damage. Spreading depression is a common epiphenomenon of these neurogenesis-stimulating conditions. We analyzed the effect of KCl-induced spreading depression on persistent neurogenesis and the spatio-temporal distribution of cells exhibiting immunohistochemical markers for divided and early committed neurons (new neurons) in the adult rat brain. After induction of spreading depression for 48 hours, the density of mitotic cells, divided cells, and new neurons in the subventricular zone increased at days 1 to 3, days 3 to 6, and day 6, respectively \((P<0.05)\). The divided cell density in the rostral migratory stream and the stream size increased at day 12 \((P<0.001)\). Vehicle (saline) infusion or induction of spreading depression for 4 hours only did not increase the divided cell density, but the latter increased new neuron density in the subventricular zone \((P<0.001)\). Double-labeled new neuron-like cells also appeared in the caudate putamen or cortex in ectopic fashion at day 3, with dramatic increases at days 6 and 12. Administration of the NMDA receptor antagonist, MK-801, which inhibits the propagation of spreading depression, abolished the increase in new neurons in the subventricular zone and the appearance of ectopic new neuron-like cells after 48-hour KCl infusion. There was no neuronal damage, as evidenced by mature neuron density, neurite density, and apoptotic cell appearance after spreading depression for 48 hours. Spreading depression has the potential to stimulate persistent neurogenesis or to produce ectopic new neuron-like cells. \(\text{(Stroke. 2005;36:000-000.)}\)

Key Words: cell differentiation ■ cell division ■ membrane potential ■ progenitor cells

In the adult mammalian brain, neurogenesis persists in the dentate gyrus subgranular zone\(^1\) and in the forebrain subventricular zone (SVZ).\(^2,3\) Recently, chemoconvulsant-induced status epilepticus and cerebral ischemia have been found to stimulate persistent neurogenesis by increasing divided cell densities, promoting a majority of them to neuronal differentiation in adult rodents.\(^4-6\) Neuronal cell death has been considered common under these harmful conditions, but cerebral lesioning did not stimulate persistent neurogenesis.\(^7\)

Spreading depression (SD), found during epileptogenic stimulation,\(^8\) is characterized by a wave of fully reversible cellular depolarization, propagating throughout the cortex, into the thalamus, caudate putamen, and hippocampus.\(^9\) Various cerebral conditions that increase regional \([K^+]\), to the depolarizing threshold (epileptic discharges and anoxia/ischemia) induce SD.\(^8,9\) Focal cerebral ischemia caused 52 to 78 events of SD in 24 hours,\(^10\) and a specific cerebral injury caused epileptic seizure activities on electroencephalography, or 68 SDs in 48 hours.\(^11\) Thus, repetitive SDs are a common epiphenomenon of the persistent neurogenesis-stimulating conditions.

In the present study, we investigated the effect of 48-hour KCl-induced SDs on persistent neurogenesis, by analyzing cells in the SVZ, the rostral migratory stream (RMS), the normal route for differentiating new neurons to the olfactory bulb, the caudate putamen (CPu), and the frontoparietal cortex (FPC) using immunohistochemical techniques.

Materials and Methods

The experimental protocols were designed in accordance with the animal experimental guidelines established by the animal research committee at NCVC. All efforts were made to minimize suffering and the number of animals used.
Experimental Groups and Induction of SD

Sprague-Dawley rats (SLC, Kyoto, Japan), 8 to 9 weeks old, were randomly divided into 5 groups: 48-hour KCl-treated (48-hour SD), 4-hour KCl-treated (4-hour SD), 48-hour KCl treatment with administration of the noncompetitive N-methyl-D-aspartate receptor antagonist (MK-801) (Sigma, St. Louis, Mo), which inhibits propagation of SD,\textsuperscript{12} 48-hour saline-treated (vehicle), and an untreated normal control group (N).

In the treated groups, an osmotic mini-pump (Alzet 2001; Alza) with the infusion needle was implanted in the primary somatosensory area in the cortex (Figure 1a) so that 4 mol/L KCl or saline could be infused continuously at a rate of 1.0 mL/h.\textsuperscript{9,13} The reliability of this method was established in our previous study: repetitive SD waves at 36-minute intervals (average) were confirmed.\textsuperscript{13} The physiological parameters were within the normal range at the end of and for at least 21 days after 48 hours of KCl-induced SD (unpublished data).

Preparation of Tissue Sections

Brains were removed under deep anesthesia: from the 48-hour SD, or vehicle group at day 0 (=day of pump removal), 1, 3, 6, 9, or 12 (n=6, or 3 each); from the 4-hour SD (n=6), or MK-801 group (intraperitoneal injections with 2 or 4 mg/kg per shot (n=6 each), 1 hour before and 24 hours after KCl-pump implantation) at day 6; and from the N group (n=10). The protocol specified that the 4-hour SD or MK-801 group be euthanized at day 6, because the density of new neurons in the 48-hour SD group increased at day 6 in our pilot study.

Each brain was cut into 2-mm-thick coronal slabs and immersed in methanol Carnoy’s solution. The paraffin-embedded tissues were sliced into 7 sets of two 3-μm-thick coronal sections, 12 μm apart, at the SVZ (Figure 1b). In these sections, 3 sets were used for analysis of the CPu and FPC (Figure 1b). At RMS (Figure 1c), 2 sets were obtained in the same manner. This slice thinness precludes any pseudo-positive double-labeled images.

Immunohistochemistry and BrdUrd Administration

Up to the time of brain removal, all rats received 5-bromo-2′-deoxyuridine (BrdUrd) (B-5002; Sigma) via drinking water (1 mg/mL)\textsuperscript{14} starting on the day of pump implantation for a maximum of 120 hours (<120 hours if brain removal occurred earlier). BrdUrd is incorporated into the dividing cell during the S-phase and labels...
every divided cell. It was established that the volume of drinking water did not differ significantly among the groups, and ranged from 80 to 110 mg/kg per day. To confirm the reliability of this method, BrdUrd-labeled cell densities in the SVZ after intraperitoneal injections of 50 mg/kg BrdUrd, every 12 hours for 120 hours, beginning at KCl pump implantation (BrdUrd control) (n=6), were also analyzed at day 6. In the MK-801 group, intraperitoneal BrdUrd administration was performed, because the drug temporarily impaired drinking behaviors in our pilot study.

The primary antibodies used were monoclonal mouse anti-PCNA (clone PC10; Upstate Biotechnology, Lake Placid, NY), 1:100, for detection of mitotically active cells;15 monoclonal mouse anti-BrdUrd (clone 3D4; BD Biosciences, San Jose, Calif), 1:150, for divided cells;16 polyclonal rabbit anti-class III β-tubulin (β-tubulin III) (PRB-435P; BAbCO, Richmond, Calif), 1:200, for cells committed to a neuronal lineage,17 polyclonal goat anti-double cortin (C-18; Santa Cruz Biotechnology, Santa Cruz, Calif), 1:100 for migrating neuroblasts,18 and polyclonal rabbit anti-GFAP (Dako, Glostrup, Denmark), 1:200 for astrocytes. Immunoreactivity was revealed by the labeled streptavidin-biotin-HRP method (LSAB kit; Dako) with hematoxylin as counter-stain, or by immunofluorescence (FITC or TRITC) using confocal laser scan microscopy. Individual control staining in the absence of primary antibodies abolished immunoreactivity (data not shown).

**Cell Densities in the SVZ**

PCNA⁺ cells, cells in mitosis (with visualized chromosomes), BrdUrd⁺ cells, or BrdUrd⁻/β-tubulin III double-labeled (BrdUrd⁻/β-tubulin III⁺) cells in the SVZ within the predetermined frame (1200×600 μm) (Figure 1b) were counted at ×600 (Mac Scope; Mitani Co) in the 48-hour SD, vehicle, and N groups. The 4-hour SD, MK-801, and BrdUrd control groups were also analyzed for BrdUrd⁻/β-tubulin III⁺ cell density. The size of the targeted SVZ was measured for calculation of cell density. The total (hematoxylin⁻) cell number in the SVZ was counted for calculation of each cell ratio.

**Cell Density in the RMS, Caudate Putamen, or Cortex**

The density of BrdUrd⁻ cells in the RMS (Figure 1c) was calculated in the same manner as in the SVZ. The sectional area of the RMS was measured for each sample. In the CPu or FPC (Figure 1b), the density and the distribution of BrdUrd⁻/β-tubulin III⁻ cells were analyzed at ×600 in the 48-hour SD, vehicle, or MK-801 group.

**Damage to the Cortex**

Brain slices were stained using monoclonal mouse anti-NeuN (Chemicon), 1:100, for detection of mature neurons, Bodian’s silver method for neurofilaments in neurites, or terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL; ApopTag, Serologicals Co) for apoptotic cells, at days 1, 3, 6, and 12 after 48-hour SD. The total number of NeuN⁻ cells in the predetermined area in supplementary somatosensory area (Figure 1b) was counted at ×100. Optical densities on Bodian’s silver stain were measured on histograms of the predetermined area in supplementary somatosensory area (presented as % of N group).

**Quantification and Statistical Analysis**

Cell density was calculated as the number of labeled cells divided by the analyzed area multiplied by the section thickness, with corrections for cell (nuclear) splitting between the sections (expressed per mm³). The mean target nuclear diameter in each location was used for correction.20 Statistical analysis was performed using 2-way (in SD and vehicle groups) or 1-way (in SD group) ANOVA. If multiple comparisons were indicated, the Bonferroni t test was applied. Data are presented as mean±SD.

**Results**

**Cell Densities in the SVZ**

PCNA⁺ cells, or cells in mitosis, increased after 48-hour SD, but not with the vehicle (Figure 1d to 1f), or in the contralateral SVZ (data not shown).

BrdUrd⁺, or BrdUrd⁻/β-tubulin III⁺ cell density increased after 48-hour SD, whereas remained unchanged in vehicle group (Figure 2a and 2b), being independent of the duration of BrdUrd administration, ranging from 48 to 120 hours. The mean density of BrdUrd⁻/β-tubulin III⁺ cells in the BrdUrd control was consistent with that after oral BrdUrd administration. The MK-801 treatments significantly reduced BrdUrd⁻/β-tubulin III⁺ cell densities at day 6 in the SVZ after 48-hour KCl infusion (Figure 2c). In the contralateral SVZ, the BrdUrd⁻/β-tubulin III⁺ cell densities at day 6 after 48-hour KCl infusion with the MK-801 treatment (2 mg or 4 mg/d) were consistent with those in the vehicle group at day 6 data (not shown).

In the 4-hour SD group, BrdUrd⁺ cell density in the SVZ remained unchanged (data not shown), but the density of BrdUrd⁻/β-tubulin III⁺ cells was significantly increased; 235±54, compared with the N group; 135±44 (×10³/mm³-SVZ, P<0.001).

**Total Cell Density and Cell Ratio in the SVZ**

The Table shows the alteration of total cell density and the individual cell ratio after 48-hour SD.

**RMS Size and Cell Density**

RMS size and BrdUrd⁺ cell density in the RMS increased at day 12 after 48-hour SD (Figure 2d).

**Cells Expressing GFAP in the SVZ**

Cells in the cellular band underlying the ventricular ependymal cell layer expressed GFAP (Figure 3a). BrdUrd⁺ cells in cell clusters, surrounded by GFAP⁺ cells, were essentially GFAP-negative.

**Ectopic BrdUrd⁻/β-tubulin III⁺ Cells**

BrdUrd⁻/β-tubulin III⁺ cells were found in the CPu or FPC after 48-hour SD (Figure 3b), but not in the vehicle group. The spatio-temporal distributions are illustrated in Figure 4a. The density increased at day 6 and/or day 12 in the CPu, or FPC restricted to the cortical layer V-VI and the anterior cingulate cortex. The MK-801 treatments abolished the ectopic BrdUrd⁻/β-tubulin III⁺ cell appearance at day 6 (data not shown). After 48-hour SD, doublecortin was detected in many divided cells in, or in areas close to, the SVZ (≈<100 μm) (Figure 3c), but not in cells in the CPu or FPC during the observation period (data not shown).

**Damage to the Cortex**

The cortical lamination, the density of mature neurons, and neurite density were not affected by 48-hour SD (Figure 5a to 5d). TUNEL-positive cells were observed only at the site of the KCl needle (Figure 5e).
Discussion

The safety of prolonged (48-hour) SD for the normal brain in the morphological perspective was demonstrated, as was the safety of brief (4 to 5 hours) SD. Administration of MK-801 abolished increases in new neuron density in the SVZ after 48-hour KCl infusion (Figure 2c), indicating that SD propagation, but not KCl diffusion, activates neurogenesis in the SVZ. The volume of localized cerebral necrosis plus edema caused by 48-hour KCl infusion was measured as 5.98 mm³ (average), limited to 0.9% of the total hemispheric volume.

Cells in mitosis (dividing cells) increased in the SVZ after prolonged SD, whereas RMS size was constant, indicating that the increase in BrdUrd⁺ (divided) cell densities was caused by an increased cell division rate in the SVZ, but not by altered rostral migration/cell accumulation in the RMS. Prolonged SD may have reduced the naturally occurring cell death rate in the dividing cells in the SVZ.

Brief (4-hour) SD increased new neurons, but not divided cell density, in the SVZ. Recurrent SDs are known to increase BDNF levels in the brain, which promotes the survival and differentiation of neurons, but they do not affect mitosis of precursor cells. The increase in new neurons in the SVZ after brief or prolonged SD may be caused, at least partially, by BDNF-derived promotion of neuronal differentiation.

| Total Cell Density and Individual Cell Ratio After 48-hour SD |
|---------------|------------------|------------------|------------------|------------------|------------------|
|               | No. | d0   | d1   | d3   | d6   | d12  |
| Total cell density |     |      |      |      |      |      |
| ×10⁶/mm³—SVZ     | 3.6 (100) | 3.9 (109) | 4.8 (132) | 5.3 (145)* | 6.5 (178)* | 5.0 (139) |

PCNA⁺ cells, % | 9.1 | 10.6 | 15.1 | 17.6* | 10.0 | 10.1 |

Mitotic cells, % | 0.4 | 0.4 | 1.3* | 0.8* | 0.6 | 0.4 |

BrdUrd⁺ cells, % | 34.7 | 32.4 | 23.0 | 36.0 | 32.9 | 31.6 |

BrdUrd⁺/β-tubulin III⁺ cells, % | 3.7 | 4.5 | 7.0 | 9.4 | 10.5* | 10.0* |

*P<0.05 compared with the values in the N group.
and/or survival. Determining the precise relationship between the duration of SD and the dividing or survival potential of those cells, ie, whether there is a threshold SD period, or whether these responses are simply exaggerated by extending the duration of SD, requires further investigation.

Figure 3. Confocal laser scan images for GFAP in the SVZ (a), new neuron-like cells in the CPs or FPC (b), and doublecortin in the SVZ (c) after 48-hour SD. a, BrdUrd /β-tubulin III (green) cells forming cell clusters and surrounding GFAP /β-tubulin III (red) cells at day 6 in the SVZ. b, BrdUrd /β-tubulin III (green) cells in the CPs at day 12 (upper panels), in the FPC at day 12 (middle panels), or in the CPs at day 6 (lower panels). c, BrdUrd /doublecortin (red) cells in the SVZ (upper panel), and cells close to the SVZ (arrows, and lower panels) at day 12. Magnification, ×600, originally. Bars: (a) 20 μm, (b) 10 μm, (c) upper panel, 20 μm, lower panels, 10 μm.

Figure 4. Distribution of ectopic BrdUrd /β-tubulin III cells. a, BrdUrd /β-tubulin III cells (dots) in the CPs or FPC after 48-hour SD. Each diagram is a superimposed composite of 6 brain sections (one section per rat). b, BrdUrd /β-tubulin III cell densities after 48-hour SD, which were not detected in the vehicle group, revealed significant differences in the CPs (P<0.001) and FPC (P<0.002).

After prolonged SD, the delayed increase in divided cells in the RMS or RMS size (Figure 2d) is considered to be the
result of the earlier increase in dividing cell density in SVZ. It is unknown why the divided cell ratio in the SVZ did not increase at days 3 to 6 (Table) at the time when the total or divided cell density increased. The activated neurogenesis in SVZ may have reduced the rostral migration temporarily, resulting in accumulation of earlier divided (BrdUrd-negative) cells in the SVZ.

BrdUrd/GFAP cells in clusters (hot spots) in the SVZ (Figure 3a) are thought to be neural (immature) precursors and their daughter cells (newborn neuroblasts). Neural precursors in hot spots have been considered as an intermediate proliferating population between newborn neuroblasts and GFAP neural progenitors (primary precursors). Neural progenitors, which can give rise to neurospheres in vitro, are relatively quiescent in vivo.

The structures that produced ectopic new neuron-like cells (double-labeled by markers for divided and early committed neurons)—the caudate putamen, the cortical layer V-VI, and the anterior cingulate cortex—may have the potential for accepting new neurons from the SVZ, as was observed after induced apoptosis or cerebral ischemia. However, these ectopic new neuron-like cells did not express the marker for migrating neuroblasts, at least for 12 days, suggesting that these cells are not migrating or differentiating as new neurons in the SVZ. Because the SVZ is one of the most primitive structures in the phylogeny of the central nervous system, these relatively primitive structures may possess latent neural progenitors. Neural progenitors (in vitro) have been identified in the striatum or cortex of the adult rat brain. Although the physiological significance of SD is currently unknown, SD may be an intrinsic mechanism functioning as an activator of endogenous neurogenesis. Elucidation of the survivability and reconstructive potential of the ectopic new neuron-like cells produced by SDs is needed, along with efforts to develop a practical SD induction method to facilitate therapeutic reconstruction of an impaired central nervous system.

Acknowledgments
This study was supported by a grant-in-aid for Scientific Research, from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan. We are thankful for the valuable assistance of Tomoko Yasuda, Ayako Yamada, Natsue Sakata, Miho Kurimoto, and Koji Nishimori. We also gratefully acknowledge the advice of Katsuyuki Simizu and Manabu Deguchi (Applied Medical Research), and Keiji Machino and Takemasa Bando (Bando).

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Stroke. published online June 2, 2005;
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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