Cell division in the cerebral cortex of adult rats after photothrombotic ring stroke

Weigang Gu, Thomas Brännström, Roland Rosqvist, Per Wester

Abstract Neurogenesis has been shown to occur in the cerebral cortex in adult rats after ischemic stroke. The origin of the newborn neurons is largely unknown. This study aimed to explore cell division in the poststroke penumbral cortex. Adult male Wistar rats were subjected to photothrombotic ring stroke. After repeated delivery of the DNA duplication marker BrdU, the animals were sacrificed at various times poststroke. BrdU was detected by immunohistochemistry/immunofluorescence labeling, as was the M-phase marker Phos H3 and the spindle components α-tubulin/γ-tubulin. DNA damage was examined by TUNEL staining. Cell type was ascertained by double immunolabeling with the neuronal markers Map-2ab/β-tubulin III and NeuN/Hu or the astrocyte marker GFAP. From 16h poststroke, BrdU-immunolabeled cells appeared in the penumbral cortex. From 24h, Phos H3 was colocalized with BrdU in the nuclei. Mitotic spindles immunolabeled by α-tubulin/γ-tubulin appeared inside the cortical cells containing BrdU-immunopositive nuclei. Unexpectedly, the markers of neuronal differentiation, Map-2ab/β-tubulin III/NeuN/Hu, were expressed in the Phos H3-immunolabeled cells, and NeuN was detected in some cells containing spindles. This study suggests that in response to a sublethal ischemic insult, endogenous cells with neuronal immunolabeling may duplicate their nuclear DNA and commit cell mitosis to generate daughter neurons in the penumbral cortex in adult rats.

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reproducible fashion. Microvascular platelet thrombi appear in the cortical lesion (Gu et al., 1999b; Wester et al., 1995), reminiscent of clinical thromboembolic stroke. Delayed, but consistent, spontaneous reperfusion of the cortical penumbra can be presaged in the ring model by manipulation of the irradiating laser beam intensity (Gu et al., 1999a), by which local cerebral blood flow in the penumbra first decreases to 59, 34, 26, and 33% of baseline values at 1, 2, 4, and 48h after irradiation and then gradually recovers to 56 and 87% of baseline values at 72 and 96h (Gu et al., 1999c). Neuronal necrosis and apoptosis prevail in the cortical penumbra at 24–48h postischemia (Gu et al., 1999b; Hu et al., 2002), and angiogenesis is meanwhile initiated to facilitate late spontaneous reperfusion (Gu et al., 2001). Cortical neurogenesis is observed at 72h poststroke in the reperfused penumbral cortex (Gu et al., 2000), where remarkable morphological recovery takes place (Gu et al., 1999b). 5-Bromodeoxyuridine (BrdU)-immunolabeled cortical neurons account for 3–6% of the BrdU-immunolabeled cortical cells and they survive up to 100 days poststroke (Gu et al., 2000).

The ring stroke concept of penumbral inversion, in which the compromised tissue lies interior rather than exterior to the ischemic locus, appears to facilitate concentration of pathogenic as well as recovery factors. It provides progressive metabolic isolation owing to the radial encroachment of ischemia into the central zone, for which recovery probably is initiated by locally recruitable factors. It is indeed the interaction of these factors that is believed to occur in a conventional penumbra (Jiang et al., 2001), except the ring stroke model allows these events to occur in a highly localizable cortical region. Similar dramatic morphological protection of nerve cells from ischemia is also reported in the setting of spinal cord ischemia after hypothermia (Wang et al., 2005), where rescue of ventral horn motor neurons from imminent death by apoptosis is associated with up-regulation of anti-apoptotic Bcl-2 and down-regulation of proapoptotic p53 in the hypothermic animals; a similar phenomenon is seen also in photothrombotic ring stroke (Hu et al., 2004). Therefore, the ring stroke model reproducibly elicits a balance between death and recovery processes that mimics what is believed to occur during microfocal evolution of the clinical penumbra.

The origin of the newborn cortical neurons in photothrombotic ring stroke in rats has not been elucidated. In this stroke model, BrdU-immunolabeled cells appear initially in the ischemic penumbral cortex (Gu et al., 2000). This indicates that the newborn neurons might have a cortical origin, in contrast to a possible tangential migration of the neural stem cells from the subventricular zone (SVZ) in rats after middle cerebral artery occlusion (Jin et al., 2003). However, the reliability of using only BrdU as a cell proliferation marker to study neurogenesis has been discussed (Nowakowski and Hayes, 2000; Wojtowicz and Kee, 2006). Further studies of cell division in the poststroke adult brain are thus needed.

The present study aims to explore possible cell mitosis in the ischemic penumbral cortex in adult rats in the context of the photothrombotic ring stroke with spontaneous reperfusion (Gu et al., 1999a).

## Results

### BrdU- and phosphohistone H3 (Phos H3)-immunolabeled cells in the poststroke penumbral cortex

Rats were sacrificed at 4, 10, 16, 24, 48, and 72h and 7 and 14 days after stroke induction and the brain sections were examined with BrdU single immunohistochemistry and immunofluorescence (Gratzner, 1982). In the native control brains (without BrdU injection), no BrdU immunolabeling was detected. In the sham-operated group and in 4- and 10-h poststroke rats, a few BrdU-immunolabeled cells were seen randomly scattered in the brain sections. At 16h poststroke, BrdU-immunolabeled cells were consistently identified in the ischemic penumbral cortex. These cells did not seem to have any specific spatial correlation with microvessels in the cortex. When consecutive sagittal brain sections were examined at this time, a few BrdU-immunolabeled cells were observed on the tangential migrating pathway from the SVZ toward the olfactory bulb, but not toward the penumbral cortex. At 24h, 48h, 72h, 7 days, and 14 days poststroke, the number of BrdU-immunolabeled cells increased gradually in the poststroke penumbral cortex. A few BrdU-immunolabeled cells were seen in the ipsilateral corpus callosum at the level of the cortical lesion.

To screen for the possible appearance of mitotic cells, and to determine the initial time point at which cortical cells might transit from the S phase into the M phase, the appearance of the M-phase-specific marker phosphorylated histone H3 (Hans and Dimitrov, 2001; Hendzel et al., 1997) was investigated in the brain sections. In the native and sham-operated brains, and in rats at 4, 10, and 16h poststroke, no Phos H3-immunolabeled cells were observed. From 24h poststroke, Phos H3-immunolabeled cells appeared in the ischemic penumbral cortex. The cell density reached its maximum at 48h and 72h poststroke and then declined at 7 days (Table 1). At 14 days after stroke, no Phos H3-immunolabeled cells were observed. The Phos H3-immunolabeled cells were randomly distributed through cortical layers I–VI (Figs. 1A and 1B), more frequently in the penumbral cortex. A few Phos H3-immunolabeled cells were also seen in the corpus callosum (Fig. 1C) and the hippocampus (Fig. 1A), but such cells were rarely seen in the ischemic ring lesion destined to pan necrosis at 24 and 48h poststroke. At 72h poststroke, a few Phos H3-immunolabeled nuclei appeared in the

<table>
<thead>
<tr>
<th>Time poststroke</th>
<th>Phos H3-positive cells</th>
<th>Map-2ab-positive cells</th>
<th>Phos H3 and Map-2ab double-positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td>16 ± 4</td>
<td>42,015 ± 2,992</td>
<td>11 ± 5</td>
</tr>
<tr>
<td>48 h</td>
<td>1810 ± 416</td>
<td>29,900 ± 11,537</td>
<td>770 ± 33</td>
</tr>
<tr>
<td>72 h</td>
<td>1590 ± 312</td>
<td>30,911 ± 7,282</td>
<td>320 ± 35</td>
</tr>
<tr>
<td>7 days</td>
<td>17 ± 13</td>
<td>41,391 ± 7,406</td>
<td>3 ± 2</td>
</tr>
</tbody>
</table>

Data are expressed as cells/mm² (mean ±SD).
ischemic ring lesion where gliosis now prevailed (Gu et al., 1999b).

To study the temporal and spatial correlation between BrdU nuclear incorporation and Phos H3 expression, BrdU and Phos H3 double-immunofluorescence labeling was performed. At 4, 10, and 16h poststroke, none of the BrdU-immunolabeled cortical cells was doubly immunopositive. At 24, 48, and 72h poststroke, an increasing number of BrdU-immunolabeled cells became Phos H3 immunopositive (Figs. 1D and 1E).

**Mitotic spindles in the ischemic cortex**

To detect the possible appearance of cell mitosis in the poststroke rat brains, we used the spindle components α-tubulin (Wittmann et al., 2001) and γ-tubulin (Lajoie-Mazenc...
et al., 1994) to label the mitotic spindles by means of immunocytochemistry and immunofluorescence.

In the sham-operated brains and in the brains at 4, 10, and 16h poststroke, no α-tubulin-immunolabeled spindles were seen. At 24h poststroke, α-tubulin-immunolabeled spindles appeared in the ischemic penumbral cortex. These cells were distributed in a temporal and spatial pattern similar to that of the Phos H3-immunopositive cells. The spindles appeared in various shapes. In some cells, the α-tubulin-immunolabeled microtubules were confined to the cytoplasm, and the nuclear DNA appeared in a single nucleus. Sometimes, the α-tubulin appeared as a bipolar spindle (Figs. 2A–2F), and the nuclear DNA was converted into chromosomes that were scattered randomly within the cell. In some cells, the nuclear DNA appeared as double nuclei at the opposite ends of a single cell (Figs. 2G–2J).

In agreement with the previous report that γ-tubulin specifically labeled the metaphase spindles and the spindle poles in animal cells (Lajoie-Mazenc et al., 1994), we observed that γ-tubulin labeled the mitotic spindles in the poststroke rat brains. These spindles were distributed in a temporal and spatial pattern similar to that of α-tubulin. In some cells, γ-tubulin labeled the spindle poles (Fig. 3A) while the cell nucleus remained intact. In others, γ-tubulin-immunolabeled microtubules penetrated throughout the cell nucleus (Fig. 3B). Furthermore, the γ-tubulin was organized into bipolar spindles, and the nuclear DNA was transformed into chromosomes (Fig. 3D) or twin nuclei (Fig. 3F).

To examine if the DNA synthesis marker BrdU was incorporated into the nuclei of cells containing mitotic spindles, we performed double-immunofluorescence labeling of BrdU and α-tubulin/γ-tubulin. The majority of the

Figure 2 (A–F) α-Tubulin and NeuN double immunofluorescence in the penumbral cortex at 24 h poststroke. (A, C, E) Single-channel confocal images of α-tubulin immunofluorescence in three consecutive optical sections (interval: 0.35 μm). In a large cortical cell in layer III (delineated), α-tubulin (green) appears as a bipolar spindle (arrows). (B, D, F) α-Tubulin and NeuN double-channel confocal images in the same scanning sections as (A, C, E). NeuN (red) immunoreactivity is detected in the cytosol of the cell, in which the nuclear DNA has been transformed into randomly scattered chromosomes (blue, DAPI). (G–J) α-Tubulin and NeuN double immunofluorescence in layer IV of the penumbral cortex at 48 h poststroke. (G) In an α-tubulin single-channel projection image, a large cell is α-tubulin-immunolabeled (green), inside which two cell nuclei (arrows, blue) are located at the opposite ends. (H) α-Tubulin and NeuN double-channel image from (G). The α-tubulin-immunolabeled cell (green) shown in (G) is NeuN (red) double immunopositive, which produces a yellow color in the merged image (framed). In the same field, two NeuN (red) singly immunolabeled cortical neurons (arrowheads in G and H) and two nonneuronal cells (blue, DAPI) are seen. (I and J) 3D analysis of the upper and lower framed area in (H). The DAPI-labeled cell nuclei (arrow, blue) are localized inside the α-tubulin and NeuN double-labeled cytoplasm under 3D analysis.
cells that contained α-tubulin- or γ-tubulin-immunolabeled spindles exhibited BrdU-immunolabeled cell nuclei \( (\text{Fig. 3B}) \).

To examine if the cortical cells containing mitotic spindles also expressed the M-phase marker Phos H3 in their nuclei, we performed Phos H3 and γ-tubulin double immunofluorescence. A Phos H3-immunolabeled cell nucleus was observed frequently in cortical cells that contained the γ-tubulin-immunolabeled spindle structure \( (\text{Fig. 3A}) \).

To examine if α-tubulin and γ-tubulin labeled the same mitotic spindle as previously reported in animal cells \( (\text{Lajoie-Mazenc et al., 1994}) \), α-tubulin and γ-tubulin double immunofluorescence was performed. In the same spindle, γ-tubulin labeled the same microtubules as α-tubulin \( (\text{Fig. 3G}) \), in agreement with the previous report \( (\text{Lajoie-Mazenc et al., 1994}) \).

### Separation of the M-phase markers from TUNEL

To examine if the mitotic cells exhibited any detectable DNA damage, TUNEL and Phos H3, TUNEL and α-tubulin, and TUNEL and γ-tubulin double immunofluorescence was used. At 24 and 48h after stroke, numerous cells were TUNEL positive in the penumbral cortex. The Phos H3- \( (\text{Fig. 3H}) \), α-tubulin- \( (\text{Fig. 3I}) \), and γ-tubulin- \( (\text{Fig. 3J}) \) immunolabeled cells were dispersed among but distinctly separated from the

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**Figure 3**  
(A) Phos H3 and γ-tubulin double immunofluorescence in the penumbral cortex at 48 h poststroke. In a maximal projection confocal image of a cell from layer II, the Phos H3-immunopositive nucleus (red, Alexa Fluor 546) is superimposed on a γ-tubulin-immunolabeled spindle (green, Alexa Fluor 488) with the pole bodies at the opposite ends (arrows, green) and the linking microtubules stretching in between.  
(B) 3D confocal analyses of BrdU and γ-tubulin double immunofluorescence in a cortical cell at 24 h poststroke. The γ-tubulin appears as a spindle (green) that is colocalized with the BrdU-immunolabeled nucleus (red) in the same cell. The γ-tubulin-immunolabeled microtubules (green) are penetrating through the BrdU-immunolabeled nucleus (red), which in combination produces a yellow color in the nucleus (arrow).  
(C) In a maximal projection confocal image, many neurons are NeuN (arrow, red) singly labeled and nonneurons are DAPI counterlabeled (arrowhead, blue). One cell is NeuN and γ-tubulin double immunolabeled (framed).  
(D) 3D confocal analysis of the cell framed in (C). The γ-tubulin (green) appears as a spindle while NeuN (red) labels the central part of the cytosol. The cell DNA (blue, DAPI) is pulled toward the opposite ends (arrows).  
(E and F) GFAP and γ-tubulin double immunofluorescence in layer II in the penumbral cortex at 72 h poststroke. (E) In a section image, a γ-tubulin-immunolabeled spindle (green) is surrounded by GFAP-immunopositive cytoplasm (red) in a cortical cell. (F) 3D confocal analysis of the same optical section as (E) shows colocalization of γ-tubulin-immunolabeled spindle (green) and the GFAP-immunolabeled cytoplasm (red) in the same cell. The DNA (blue, DAPI) appears as two separated nuclei at the opposite ends (arrows).  
(G) 3D confocal analyses of γ-tubulin and α-tubulin double immunofluorescence in a spindle structure in the cortex. The α-tubulin (green) is colocalized with the γ-tubulin (red) in the same microtubules of a spindle (arrow, yellow).  
(H) Confocal microscopy of TUNEL and Phos H3 double immunofluorescence in the cortical penumbra at 48 h after stroke. The Phos H3-immunolabeled cells (arrows, green) are separate from the TUNEL-positive cells (arrowheads, red).  
(I) Confocal microscopy of TUNEL and α-tubulin double immunofluorescence in the penumbral cortex at 48 h after stroke. An α-tubulin-immunolabeled cell (arrow, green) is separate from the TUNEL-positive cells (arrowheads, red).  
(J) Confocal microscopy of TUNEL and γ-tubulin double immunofluorescence in the cortical penumbra at 48 h after stroke. The γ-tubulin-immunolabeled cells (arrows, green) are separate from the TUNEL-positive cells (arrowheads, red).
TUNEL-positive cells. At 72h poststroke and later, TUNEL-positive cells were scarcely detected in the reperfused cortical penumbra, whereas Phos H3-, α-tubulin-, and γ-tubulin-immunolabeled cells were frequently seen.

**Lineage of the dividing cells**

To identify a possible cell lineage of the mitotic cells, we used the markers Map-2ab, β-tubulin III, NeuN, and Hu for neuronal differentiation and the astrocyte marker GFAP to perform double immunohistochemistry and immunofluorescence. In some cortical cells, the Phos H3-immunolabeled cell nucleus was enveloped by β-tubulin III (Figs. 4A and 4B), Map-2ab (Fig. 4C), or GFAP-immunopositive cytoplasm (Fig. 4D). Under 3D confocal analysis, Phos H3 was colocalized with NeuN (Figs. 4E, 4F, and 4H) or Hu (Figs. 4I and 4J) in the same cell. In these cells, NeuN labeled the cell nucleus and the perinuclear cytoplasm, a cell labeling pattern that agrees with the original description of NeuN immunolabeling in normal cortical neurons (Mullen et al., 1992; Wolf et al., 1996). In some cortical cells that contained α-tubulin- (Figs. 2A–2F and 2G–2J) or γ-tubulin- (Figs. 3C and 3D) immunolabeled spindles, NeuN immunoreactivity was detected. When the nuclear DNA was transformed into chromosomes...
that were dispersed inside the cytoplasm, NeuN labeled the cytosol correspondingly (Figs. 2A–2F, 2G–2J, and 3C and 3D). In some cells that contained α-tubulin- or γ-tubulin-immunolabeled spindles (Figs. 3E and 3F), GFAP immunoreactivity was detected.

Cell counting

As shown in Table 1, Phos H3 and Map-2ab double-immunopositive cells appeared in the penumbral cortex at 24h poststroke. They maximized at 48 and 72h and then declined at 7 days poststroke.

Discussion

The most significant finding of this study is that cortical cells in the central penumbra, in response to ischemic ring stroke, begin to synthesize nuclear DNA and then commit cell mitosis. These data provide morphological evidence to support the previous finding that the sustained appearance of BrdU-incorporating neurons in the poststroke cerebral cortex in this stroke model represents cortical neurogenesis (Gu et al., 2000) rather than DNA repair (Nowakowski and Hayes, 2000).

Because a cell proliferation-specific marker, BrdU, is incorporated into cell nuclei when cells duplicate their DNA (S phase), this process can be detected immunohistochemically (Gratzner, 1982). As soon as the DNA is duplicated, massive phosphorylation of the nuclear protein histone H3 takes place, initiating cell mitosis (Hans and Dimitrov, 2001; Hendzel et al., 1998; Van Hooser et al., 1998). Immediately after cell mitosis, the phosphorylated histone H3 becomes quickly dephosphorylated (Hendzel et al., 1998). Therefore, Phos H3 detects M-phase cells specifically (Hendzel et al., 1998). In the present study, nuclear BrdU incorporation and Phos H3 expression appear subsequently in the penumbral cortex at 16 and 24h poststroke, respectively. This marks the initial time points at which cortical cells enter the S phase and then the M phase. Therefore, the length of the S phase is approximately 8h and the length of the whole cell cycle is about 8–10h in this stroke model, which agrees with the cell-cycle calculation in mouse brain (Nowakowski et al., 1989, 2002). The concurrence of the mitotic cells with TUNEL-labeled cells but their distinct spatial separation in the same penumbral cortical region even distinguishes poststroke cell mitosis from cell apoptosis. These data are in agreement with the previous report that Phos H3 labels specifically the mitotic cells rather than apoptotic cells (Hendzel et al., 1998). Such a concurrence of cell regeneration and degeneration is normally seen in the olfactory bulbs, where dead neurons are replaced by virtue of continuous neurogenesis (Harzsch et al., 1999). Thus, poststroke cell division in our present study differs from the cell-cycle reentry of CA1 neurons in the hippocampus in adult mice (Kuan et al., 2004). In that study, CA1 neurons started to incorporate BrdU after cerebral hypoxia and ischemia. However, the BrdU-incorporating neurons failed to express Phos H3, which was needed for initiation of cell mitosis. Instead, these neurons became TUNEL positive and were degraded by apoptosis. In human brains with Alzheimer disease, neurons in the hippocampus were reported to reenter the cell cycle and to express Phos H3 (Ogawa et al., 2003). However, this Phos H3 activation was dislocated into the cytoplasm rather than inside the nucleus (Ogawa et al., 2003), resulting in an abortive cell-cycle reentry. In our study, nuclear BrdU incorporation in the penumbral cortex is followed by Phos H3 activation in the same nuclei and spindle formation in the same cells and then nuclear division, the formation of twin nuclei, and finally splitting of the cells. These data suggest that cell mitosis in the poststroke penumbral cortex may be regenerative.

Detection of the neuronal markers of differentiation or of the astrocyte marker in the dividing cells suggests their differentiation toward neurons or astrocytes. It is not clear why the neuronal differentiation markers are expressed in cells in metaphase. The quick appearance of S-phase cells inside the penumbral cortex rather than in other parts of the brain suggests that the dividing cells may be endogenous cortical cells. However, stem cell migration from the SVZ (Ohab et al., 2006) or migration of hematopoietic stem cells into the ischemic cortex might occur as well (Hess et al., 2004). There is also an option that the somatic cortical neurons may undergo some kind of reprogramming and thus function as pluripotent stem cells, as has recently been described (Nakagawa et al., 2008).

The dominating subtype of the Phos H3-immunolabeled cells in the cortical penumbra shifts from neurons at 48h poststroke to nonneurons at 72h. This is probably due to the proliferation of macrophages, microglias, astrocytes (Gu et al., 1999b), and endothelial cells (Gu et al., 2001) at 72h poststroke. The recession of Phos H3 immunolabeling at 7 days poststroke is associated with a substantial numerical increase in the BrdU-incorporating neurons (Gu et al., 2000) and disappearance of the TUNEL-labeled cells in the same cortex. Therefore, transit of the mitotic cells into interphase in the penumbral cortex is associated with cell regeneration, rather than cell death. Nevertheless, certain neurons may die at S phase or early M phase in this stroke model. The proportion of this cell population is difficult to assess and is beyond the scope of the present study. However, at 18 months after photothermotic ring stroke, the BrdU-immunolabeled neurons are dispersed among non-BrdU-immunolabeled neurons in the penumbral cortex (unpublished data), indicating their long-term survival during the poststroke recovery period.

This study of poststroke cell division collected different instances of cell mitosis from various cortical cells of different animals. A continuous recording of single-cell mitosis in the poststroke rat brain should be attempted in the future.

Materials and Methods

Animal preparation

Animal care and all experimental procedures were in accordance with the European Communities’ Council Directive (86/609/EEC). The experimental protocol was approved by the Ethics Committee for Animal Research at Umeå University, Sweden. Nonfasted, 12-week-old male Wistar rats (BK, Sollentuna, Sweden) were subjected to either photothermotic or sham operation (Gu et al., 1999a). To label maximally the cells under DNA duplication, BrdU (10mg/kg; Sigma, St. Louis, MO, USA) was injected intraperitoneally
every 4 h after stroke induction up to 72 h after stroke, then
two times daily, and was ended at poststroke day 7. The rats
were sacrificed at 4, 10, 16, 24, 48, and 72 h and 7 and 14 days
after stroke (n = 4 in each group) or 7 days after sham
operation (n = 4) (Gu et al., 2000). Normal rats without
operation and BrdU delivery were used as BrdU-negative
controls (n = 4).

Immunohistochemistry and Immunofluorescence

Immunohistochemistry was performed on 10-μm-thick par-
affin-embedded brain sections as described previously (Gu et al.,
2000). Single-label immunohistochemistry was con-
ducted using Vectastain ABC-AP-Vector-Blue or Elite Vectas-
tain ABC-Peroxidase-Vector-VIP or DAB kits (Vector
Laboratories, Burlingame, CA, USA). Double immuno histo-
chemistry was conducted by sequential detection of the first
primary antibodies with Vectastain ABC-AP-Vector-Blue and
then the second primary antibodies with Elite Vectastain
ABC-Peroxidase-Vector-VIP. The primary antibodies used in
the study were the DNA duplication marker mouse anti-BrdU
(Becton–Dickinson, San Jose, CA, USA), the neuron-specific
markers mouse anti-Map 2ab (Boehringer Mannheim, India-
napolis, IN, USA) and mouse anti-β3-tubulin III (Promega,
Madison, WI, USA), the astrocyte-specific marker rabbit anti-
GFAP (DAKO, A7S, Glostrup, Denmark), and the mitosis-
specific marker rabbit anti-Phos H3 (Upstate Biotechnology,
Lake Placid, NY, USA).

Immunofluorescence labeling was performed on 10-μm-
thick frozen brain sections. The brain sections were fixed in
4% paraformaldehyde–PBS solution for 30 min and then
washed twice with 0.01 M PBS for 3 min. For BrdU immuno-
labeling, the sections were pretreated with 2 M HCl for 30 min
to denature the DNA. The slides were then immersed in 1%
Trition X-100–PBS for 30 min, washed twice in 0.01 M PBS for
3 min, and blocked with 5% normal goat serum for 30 min.
After overnight incubation with the first primary antibodies
diluted in 0.1% BSA in PBS, the sections were washed in 0.01 M
PBS three times, 3 min each time. After a 20-min blocking
period in 5% normal goat serum in PBS, the brain sections
were incubated for 2 h with the corresponding secondary
antibody conjugated with Alexa Fluor 546 (Molecular Probes,
Eugene, OR, USA) and then rinsed in 0.01 M PBS. When
double-immunofluorescence labeling was performed, the first
primary antibody was detected as mentioned above. The
second primary antibodies, which were raised in animal
species different from those of the first primary antibodies,
were stained using the same procedure as for the first primary
antibodies described, but detected with their corresponding
secondary antibodies conjugated with Alexa Fluor 488
(Molecular Probes). After a short rinse in PBS, the sections
were mounted in Vectashield medium (Vector Laboratories).
When sections were double labeled for mouse anti-β3-tubulin
(Accurate Chemical & Scientific Corp., Westbury, NY, USA),
the Fab blocking technique was used (Wessel and McClay,
1986). Briefly, brain sections were first labeled with mouse
anti-β3-tubulin (Accurate Chemical & Scientific Corp.) and detected with Alexa Fluor 488 goat anti-
mouse IgG F(ab′)2 fragment conjugate. To detect if cross-
reaction between the two primary antibody detecting
systems occurred (Wessel and McClay, 1986), the double-
labeled brain sections were compared with NeuN or γ-tubulin
single-immunofluorescence labeling. In the NeuN and γ-
tubulin double-immunolabeled cells (Figs. 3C and 3D), NeuN
and γ-tubulin marked different cell structures as they
appeared in the corresponding NeuN or γ-tubulin single-
immunofluorescence labeling. Thus the Fab blocking step
stopped sufficiently any cross-reaction between the two
primary antibody detecting systems (Gu et al., 2000). The
primary antibodies used in the present study were the
neuron-specific markers mouse anti-NeuN (Chemicon) and
mouse anti-HuC/HuD (Molecular Probes); an astrocyte-
specific marker, rabbit anti-GFAP (DAKO, A7S, Glostrup, Denmark), and the mitosis-
specific marker rabbit anti-Phos H3 (Upstate Biotechnology,
Lake Placid, NY, USA).

In situ apoptosis detection

The single and double immunofluorescent brain sections
were scanned with a laser-scanning confocal microscope
(Leica SP2) equipped with an argon laser (488 nm excita-
tion), HeNe laser (543 nm excitation), and multiphoton laser
(Tsunami) for excitation of DAPI. To eliminate potential
bleedthrough among the different fluorophores, a separate
fluorophore detection procedure was employed in the

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To examine the specificity of the immunolabeling, primary
antibodies were omitted from the staining procedure in a set
of control experiments. Native animals without BrdU injec-
tions were used as anti-BrdU negative controls (n = 4).

Confocal microscopy

The single and double immunofluorescent brain samples
were scanned with a laser-scanning confocal microscope
(Leica SP2) equipped with an argon laser (488 nm excita-
tion), HeNe laser (543 nm excitation), and multiphoton laser
(Tsunami) for excitation of DAPI. To eliminate potential
bleedthrough among the different fluorophores, a separate
fluorophore detection procedure was employed in the
scanning, i.e., one employing sequential laser excitation in each optical plane. When one fluorophore was scanned, all other channels were closed (i.e., zeroed). Confocal parameters (e.g., pinhole sizes) were adjusted to minimize the thickness of the optical sections. The Huygens System 2 deconvolution algorithm (Scientific Volume Imaging b.v., Hilversum, the Netherlands) was used to process the acquired sets of images and Imais (Bitplane) software to render volume.

Cell counts

Three coronal brain sections through the ischemic lesion were randomly selected from each brain at 24, 48, and 72 h poststroke (n = 3 in each group) and double immunolabeled with Phos H3 and Map-2ab. Cell counting was performed in the postischemic cerebral cortex under a CAST-Grid system (Olympus, Albertslund, Denmark) (Gundersen et al., 1988). The region of interest was defined as the cortical region confined between two artificial delineations from the lateral and interior edges of the ischemic ring lesion down to the corpus callosum. Both the ischemic cortical ring lesion and the centrally located region at risk were thus included in the stereological cell counting. In the 48-h ischemic group, an average of 850 optical dissectors per brain were counted in the cortex ipsilateral to the lesion. In the 72-h ischemic group, an average of 710 optical dissectors per brain were counted in the ipsilateral cortex. In the 24-h and 7-day groups, the Phos H3-immunolabeled cells were too few to be assessed accurately by optical dissectors, so Phos H3-immunolabeled cells were counted directly in the whole sections. The data are expressed as means ± SD.

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