Induced Spreading Depression Evokes Cell Division of Astrocytes in the Subpial Zone, Generating Neural Precursor-Like Cells and New Immature Neurons in the Adult Cerebral Cortex

Jing-Hui Xue, MD, PhD; Hiroji Yanamoto, MD, DMSc; Yukako Nakajo, MSc; Norimitsu Tohnai, PhD; Yoshikazu Nakano, BSc; Takuya Hori, MCE; Koji Iihara, MD, PhD; Susumu Miyamoto, MD, PhD

Background and Purpose—New immature neurons appear out of the germinative zone, in cortical Layers V to VI, after induced spreading depression in the adult rat brain. Because neural progenitors have been isolated in the cortex, we set out to determine whether a subgroup of mature cells in the adult cortex has the potential to divide and generate neural precursors.

Methods—We examined the expression of endogenous markers of mitotic activity, proliferating cell nuclear antigen, and vimentin as a marker for neuronal progenitor cells, if any, in the adult rat cortex after spreading depression stimulation. Immunohistochemical analysis was also performed using antibodies for proliferating cell nuclear antigen, for vimentin, and for nestin. Nestin is a marker for activity dividing neural precursors.

Results—At the end of spreading depression (Day 0), glial fibrillary acidic protein-positive cells in the subpial zone and cortical Layer I demonstrated increased mitotic activity, expressing vimentin and nestin. On Day 1, nestin+ cells were found spreading in deeper cortical layers. On Day 3, vimentin-/nestin+ neural precursor-like cells appeared in cortical Layers V to VI. On Day 6, new immature neurons appeared in cortical Layers V to VI. Induced spreading depression evokes cell division of astrocytes residing in the subpial zone, generating neural precursor-like cells.

Conclusions—Although neural precursor-like cells found in cortical Layers V to VI might have been transferred from the germinative zone rather than the cortical subpial zone, astrocytic cells in the subpial zone may be potent neural progenitors that can help to reconstruct impaired central nervous system tissue. Special caution is required when observing or treating spreading depression waves accompanying pathological conditions in the brain. (Stroke. 2009;40:00-00.)

Key Words: astrocytes ■ ischemia ■ neural stem cells ■ neurophysiology ■ neuroregeneration ■ stroke management ■ stroke recovery

Status epilepticus and cerebral ischemia stimulate persistent neurogenesis by increasing divided cell density in the germinative zone and promoting a majority of them to neuronal differentiation.1,2 Spreading depression (SD), consisting of transient increase in [Ca++]i, is triggered by epileptic seizures, cerebral injury, or ischemic stroke.3 SD has been also associated with migraine with aura.4,5 Previously, we found that induced multiple SD waves in the adult rat brain under normal condition activates cell division of astrocytes residing in the subventricular zone (SVZ), enhancing persistent neurogenesis toward the olfactory bulb.6 Furthermore, induced SD generates new immature neurons, at an ectopic location, in cortical Layers V to VI.6 Recently, it was found that induced SD activates neurogenesis in the hippocampal subgranular zone.7 In the embryonic ventricular zone, spontaneously occurring propagations of “calcium waves” accelerate cell division of radial glial cells (neural progenitors).8 “Calcium waves” initiated at the sperm entry site in mammalian eggs have been related to levels of cell cycle regulating factors.9 These evidences suggest that SD, SD-like depolarizing waves, or calcium waves are an endogenous activation signal for germinative cells and neural progenitors to divide.

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In the adult mammalian brain, outside of the germinative zone, neural progenitors have been isolated from the septum, striatum, subcortical white matter, and cortex. We hypothesized that neural progenitors may reside permanently in the cortex and investigated whether a subgroup of cells in the adult cortex undergoes mitosis after induced SD expressing the marker for neural progenitors. The absence of mitotic figure and neural progenitor-like cells in the cortex after induced SD confirms that new immature neurons migrate from outside the cortex. We analyzed mRNA levels for proliferating cell nuclear antigen (PCNA), an endogenous marker for mitotic activity, and vimentin, a cytoskeletal intermediate filament protein primarily localized in radial glial cells in the embryonic brain.

Nestin belongs to another class of cytoskeletal intermediate filament protein recognized as a general marker of proliferating neural precursor cells. Neural precursor cells express nestin abundantly as do radial glia in the embryonic central nervous system, but most of these disappear after completion of the development of the central nervous system. In the adult SVZ, actively dividing cells generating clusters of neuroblasts are neural precursors, an intermediate proliferating population between glial fibrillary acidic protein-positive neural progenitors and neuroblasts. We also studied the temporospatial distribution of cells expressing PCNA, vimentin, nestin, and doublecortin (DCX) in the adult rat cortex after induced SD.

Methods
The experimental protocols were approved by the Experimental Animal Research committee at the National Cardio-Vascular Center. Every effort was made to minimize the suffering and number of animals used. In all, 116 male Sprague-Dawley rats (SLC, Kyoto, Japan), 8 to 9 weeks old, were used. All rats were allowed free access to food and water before and after all procedures.

Induction of SD
SD was generated by a continuous microinfusion of 4 mol/L KCl at a rate of 1.0 μL/hour for 48 hours into the left cortex (Figure 1). Cortical direct current potential was monitored starting 3 hours after the pump implantation (n=5) using an Ag/AgCl electroencephalogram needle electrode.

Northern Blot Analysis
Setting Day 0 as the day of KCl pump removal, groups of 6 rats were euthanized (under deep anesthesia) on Days 1, 3, 6, and 12 after 48-hour SD as were saline infusion control rats on Days 1, 3, 6, and 12 after a 48-hour vehicle infusion (n=4) and untreated normal controls that received no infusion (n=6). Total RNA was separated from the left cortex using the standard agarose gel electrophoresis technique. A cDNA probe for PCNA and vimentin was prepared by reverse transcription–polymerase chain reaction. The hybridized signals ([32P]-dCTP) were quantified (Image Gauge; Fujifilm). Signals were compared among the groups. A DNA probe complementary to 28S rRNA was used to confirm equal loading (5 μg) of the RNA samples.

Immunohistochemistry
On Days 0, 1, 3, 6, and 12 after 48-hour KCl infusion (n=6 each), after 48-hour vehicle infusion (n=4 each), and without the pump...
Expression levels of PCNA and vimentin mRNA. Signals for PCNA and vimentin mRNA increased in the cortex after induced SD, but not after vehicle infusion. N indicates untreated normal control.

implantation (n = 4), brains were removed, cut into 7 2-mm-thick coronal slabs, and immersed in methanol Carnoy’s solution. The paraffin-embedded slab rostral to the KCl injection site was sliced into 7 sets of 6 3-μm-thick coronal sections 21 μm apart (Figure 1B). In addition, 3 sets of 6 3-μm-thick coronal sections, 21 μm apart, were also obtained using a separate set of animals on Days 1, 3, 6, and 12 after a 48-hour KCl infusion (n = 4 each). The slice thinness precludes any pseudopositive double-labeled images.

Expression of PCNA, nestin, vimentin, glial fibrillary acidic protein (GFAP), and neuronal nuclei was visualized using monoclonal antibodies against PCNA (1:80; mouse-PC10; Upstate Biotechnology), nestin (1:100; mouse-R-401; Becton, Dickinson and Company), vimentin (1:150; mouse-V9; Sigma), GFAP (1:100; mouse-GF-2; Dako), and neuronal nuclei (1:200; mouse-antineuronal nuclei; Chemicon). The immunostaining techniques in details were described elsewhere. Hematoxylin was used as a counterstain.

To identify apoptotic cells, and migrating neuroblasts, brain slices were stained using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL; ApopTag; Serologicals Co), or DCX (1:200; guinea pig-anti-DCX; Chemicon), respectively, at Days 1, 3, 6, and 12 after a 48-hour KCl infusion.

5-Bromo-2’-deoxyuridine Administration
All rats undergoing immunohistochemical study received the S-phase marker, 5-bromo-2’-deoxyuridine (BrdU; B-5002; Sigma) through drinking water (1 mg/mL) starting on the day of pump implantation.

Double immunostaining was performed using monoclonal mouse anti-BrdU (1:150; 3D4; BD Biosciences) for divided cells and polyclonal rabbit anticlass III β-tubulin (β-tubulinIII; 1:200; PRB-435P; BabCO) for cells committed to a neuronal lineage.

Quantification and Statistical Analysis
The numbers of PCNA+ cells, cells in mitosis with visualized chromosomal vimentin+, nestin+ cells, and BrdU+/β-tubulinIII+ cells were counted at 21-μm intervals, 6 sections per animal at each time point, at 600× magnification using image analysis software (Mac Scope; Mitani Co, Osaka, Japan). The targeted region, within a 1200×600-μm square, was set in cortical Layers I to VI (Figure 1B). The area within a 50-μm distance from the pia mater was defined as the subpial zone (SPZ). Cell densities/mm² were calculated after stereological corrections. Statistical analysis was performed using one-way analysis of variance. If multiple comparisons were indicated, the Bonferroni i test was applied. Data are presented as mean ± SEM.

Induction of SD
Multiple SD waves, 7±1 episodes/hour, appeared on the direct current potential monitor (Figure 1).

Northern Blot Analysis
The probe detected the PCNA or vimentin mRNA in a single band (Figure 2). The baseline signals in normal cortex increased by 2.2-fold or 2.1-fold on Day 1. By contrast, these signals did not increase throughout the observation period in the vehicle group (Figure 2).

Immunohistochemistry
The laminar structures visualized by neuronal nuclei stain were not affected by induced SD (Figure 3A). On Day 0, PCNA+ cells were found primarily in the SPZ or cortical Layer I and to a lesser extent in cortical Layers II to VI, and cells in mitosis were observed in the SPZ (Figures 3B, 4A, and 5A). On Day 1, PCNA+ cells or dividing cells were found spreading in cortical Layers I to VI or in cortical Layers I to IV, respectively. On day 3, the total number of these cells decreased significantly (Figure 4A–B). There was no expression of PCNA or mitotic figure in the cortex of untreated normals (Figure 3B), in the contralateral hemisphere after induced SD, or rats treated with vehicle infusion (data not shown). PCNA+ cells and mitotic figure were identified as GFAP+ and Neu/U− (Figures 3E and 5B–E).

In normal cortex, immunoreactivity toward vimentin was detected only in the arachnoid tissues and pia matter (Figure 3C). On Day 0, cells in the SPZ and cortical Layer I expressed vimentin (Figures 3C and 6A–B). On Day 1, the vimentin+ cells decreased in the SPZ and increased in the deeper cortical Layer I. Within the SPZ, the vimentin+ cell density decreased from Day 0 to 3 and increased from Day 3 to 6 (Figure 4C). Vimentin+ cells underwent mitosis (Figure 6C–D).

Immunoreactivity for nestin was not observed in normal or vehicle-treated cortex (data not shown). On Day 0, cells in the SPZ and cortical Layer I expressed nestin (Figures 3D and 6E). On Day 1, the predominant distribution of nestin+ cells shifted from the SPZ to cortical Layer I, and nestin+ cells were observed in cortical Layers II to VI (Figures 3D and 4D). Mitotic figures were observed in nestin+ cells (Figures 3D and 6F–H). In cortical Layers V to VI on Day 3, the nestin+ cell density was higher than the vimentin+ cell density (P < 0.001; Figure 4C–D), indicating that nestin/vimentin+ cells appeared there. Nestin+ cells disappeared on Day 6, but the appearance of vimentin+ cells lasted at least for 12 days (Figure 4C–D).

Immunoreactivity for DCX was detected in migrating neuroblasts in the SVZ or rostral migratory stream, but not in the cortex after induced SD during the observation period (Figure 3G). TUNEL-positive cells were not observed in the cortical layers during the observation period (Figure 3H).

BrdU+/β-tubulinIII+ cells were found in cortical Layers V to VI after induced SD (Figures 4E and 5F, G, K), showing significant increase on Days 6 and 12 compared with untreated normal or vehicle controls. The spatiotemporal distribution of new immature neurons is illustrated in Figure 3F.
Figure 3. Histological findings after induced SD. A, Neuronal nuclei \( (+) \) cells. Mature neurons and cortical architecture visualized in the normal cortex (N) and in the cortex after induced SD. B, The appearance of PCNA \( (+) \) cells. On Day 0 (d 0) after induced SD, PCNA \( (+) \) cells appeared primarily in cortical Layer I, spreading to deeper cortical layers on Day 1. C, The appearance of vimentin \( (+) \) cells. On Day 0, vimentin \( (+) \) cells appeared in cortical Layer I extending over cortical Layers I to IV on Day 3. D, The appearance of nestin \( (+) \) cells. Cells residing in the SPZ and a subgroup of cells in the adjacent cortical Layer I expressed nestin on Day 0, extending to cortical Layers V to VI on Day 3. A–D, magnification: \( \times 100 \), left panels; \( \times 600 \), right panels, originally. Bar: 100 \( \mu m \), left panels; 20 \( \mu m \), right panels. E, GFAP \( (+) \) or vimentin \( (+) \) cells. In normal cortex, cells in the SPZ (N, arrows) and cortical Layer I express GFAP (upper 4 panels). GFAP \( (+) \) mitotic figures in the SPZ on Day 0 (d0, arrows). A GFAP \( (+) \) cell in cortical Layer I on Day 3 (d3, arrow) extending its cellular process toward the pia mater. GFAP \( (+) \) cells underlining the pia mater on Day 6 (d6, arrows). A cellular process extending toward deeper cortical layers (large arrow). Vimentin \( (+) \) cells underlining the pia mater on Day 6 (bottom panel, arrows). A vimentin \( (+) \) cellular process extending from the pia mater (large arrow) on Day 6. F, The distribution of new immature neurons. BrdU \( (-)/\beta\)-tubulinIII \( (-) \) cells (dots) observed only in cortical Layers V to VI in the region of interest (indicated by the square in the left cortex, see Figure 1) after induced SD. Each diagram is an integrated view of 6 animals; a superimposed composite of 6 brain sections (one section from one animal) on Day 3, 6, or 12. BrdU \( (-)/\beta\)-tubulinIII \( (-) \) cells were not detected in the untreated normal or the vehicle group. E–F: magnification: \( 600\times \), originally. Bar: 20 \( \mu m \). G, DCX \( (+) \) cells and (H) analysis with TUNEL stain. DCX \( (+) \) (brown) cells were not detected in cortical Layers I to VI for 12 days after induced SD. Migrating neuroblasts in the rostral migratory stream (left) or SVZ (right; on Day 6) served as a positive control (magnified window). TUNEL \( (+) \) (brown) cells were not detected in the cortex for 12 days after induced SD (counterstain: methyl green). TUNEL \( (+) \) apoptotic cell bodies in blood clot around the needle cavity (on Day 3) served as a positive control (magnified window). G–H: magnification: \( \times 100 \), originally. Bar: 100 \( \mu m \), \( \times 600 \) in the magnified window. Bar: 20 \( \mu m \).
Figure 4. The density of PCNA+ cells, mitotic figures, vimentin+, or nestin+ cells in the cortical layers after induced SD. A, From Day 0 to 1, the PCNA+ cell density decreased in the SPZ (*P* < 0.001) and increased in the adjacent cortical Layer I (*P* < 0.002) or in cortical Layers V to VI (*P* = 0.018), but not in cortical Layers II to IV (*P* > 0.061). The density in all layers decreased from Day 1 to 3 (*P* < 0.001). B, From Day 0 to 1, the distribution of mitotic figures shifted from the SPZ toward the adjacent cortical Layer I or Layers II to IV. C, From Day 0 to 1, the vimentin+ cell density decreased in the SPZ (*P* < 0.001) but increased in the adjacent cortical Layer I (*P* < 0.008). From Day 3 to 6, the density in cortical Layers II to IV increased (*P* < 0.001), but decreased from Day 3 to 6 (*P* < 0.001). From Day 3 to 6, the density in the SPZ increased (*P* = 0.003). D, From Day 0 to 1, the nestin+ cells density in the SPZ decreased (*P* < 0.001) but increased in the adjacent cortical Layer I (*P* < 0.001). The density in all layers increased from Day 0 to 3 (*P* < 0.002), an effect that disappeared at Day 6. E, BrdU+/β-tubulinIII+ cells increased in cortical Layers V to VI on Days 6 and 12 compared with untreated normal animals or vehicle control (*P* < 0.05). N indicates untreated normal control; d1, 3, 6, and d12, 1, 3, 6, and 12 days after SD. Asterisk: *P* < 0.05; double asterisk: *P* < 0.01; triple asterisk: *P* < 0.001; significantly different from untreated controls.
Induced SD stimulated GFAP+/H11001 astrocytes residing in cortical Layer I to express vimentin (the marker for radial glia or neural progenitor cells) and/or nestin (the marker for neural progenitor or precursor cells) and divide. Cell division in vimentin+/H11001 and/or nestin+/H11001 cells from Day 0 to 1 was considered to be the cause of increased vimentin+ or nestin+ cell density in all layers on Day 3. The appearance of vimentin+ cells for at least 12 days after the enhanced gene expression on Day 1 demonstrated a relatively long life for vimentin.

From Day 0 to 3, the vimentin+/H11001 cell density in the SPZ decreased but increased in the other cortical layers. Because there was no sign of active apoptosis in the SPZ from Day 1 to 3, the acute decrease in vimentin+/H11001 cell density was not considered to be caused by apoptotic cell loss in the SPZ but the transfer of these cells to other cortical areas.

Actively dividing neural progenitors in the embryonic ventricular zone have the characteristics of radial glia.22 Radial glial cells and GFAP+ neural progenitors in the SVZ express vimentin.17,23 Radial glial cells divide asymmetrically, producing neural precursor cell(s) and a new radial glial cell.24 The radial process of radial glia serves as a scaffold connected to the embryonic ventricular surface and the pia mater in embryonic cortical Layer I to guide their daughter

**Figure 5.** Phenotype of PCNA+ or mitotic cells and early committed or mature neurons. A, A PCNA+ (brown) cell (on the left) is extending a cellular process toward the pia mater (on the right) on Day 0 after induced SD. B, PCNA+ (brown) cells express GFAP (red) in cortical Layers II to IV on Day 1. C–D, GFAP+ (red) cells with visualized chromosomes in cortical Layers II to IV. E, Cells in mitosis were negative for neuronal nuclei. Representative images of new immature neurons (F–G, arrow) expressing β-tubulin (red) and BrdU (brown) in cortical Layers V to VI. Native mature neurons (H–I, arrow) expressing β-tubulin (red) but not BrdU (brown). Representative confocal laser scan images of native mature neurons (J, arrows) expressing β-tubulin (red) but not BrdU (green). A new immature neuron (K) expressing β-tubulin (red) and BrdU (green). Magnification 600×, originally. Bar: 10 μm.

**Discussion**

Induced SD stimulated GFAP+ astrocytes residing in cortical Layer I to express vimentin (the marker for radial glia or neural progenitor cells) and/or nestin (the marker for neural progenitor or precursor cells) and divide. Cell division in vimentin+ and/or nestin+ cells from Day 0 to 1 was considered to be the cause of increased vimentin+ or nestin+ cell density in all layers on Day 3. The appearance of vimentin+ cells for at least 12 days after the enhanced gene expression on Day 1 demonstrated a relatively long life for vimentin. From Day 0 to 3, the vimentin+ cell density in the SPZ decreased but increased in the other cortical layers. Because there was no sign of active apoptosis in the SPZ from Day 1 to 3, the acute decrease in vimentin+ cell density was not considered to be caused by apoptotic cell loss in the SPZ but the transfer of these cells to other cortical areas.

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cells to migrate into cortical Layers II to VI.\(^{24}\) The long processes of radial glia cells remain attached to these surfaces even during mitosis.\(^{25}\) When the neuronal production is complete, after “final asymmetrical” cell division with loss of vimentin, most of the radial glial cells transform into mature astrocytes, and only a few radial glial cells remain into adulthood as relatively quiescent neural progenitors in the SVZ.\(^{17,24}\)

The vimentin\(^+\) cell density increased from Day 3 to 6 without a prior increase in mitotic cell density in the SPZ (Figure 4A), suggesting that vimentin\(^+\) cells spreading over cortical Layers II to IV moved to the SPZ. To clarify translocation mechanisms, if any, including scaffold structures involving the pia mater, requires further investigation.

After induced SD, DCX, the marker for migrating neuroblasts, was not detected in the cortex or the corpus callosum after induced SD, indicating that new immature neurons (newborn neuroblasts) do not migrate from the germinative zone to the cortex. It has also been reported that newly generated immature neurons found in the normal adult rat neocortex do not express DCX, indicating that new neurons are derived from neural precursors in the cortex under normal conditions.\(^{26}\) Taken together, these observations make it appear likely that β-tubulinIII\(^+\)/BrdU\(^+\) cells (divided and early committed neurons, ie, new immature neurons) appearing in the adult cortex were generated from nestin\(^+\)/vimentin\(^-\) (neural precursor) cells appearing at the same location.

It is interesting that vimentin\(^+\) radial glia-like cells appeared in cortical Layer I, the oldest structure in the cortex, under which new neurons appear in the embryonic period generating cortical Layer VI to II in an inside-out gradient.\(^{27}\) Besides the SPZ in the cortex, actively dividing cells in “cerebellar” SPZ continue to generate neuroblasts beyond puberty in rabbits.\(^{28}\) In the developing dentate gyrus, the SPZ in the hippocampus has been identified as a transient neurogenic zone to form subgranular zone.\(^{29}\) Thus, the SPZ in the cerebellum and the hippocampus carry germinative cells, at least temporarily, in the developing brain. A germinative cell population, if resident in the SPZ in the adult cerebral cortex and even surviving in the vicinity of cortical lesion development after stroke or cerebral injury, may help to reconstruct injured cortex by providing new neurons.

Compared with the present results, SD waves were less frequent (1.7±0.5 /h) when direct current potential was monitored a day after KCl pump implantation in our previous study.\(^{18}\) In line with this, the frequency of SD waves decreased 6 hours after a continuous KCl injection, from 12 per hour to 2 per hour.\(^{30}\) Therefore, we estimate the total number of SD waves as 84±26/48 hours in the present study.

SD waves are frequently observed in the cortex of patients with ischemic stroke, subarachnoid hemorrhage, or traumatic cerebral injury.\(^ {31,32}\) SD or SD-like waves triggered by experimental focal ischemia, 52 to 78 acute events in 24 hours, have recruited penumbral tissue into the infarct core.\(^ {33}\) In contrast, KCl-induced multiple SD waves can induce ischemic tolerance through brain-derived neurotrophic factor, a gene-dependent mechanism.\(^ {19}\) Increase in BDNF levels and appearance of immature new neurons induced by multiple SD waves may play a beneficial role to the brain function. Thus, special caution is required when inducing, observing or treating SD waves in the brain.

Recently, GFAP-δ, an isoform of GFAP, was found in astrocytes residing in the SVZ, the subgranular zone in the hippocampus, and the SPZ in the adult human cortex.\(^ {34}\) GFAP-δ, unlike GFAP-α, plays no role in astrogliosis, but has great benefits in migration.\(^ {34}\) This indicates that astrocytes in the SPZ are a different cell population from the other astrocytes in the brain.

Although it is still uncertain from the present data whether nestin\(^+\)/vimentin\(^-\) cells appearing in cortical Layers V to VI are neural precursors originating in cortical Layer I, we propose that the astrocytes residing in the SPZ in the adult cortex are latent or quiescent neural progenitors. We also propose that multiple SD waves are an endogenous activation signal for germinative cells in the brain to divide.

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Disclosures

None.

References

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