Neurobiology of Disease

Hypoxia–Ischemia Induces DNA Synthesis without Cell Proliferation in Dying Neurons in Adult Rodent Brain

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Recent studies suggest that postmitotic neurons can reenter the cell cycle as a prelude to apoptosis after brain injury. However, most dying neurons do not pass the G_1/S-phase checkpoint to resume DNA synthesis. The specific factors that trigger abortive DNA synthesis are not characterized. Here we show that the combination of hypoxia and ischemia induces adult rodent neurons to resume DNA synthesis as indicated by incorporation of bromodeoxyuridine (BrdU) and expression of G_1/S-phase cell cycle transition markers. After hypoxia–ischemia, the majority of BrdU- and neuronal nuclei (NeuN)-immunoreactive cells are also terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL)-stained, suggesting that they undergo apoptosis. $BrdU^+$ neurons, labeled shortly after hypoxia–ischemia, persist for >5 d but eventually disappear by 28 d. Before disappearing, these $BrdU^+/NeuN^+/TUNEL^+$ neurons express the proliferating cell marker Ki67, lose the G_1-phase cyclin-dependent kinase (CDK) inhibitors p16INK4 and p27Kip1 and show induction of the late G_1/S-phase CDK2 activity and phosphorylation of the retinoblastoma protein. This contrasts to kainic acid excitotoxicity and traumatic brain injury, which produce TUNEL-positive neurons without evidence of DNA synthesis or G_1/S-phase cell cycle transition. These findings suggest that hypoxia–ischemia triggers neurons to reenter the cell cycle and resume apoptosis-associated DNA synthesis in brain. Our data also suggest that the demonstration of neurogenesis after brain injury requires not only BrdU uptake and mature neuronal markers but also evidence showing absence of apoptotic markers. Manipulating the aberrant apoptosis-associated DNA synthesis that occurs with hypoxia–ischemia and perhaps neurodegenerative diseases could promote neuronal survival and neurogenesis.

Key words: **neurogenesis; apoptosis; cell cycle; BrdU; ischemia; hypoxia**

Introduction

Neurons in the adult mammalian brain normally do not divide again once they are born. However, increasing evidence suggests that some postmitotic neurons attempt to reenter the cell cycle after brain injury, as indicated by the induction of cell cycle-associated proteins in multiple experimental paradigms (Copani et al., 2001; Liu and Greene, 2001; Herrup and Arendt, 2002). Moreover, the inhibition of G_1-phase cyclin-dependent kinases (CDKs) is cytoprotective against nerve growth factor deprivation and cerebral ischemia (Park et al., 1997; Osuga et al., 2000). The expression of cell cycle-associated proteins also often predicts the sites of neuronal death in Alzheimer's disease (Busser et al., 1998). These observations suggest that aberrant cell cycle reentry may be an important mechanism of apoptosis in neurological disorders.

If cell cycle reentry is a common mechanism of neurodegeneration, it raises the possibility that damaged neurons could pass the G_1/S-phase checkpoint to resume DNA synthesis. This possibility is supported by several reports of *de novo* DNA synthesis after neuronal injury in tissue cultures or mutant mice (Sanes and Okun, 1972; Al-Ubaidi et al., 1992; Smith et al., 2000; Klein et al., 2002; de Bruin et al., 2003). Furthermore, neurofibrillary tangles in specimens from Alzheimer's disease patients are labeled by an antibody for mitotic phosphoepitopes (Vincent et al., 1996). *In situ* hybridization also demonstrates the presence of polyploid neurons in the brains of the Alzheimer's disease patients (Yang et al., 2001). Together, these results suggest that the coupling of abortive DNA replication and neuronal death may occur in both experimental preparations and human neurodegenerative diseases.

However, most neurons that die after cerebral ischemia and other types of acute brain injury do not appear to resume DNA synthesis. For example, although transient middle cerebral artery

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occlusion (MCAO) induces aberrant cell cycle reentry, the incorporation of a thymidine analog, bromodeoxyuridine (BrdU), is only found in <1% of the terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL)-positive cells (Katchanov et al., 2001; Wen et al., 2004). One interpretation of the small percentage of TUNEL- and BrdU-double-labeled cells is that most neurons are inherently incapable of passing the G_1/S-phase checkpoint even after reentering the cell-cycle. Alternatively, the injured neurons may proceed into necrosis too quickly to enter the S-phase after the MCAO model of stroke (Benchoua et al., 2001). Furthermore, the MCAO model of stroke may fail to trigger the specific signaling responses to overcome the G_1/S-phase checkpoint and induce DNA synthesis.

In the present study, we show that the combination of cerebral hypoxia and ischemia (the Levine/Vannucci model of stroke) can induce large numbers of neurons in the adult rodent brain to enter the S-phase and resume DNA synthesis (Levine, 1960). We also show that although kainic acid excitotoxicity and traumatic brain injury result in many apoptotic neurons, they do not induce DNA synthesis. Finally, although damaged neurons enter the S-phase after cerebral hypoxia–ischemia, they eventually die via apoptosis without giving rise to new neurons. These results suggest that the combination of hypoxia and ischemia is a unique and powerful stimulus of cell cycle reentry and DNA synthesis for neurons in adult brains.

Materials and Methods

Cerebral hypoxia and ischemia model. Adult C57BL/6 mice (25–30 gm body weight) were anesthetized with isoflurane and subjected to permanent occlusion of the unilateral common carotid artery. After recovering from the anesthesia, mice were exposed to hypoxia inside a controlled atmosphere chamber (model 855-AC; PlasLabs) that was infused with 7.5% oxygen. An oxygen analyzer (model 600; ESD) was used to monitor the oxygen concentration inside the chamber. To induce cerebral hypoxia–ischemia in rats, both common carotid arteries were reversibly occluded in 1-month-old rats followed by exposure to 7.5% oxygen inside a computer-controlled hypoxic chamber (In Vivo 400 workstation and Ruskinn gas mixer). The animal procedures were approved by the Institutional Animal Care and Use Committee and conform to the National Institutes of Health *Guide for Care and Use of Laboratory Animals*.

Adrenalectomy, kainic acid excitotoxicity, and traumatic brain injury models. Adult male Sprague Dawley rats (300 gm body weight) were bilaterally adrenalectomized using aseptic procedures with isoflurane anesthesia. To produce status epilepticus, 8-week-old male FVB/N mice were injected intraperitoneally with kainic acid (30 mg/kg) dissolved in 0.1 M phosphate buffer. For traumatic brain injury, adult male Sprague Dawley rats (300–350 gm body weight) were anesthetized using isoflurane and fixed in a steretotaxic apparatus. A craniectomy was performed on the left side of the brain, midway between lambda, bregma, and the temporal ridge using a trephine, the dura being left intact. Focal brain injury was produced using a piston impact model (2.7 mm deep, 4 m/sec velocity, 100 msec duration). These animal procedures were approved by the Institutional Animal Care and Use Committee and conform to the National Institutes of Health *Guide for Care and Use of Laboratory Animals*.

Histology. The antibodies used in this study included mouse monoclonal anti-BrdU (catalog #347580; BD Biosciences, Franklin Lakes, NJ), rat anti-BrdU (MCA2060; Serotec, Oxford, UK), anti-neuronal nuclei (NeuN) (MAB377; Chemicon, Temecula, CA), anti-Ki67 (clone TEC-3; Dako, Carpinteria, CA), anti-phosphohistone H3 (Ser-10, catalog #05-598; Upstate Biotechnology, Lake Placid, NY), anti-p16INK4 (sc-1207; Santa Cruz Biotechnology, Santa Cruz, CA), anti-phosphorylated retinoblastoma (RB; Ser-795, catalog #9301; Cell Signaling), anti-GFAP (catalog #20334; Dako), anti-β-tubulin III (MMS-435P; Covance), and anti-CD11b/OX42 (CBL-1512; Cymbus Biotechnology). BrdU immunocytochemistry was performed using a standard protocol (Miller and Nowakowski, 1988). TUNEL labeling was performed using the alkaline phosphatase (AP)-conjugated sheep anti-digoxigenin (DIG) Fab fragment (catalog #1-093-274; Roche Molecular Biochemicals, Indianapolis, IN; 1:1200 dilution) as a primary antibody to detect DIG-11-dUTP added to the DNA breaks by the terminal deoxynucleotidyl transferase (TdT) followed by using biotinylated anti-sheep antibody and Texas Red or fluorescein-conjugated streptavidin to amplify the signal. For immunofluorescent double labeling of BrdU and TUNEL signals, the TdT-mediated incorporation of DIG-11-dUTP was performed first, followed by treatment of sections with 0.07N NaOH to expose the single-stranded DNA and then simultaneous application of the AP-conjugated sheep anti-DIG Fab fragment and mouse anti-BrdU antibody. Secondary amplification was performed using biotinylated anti-sheep antibody followed by Texas Red-conjugated streptavidin and fluorescein-conjugated anti-mouse antibody. Negative controls for BrdU and TUNEL double labeling were performed by omitting either the BrdU antibody or the TdT enzyme in the TUNEL reaction. Double labeling of BrdU and cell-type markers were performed by 0.07N NaOH treatment first, followed by application of the AP-conjugated sheep anti-DIG Fab fragment and mouse anti-NeuN, rabbit anti-GFAP, or mouse anti-OX42 antibody, respectively. Secondary amplifications of the immunosignals were identical to BrdU–TUNEL double labeling with the only difference of fluorescein-conjugated species-specific antibodies. Epifluorescence images were collected on a Leica (Nussloch, Germany) DMIRE2 microscope. Confocal fluorescence images were collected using a Zeiss (Thornwood, NY) LSM510 microscope. Green and red fluorophores were excited using the argon ion (488 nm) and HeNe (543 nm) visible lasers, respectively. To increase the confidence in the colocalization analysis, care was taken to collect potentially overlapping emissions separately using the "multitrack" function. For orthogonal projections, a stack of 40–50 confocal images that were 0.45 μm apart were typically collected and analyzed.

Biochemistry. Glutathione *S*-transferase-RB-C-terminal domain fusion protein was used as substrate to measure the kinase activity of the anti-CDK2 immunoprecipitate. Antibodies used in this study were anti-CDK2 (sc-163; Santa Cruz Biotechnology), anti-p27Kip1 (sc-528; Santa Cruz Biotechnology), anti-Cyclin D1 (catalog #06-137; Upstate Biotechnology), and, anti-histone H1 (sc-8030; Santa Cruz Biotechnology).

Results

Adrenalectomy, but not the kainic acid excitotoxicity or traumatic brain injury, induces DNA synthesis in apoptotic cells

We first examined the naturally occurring neurogenesis in the adult dentate gyrus to validate our BrdU and TUNEL double-labeling procedures. In unchallenged, normal control mice injected with BrdU (50 mg/kg, i.p.), BrdU-positive cells were detected in the subgranular zone of the hippocampal dentate gyrus, and they were nonpyknotic and negative for TUNEL staining (Fig. 1*A–D*). This result is consistent with that from previous studies (e.g., Cooper-Kuhn and Kuhn, 2002) and validates the specificity of our BrdU and TUNEL double-labeling procedures.

We then explored the possibility of death-associated DNA synthesis by applying TUNEL and BrdU double labeling to three experimental models of brain injury. First, we examined adrenalectomy-induced degeneration of granule cells in the rat dentate gyrus (Sloviter et al., 1989). Previous studies showed that the removal of both adrenal glands causes a gradual depletion of granule cells over 3–4 months, with dying cells displaying morphological features of apoptosis (Sloviter et al., 1993). We administered a single dose of BrdU (50 mg/kg) on either the third or sixth day after adrenalectomy and killed the animals 24 hr later for analysis ($n = 4$ for both time points). A few pyknotic nuclei were found sporadically distributed in the granule cell layer of the dentate gyrus (Fig. 1*E*). These pyknotic nuclei were both BrdU- and TUNEL-positive, suggesting that DNA synthesis by apopto-

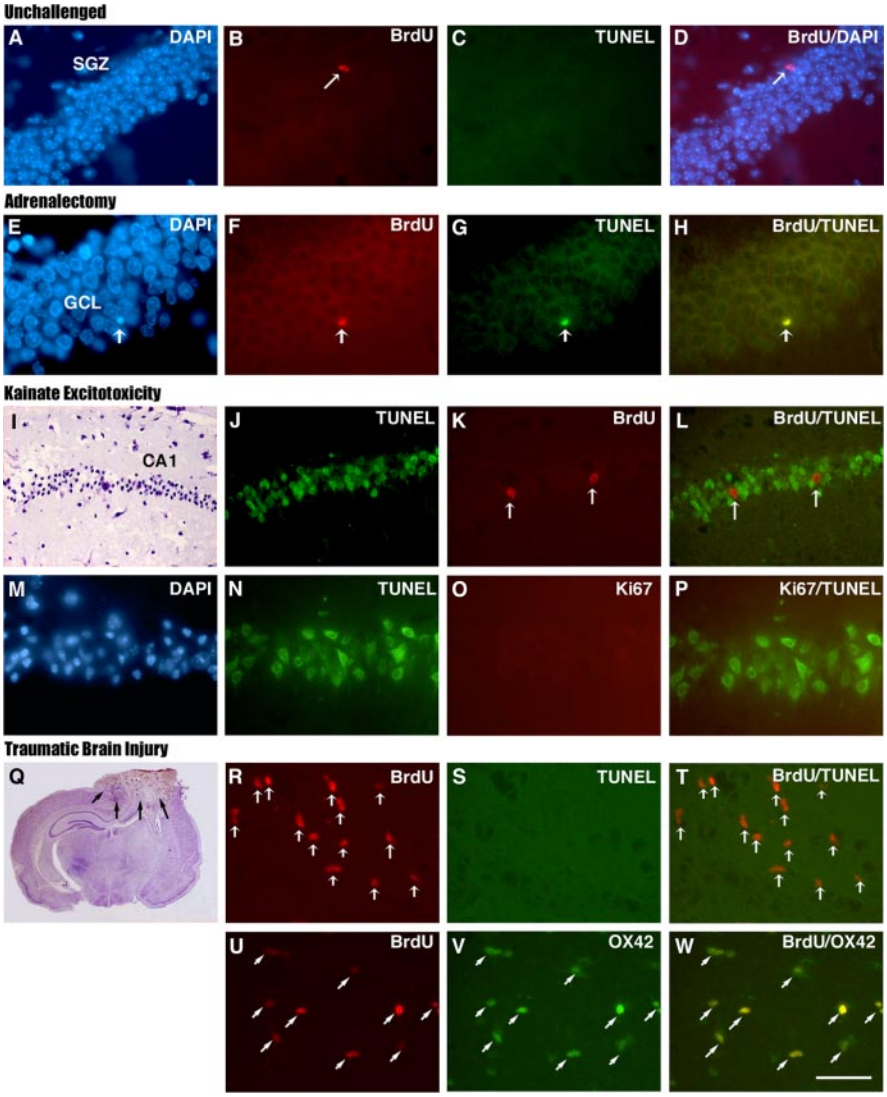

Figure 1. Not all dying neurons incorporate BrdU. *A–D*, In unchallenged mice injected with BrdU (50 mg/kg), BrdU-incorporated nuclei that were negative for TUNEL staining were detected in the subgranular zone (SGZ) of the dentate gyrus. *E–H*, In adult rats injected with BrdU (50 mg/kg) on the sixth day after adrenalectomy and killed 1 d later, BrdU incorporation was detected in the granule cell layer (GCL) of the dentate gyrus. The BrdU-positive nucleus (*F*) was pyknotic (*E*) and colabeled for TUNEL staining (*G, H*). *I–P*, In mice injected with BrdU (50 mg/kg) on the second day after the kainic acid challenge (30 mg/kg) and killed on the next day, a large number of pyknotic and TUNEL-positive nuclei were detected in the CA1– hippocampal subfield (*I, J, M, N*). In contrast, only a few BrdU-positive nuclei were detected in the CA1– hippocampal subfield (*K*), and a merged image shows that they were segregated from the TUNEL-staining signals (*L*). The TUNEL-positive cells were also negative for the proliferative cell marker Ki67 (*O, P*). *Q–W*, In rats that received six doses of BrdU (100 mg/kg, i.p.) and were killed 72 hr after a traumatic brain injury, many BrdU-positive cells were found in the area surrounding the site of the piston impact (*Q*, arrows). The BrdU-incorporated cells were negative for TUNEL labeling (*R–T*). In contrast, most BrdU-incorporating cells were positive for the macrophage/microglial cell marker OX42 (*U–W*). Scale bar: *A–H, M–P*, 150 μm; *I–L, R–W*, 100 μm.

tic neurons can be induced by adrenalectomy (Fig. 1*F–H*). The small number of TUNEL- and BrdU- positive cells may be caused by a gradual onset of cell loss but fast elimination of the corpses after adrenalectomy (Hu et al., 1997).

Next, we examined the excitotoxic death of hippocampal neurons induced by systemic injection of the kainic acid (Schauwecker and Steward, 1997; Yang et al., 1997). Kainic acid (30 mg/kg) was injected intraperitoneally to adult FVB/N mice to induce seizures ($n = 8$). BrdU (50 mg/kg) was administered on the second day after kainic acid injection, and the mice were killed 24 hr later for analysis. A large number of pyknotic and TUNEL-positive cells were found in the CA1 hippocampal subfield (Fig. 1*I,J*). However, only a few BrdU-stained cells were detected in the same area, and they were negative for TUNEL staining (Fig. 1*K,L*). Because the injection of a single dose of BrdU may not coincide with DNA synthesis after the kainic acid challenge, we examined whether the hippocampal neurons express the proliferative cell marker Ki67 (Scholzen and Gerdes, 2000). None of the TUNEL-positive cells in the CA1– hippocampal subfield were positive for Ki67 staining (Fig. 1*M–P*). This result suggests that kainic acid excitotoxicity does not induce degenerating hippocampal neurons to reenter the cell cycle.

Finally, we examined the cell death induced by traumatic brain injury. Adult rats were anesthetized by isoflurane and subjected to a unilateral piston impact while held in a stereotaxic apparatus (Strauss et al., 2000). The challenged rats were injected with BrdU (100 mg/kg) every 12 hr for 3 d and killed at 72 hr after injury for analysis ($n = 6$). Many BrdU-positive cells were detected around the site of impact, but they were negative for TUNEL staining (Fig. 1*Q–T*). In contrast, many of the BrdU-positive cells were positively stained for a macrophage–microglial cell marker, OX42, indicating that they are newly born immune response cells that had infiltrated the zone of injury (Fig. 1*U–W*).

Taken together, these results suggest that, although DNA synthesis by apoptotic neurons may occur in certain types of brain injury such as adrenalectomy, it is not a universal phenomenon and is not observed after kainate injections or experimental head trauma.

Hypoxia–ischemia induces massive cell death and BrdU incorporation in the hippocampus

We next examined whether the Levine model of cerebral hypoxia and ischemia can induce dying neurons to synthesize DNA. To perform the Levine model, adult C57Bl/6 mice (25–30 gm body weight) were anesthetized with isoflurane and subjected to permanent occlusion of the right common carotid artery. After recovering from anesthesia, the mice were exposed to 7.5% oxygen and 92.5% nitrogen for 4 hr inside a hypoxic chamber before returning to the ambient environment. Consistent with previous reports (Levine, 1960; Rice et al., 1981), we found that unilateral occlusion of the common carotid artery or hypoxia alone did not cause apparent brain damage (Fig. 2*A*). In contrast, the combination of both insults induced massive cell loss in the CA1– hippocampal subfield by 24 hr in 60% of the animals (Fig. 2*B*). By 3 d after the Levine model of stroke, the CA1– hippocampal subfield was densely populated by pyknotic nuclei that showed typical morphological features of apoptosis, including condensation of the chromatin and preser-

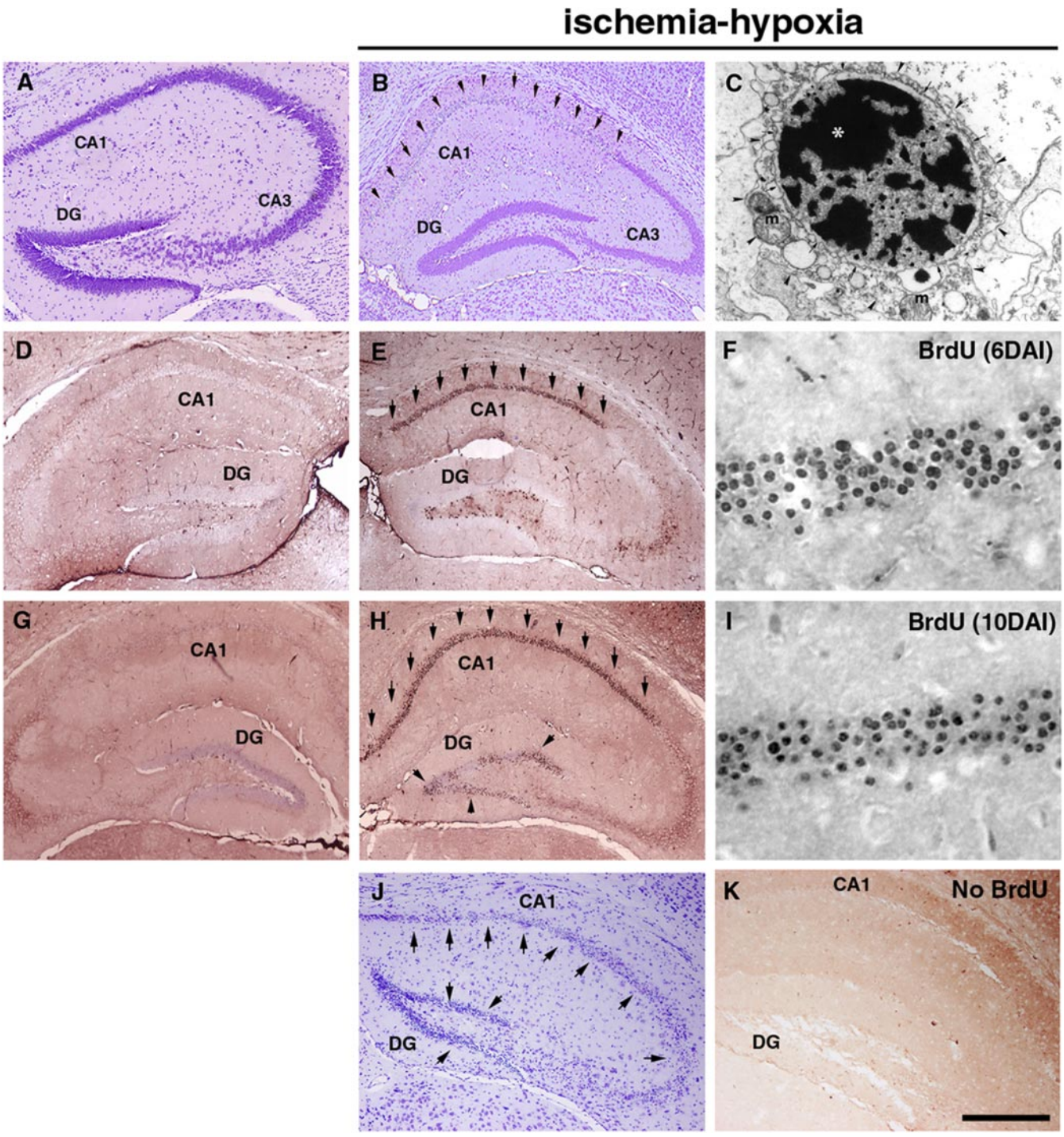

Figure 2. Cerebral hypoxia and ischemia induce neurodegeneration and BrdU incorporation in the same region of the hippocampus. *A, B*, Nissl stain shows that unilateral common carotid artery occlusion or hypoxia alone does not cause damage to the hippocampus (*A*). In contrast, the combination of both injures the CA1–pyramidal hippocampal subfield (*B*, arrows). *C*, Electron microscopy shows characteristics of apoptosis, including condensation of the chromatin (asterisk) but preservation of the mitochondria (m) and nuclear and cytoplasmic membranes (arrows) of pyknotic cells in the CA1– hippocampal subfield. *D–I*, A single dose of BrdU (50 mg/kg) injected on the 6th (*D–F*) or 10th (*G–I*) day after hypoxia–ischemia was taken up in the damaged CA1 neurons (*E, H*, arrows) but not in the unaffected neurons in the contralateral hippocampus (*D, G*). Higher magnification photomicrographs show the BrdU immunoreactivity inside the pyknotic nuclei in the CA1–hippocampal subfield (*F, I*). *J, K*, No BrdU immunoreactivity (*K*) is detected in the pyknotic nuclei in the hippocampus (*J*, arrows) of the mice that did not receive a BrdU injection after cerebral hypoxia–ischemia. Scale bar: *A, B, D, E, G, H*, 2 mm; *C*, 1 μm; *F, L*, 60 μm; *J, K*, 400 μm. DG, Dentate gyrus.

vation of the nuclear and cellular membranes (Fig. 2*C*). By killing the mice at various times after hypoxia–ischemia, we determined that the pyknotic and TUNEL-positive nuclei persist in the injured hippocampus for as long as 15 d (data not shown), a situation quite different from the most other ischemia models that produce injury to striatum or cortex (Katchanov et al., 2001).

To test whether the Levine model of stroke can induce DNA synthesis in neurons, a single dose of BrdU (50 mg/kg) was injected intraperitoneally into mice on the 2nd, 6th, 10th, and 14th days after hypoxia–ischemia. Mice were killed at 24 hr after BrdU injection for immunocytochemistry (Miller and Nowakowski, 1988). A large number of BrdU-labeled cells were detected in the

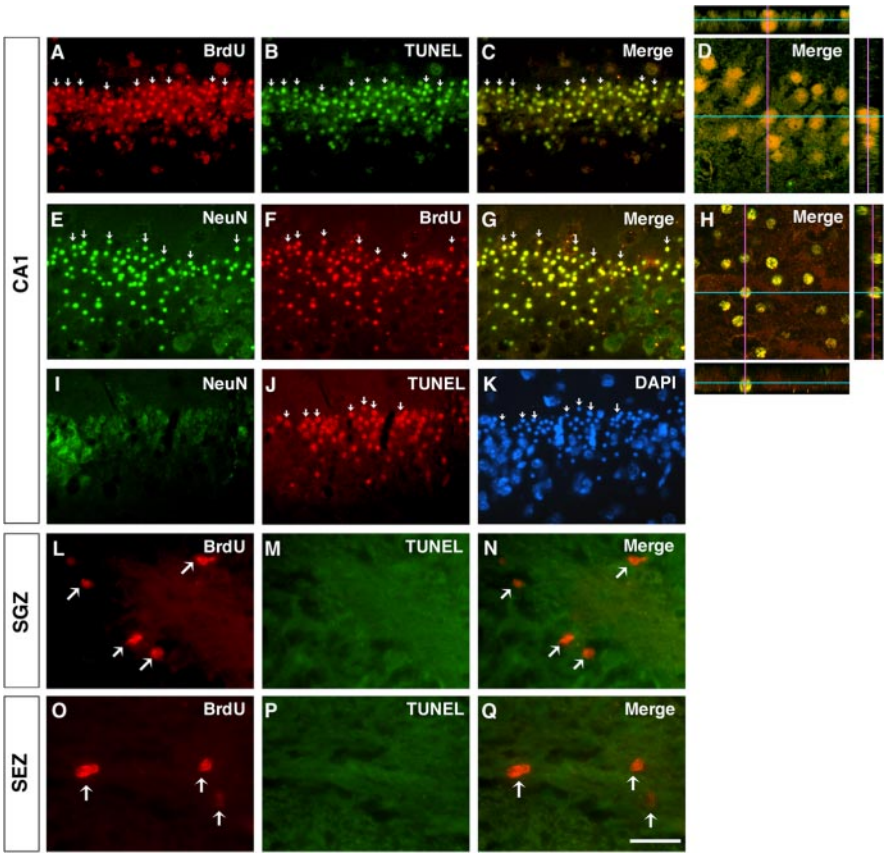

Figure 3. Colocalization of TUNEL and BrdU staining in postmitotic neurons but not in the endogenous neural progenitors after cerebral hypoxia–ischemia. *A–C*, Immunostaining for BrdU (*A*), TUNEL labeling of the damaged hippocampus (*B*), and the merged image (*C*) reveal extensive colocalization of BrdU and TUNEL signals on the seventh day after hypoxia–ischemia. *D*, Confocal images of merged BrdU and TUNEL labeling and orthogonal projections confirm the localization of both signals within the same nucleus. *E–H*, Double labeling of NeuN (*E*) and BrdU (*F*) also reveals extensive colocalization of both signals on the seventh day after hypoxia–ischemia in the merged image (*G*) and three-dimensional confocal analysis (*H*). *I–K*, Immunostaining for NeuN (*I*), TUNEL labeling (*J*), and DAPI staining (*K*) show that the pyknotic nuclei under the DAPI stain were TUNEL-positive but devoid of the NeuN antigen on the 11th day after hypoxia–ischemia. *L–N*, In contrast to BrdU–TUNEL double labeling in the injured CA1 region, endogenous neural progenitor cells in the subgranular zone of the dentate gyrus were BrdU-positive (*L*) but negative for TUNEL staining (*M, N*). *O–Q*, Endogenous neural progenitor cells in the subependymal zone of the cortex after cerebral hypoxia–ischemia remain BrdU-positive (*O*) but TUNEL-negative (*P, Q*). Scale bar: *A–C, E–G, I–K*, 80 μm; *D, H*, 20 μm; *L–Q*, 150 μm.

injured CA1–hippocampal subfield but not on the nondamaged, contralateral side of brain (Fig. 2*D–I*). The Levine model also occasionally injures the dentate gyrus, and when this happened, BrdU incorporation was found in the granule cell layer in the dentate gyrus (Fig. 2*H*, arrows). The BrdU incorporation is a consistent finding for all time points of BrdU injection after ischemia ($n > 6$ each for 2, 6, 10, and 14 d after ischemia). Furthermore, the BrdU staining is specific because our immunocytochemistry protocol does not detect the pyknotic nuclei in the mice that were challenged by hypoxia–ischemia but were not injected with BrdU ($n = 6$) (Fig. 2*J,K*).

The observed coincidence of the BrdU incorporation in the CA1 region where pyramidal neurons die suggested that cerebral hypoxia–ischemia may induce apoptotic neurons to synthesize DNA. We therefore performed BrdU and TUNEL double labeling to further test this possibility. Numerous BrdU-incorporating cells were colabeled by TUNEL staining, an indication of DNA fragmentation and apoptosis, in the injured CA1 hippocampal subfield (Fig. 3*A–C*). The colocalization of BrdU and TUNEL signals in the same nucleus was confirmed by three-dimensional confocal image analysis (Fig. 3*D*). Interestingly, the BrdU-incorporated nuclei were transiently labeled for the neuron-specific antigen NeuN in the first week after cerebral hypoxia–ischemia (Fig. 3*E–H*). By the second week after hypoxia–ischemia, the BrdU-incorporated nuclei became NeuN-negative (Fig. 3*I*) but remained TUNEL-positive (Fig. 3*J*) and pyknotic using the 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) stain (Fig. 3*K*). In contrast, no BrdU and TUNEL double-positive cells were found in the subgranular zone of the dentate gyrus or the subependymal zone that harbor endogenous neural progenitor cells (Fig. 3*L–Q*). Taken together, the extensive colocalization of BrdU and TUNEL labeling in the CA1–hippocampal neurons suggests that cerebral hypoxia–ischemia can induce apoptotic neurons, pyramidal neurons in the CA1 region of hippocampus, to resume DNA synthesis.

Apoptotic neurons display hallmarks of the G_1/S-phase cell cycle transition

We next examined possible pathways by which hypoxia–ischemia triggers BrdU-incorporation into apoptotic neurons after cerebral hypoxia and ischemia. We tested whether the apoptotic neurons exhibit features that are consistent with the G_1/S-phase transition. For immunofluorescent staining, we used DAPI as a counterstain to demonstrate dying cells that have pyknotic nuclei and have positive TUNEL staining (Fig. 3*J,K*) compared with the surviving cells with nonpyknotic nuclei.

First, we found that Ki67, a marker for proliferating cells at all phases of the cell cycle, is expressed exclusively in the pyknotic but not in the nonpyknotic nuclei in the hippocampus (Fig. 4*A–C*, compare arrows, asterisks). It is important to point out that Ki67 is specific for proliferating cells and cannot be induced by DNA damage and repair (Scholzen and Gerdes, 2000). Second, hippocampal extracts of the wild-type mice demonstrate higher CDK2 activity, a late G_1/S-phase transition marker, as early as 4 hr after hypoxia–ischemia (Fig. 4*G*). To provide a comparison, CDK2 activity was only slightly induced in *Jnk3*-null mice that are resistant to glutamate excitotoxicity and hypoxia–ischemia, suggesting that the CDK2 response is associated with brain injury (Yang et al., 1997; Kuan et al., 2003). Third, there is a consistent but modest reduction of the protein level of the G_1-phase CDK inhibitor p27kip1 in hippocampal extracts from the wild-type but again not in the *Jnk3*-null mice (Polyak et al., 1994; Toyoshima and Hunter, 1994; Sheaff, 1997) (Fig. 4*H*). The modest reduction of p27Kip1 protein suggests that it may be reduced only in a subset of cells in the hippocampus. To test this possibility and extend our analysis, we examined the expression of another G_1-phase CDK2 inhibitor, p16INK4, after hypoxia–ischemia (Serrano et al. 1993). We found that p16INK4 was selectively depleted in the pyknotic but not in the nonpyknotic cells (Fig. 4*I,L*, compare arrows, asterisks). Previous

studies have shown that null mutation of the G_1-phase CDK inhibitors *p27Kip1* and *p19INK4d* cause postnatal neuronal proliferation in these mice (Zindy et al., 1999). Thus, the observed reduction of p27Kip1 and P16INK4 may promote the G_1/S-phase transition after cerebral hypoxia–ischemia. Finally, the increased CDK2 activity observed should lead to hyperphosphorylation of the RB protein (Cobrinik et al., 1992). Indeed, intense staining of phosphorylated RB (Ser-795) was detected in the injured hippocampus but not in the nondamaged, contralateral side of the brain (Fig. 4*J,K,M,N*). Moreover, immunoreactivity for phosphorylated RB colocalized exclusively with the pyknotic nuclei, skipping the nonpyknotic cells (Fig. 4*J,M*, compare arrows, asterisks). Together, these results suggest that cerebral hypoxia–ischemia can induce hippocampal neurons to reenter the S-phase and to resume DNA synthesis.

To test how far apoptotic nuclei can progress along the cell cycle, we examined the expression of phosphorylated histone H3 as a late G_2/M-phase marker (Hendzel et al., 1997). Notably, no phosphorylated histone H3 (Ser-10) was detected in the damaged hippocampus at multiple time points after cerebral hypoxia–ischemia (Fig. 4*D,E*). In contrast, our staining protocol readily detected phosphorylated histone H3 in the ventricular zone of embryonic day 13.5 mouse cortex (Fig. 4*F*). The failure to detect phosphorylated histone H3 after cerebral hypoxia–ischemia suggests that most BrdU-incorporating neurons are unable to complete cell division.

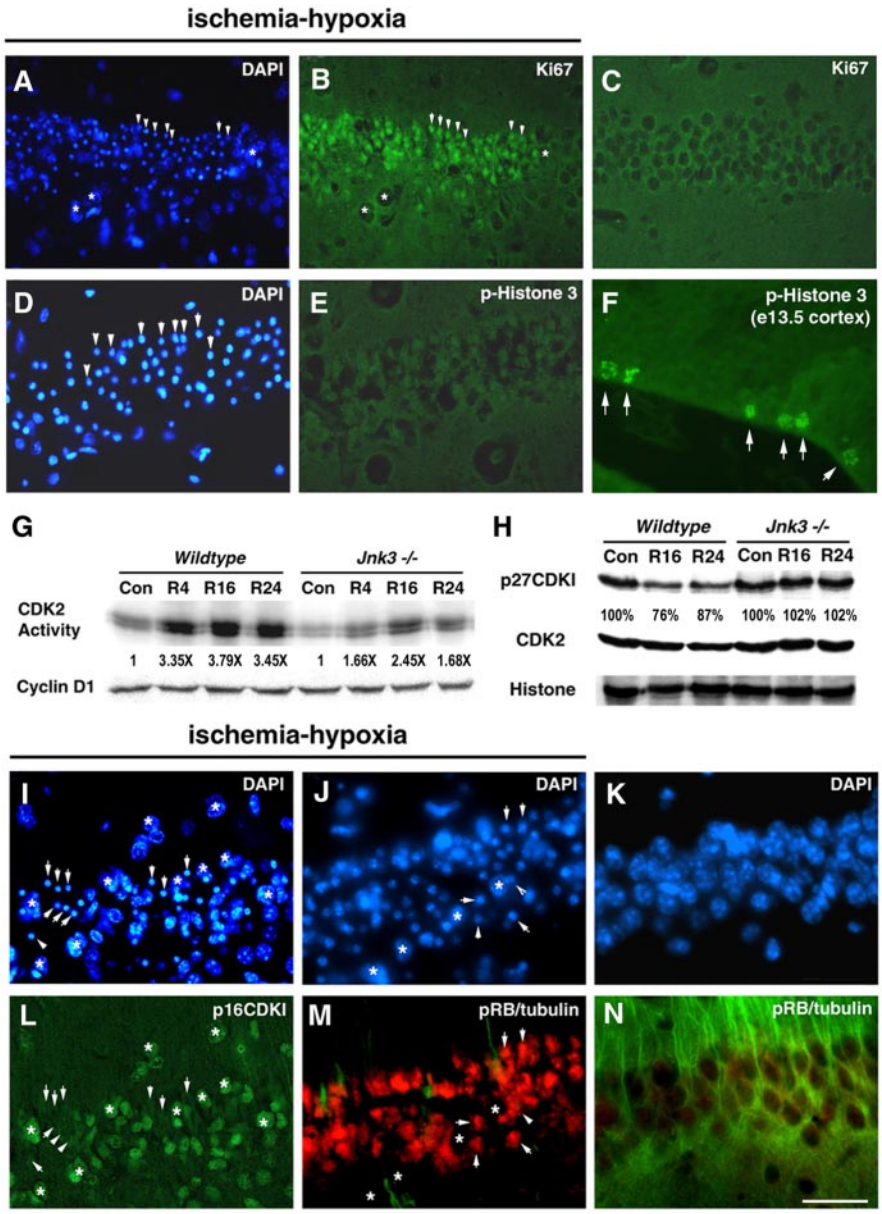

Figure 4. Apoptotic neurons enter the S-phase of the cell cycle after cerebral hypoxia–ischemia. *A–C*, The proliferative cell marker Ki67 is induced by hypoxia–ischemia in the affected hippocampus (*A, B*) but not on the opposite side (*C*). Note that the expression of Ki67 is restricted to the pyknotic neuronal nuclei (arrows) but not the nonpyknotic nuclei (asterisks) using the DAPI stain. *D–F*, The late G_2/M-phase marker phosphohistone H3 (Ser-10) immunoreactivity is not detected in the pyknotic nuclei after hypoxia–ischemia (*D, E*) but is present in the cerebral cortex ventricular zone of 13.5 d mouse embryos in control experiments (*F*, arrows). *G*, The CDK2 activity is greater in wild-type than in *Jnk3*-null mice hippocampi at 4 (R4), 16 (R16), and 24 (R24) hr after hypoxia–ischemia. There is no change in the protein level of cyclin D1. The hippocampal extracts from unchallenged mice were used as controls (Con) of the basal level of CDK2 activity and cyclin D1 protein level. *H*, The protein level of the p27Kip1 CDK inhibitor but not CDK2 is reduced at 16 and 24 hr after hypoxia–ischemia in the wild-type mice with less effect in *Jnk3*-null mice. The protein level of histone H1 was used as loading control in the immunoblots. *I, L*, The expression of p16INK4 CDK inhibitor protein is absent in the pyknotic nuclei (arrows) but not in the nonpyknotic nuclei (asterisks) after hypoxia–ischemia. *J, M*, Double labeling of phosphorylated RB (red) and neuron-specific β-tubulin III (*M*, green) in conjunction with the DAPI stain (*J*) shows the selective induction of phosphorylated RB in the condensed pyknotic nuclei (arrows) but not in the normal-sized nuclei (asterisks) after cerebral hypoxia–ischemia. *K, N*, High levels of β-tubulin III (green) and very little phosphorylated RB (*N*, red) are expressed in the hippocampal nuclei (*K*) on the contralateral side. Scale bar, 100 μm.

BrdU-incorporated neurons only temporarily exist in the hippocampus after cerebral hypoxia–ischemia

To trace the fate of BrdU-incorporated neurons after cerebral hypoxia–ischemia, we administered a single dose of BrdU (50 mg/kg) intraperitoneally to the mice on the 2nd day after stroke and killed the mice on the 7th or 28th day for the BrdU immunocytochemistry analysis. Because the systemically administered BrdU is quickly cleared from the body within 1 hr (Packard et al., 1973; Cameron and McKay, 2001), this experiment allows us to "pulse label" the hippocampal neurons that were synthesizing DNA on the second day after stroke and "chase" the outcomes of these cells at intervals of 5 and 26 d, respectively. We found that at a 5 d interval, the CA1–hippocampal subfield remained densely populated by pyknotic and BrdU-labeled nuclei (Fig. 5*A,B*). Quantification showed that 87 ± 4.3% of the CA1–hippocampal neurons were pyknotic, and 81 ± 5.1% were BrdU-positive (Fig. 5*C*) ($n = 6$). Confocal laser microscopy analysis also showed extensive colocalization of the TUNEL and BrdU signals inside the hippocampal pyramidal neuron layer (Fig. 5*D–F*). In addition, there were a few larger BrdU-positive but TUNEL-negative nuclei both inside and outside the pyramidal neuron layer that could have been macrophages or reactive astrocytes (Fig. 5*D,F*, arrows). The high percentage of BrdU- and TUNEL-double-positive nuclei at the seventh day after hypoxia–

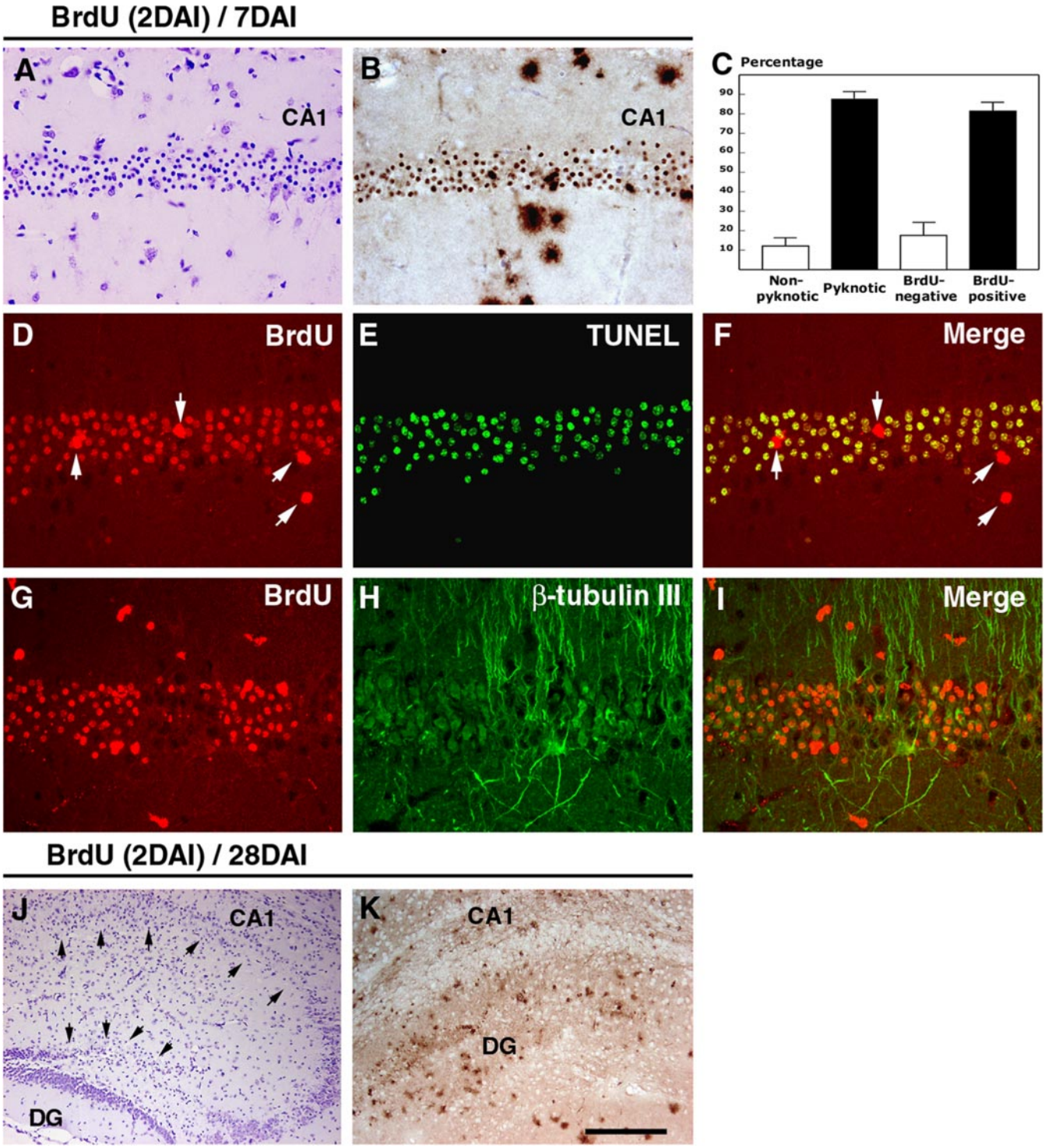

Figure 5. BrdU-incorporated neurons persist for several days but eventually die in the damaged brain. *A–C*, Nissl stain and BrdU immunocytochemistry show that the CA1– hippocampal sector is densely populated by pyknotic (87.3% of total cells; SD, 4.3%) (*A, C*) and BrdU-positive (81.6% of total cells; SD, 5.1%) (*B, C*) nuclei in the mice that received a single injection of BrdU (50 mg/kg) on day 2 and were killed on day 7 after hypoxia–ischemia (DAI). *D–F*, Confocal microscopy analysis reveals extensive colocalization of BrdU (*D, F*) and TUNEL (*E, F*) in the condensed nuclei in the CA1– hippocampal subfield, whereas noncondensed BrdU-positive nuclei were TUNEL-negative (*D, F*, arrows). *G–I*, BrdU-positive nuclei (*G, I*) did not colocalize with staining for the mature neuron marker β-tubulin III (*H, I*), suggesting that BrdU-incorporated cells do not differentiate into mature neurons. *J, K*, By the 28th day after cerebral hypoxia–ischemia, the damaged CA1– hippocampal subfield is mostly devoid of pyknotic nuclei (*J*) and not populated by BrdU-positive cells (*K*) in the mice that received a single injection of BrdU on the 2nd day after the hypoxia–ischemia. Scale bar: *A, B*, 80 μm; *D–I*, 60 μm; *J, K*, 400 μm. DG, Dentate gyrus.

ischemia suggests that neurons can persist for several days after BrdU incorporation in the damaged brain.

Interestingly, none of the BrdU-labeled nuclei were colocalized with β-tubulin-III staining, a differentiated neuron marker that quickly disappears in injured neurons (Fig. 5*G–I*). The lack of BrdU and β-tubulin-III colocalization suggests the BrdU-incorporated cells do not become mature neurons. This scenario is consistent with the extensive BrdU–TUNEL double labeling

and further supported by the disappearance of BrdU-positive cells in the hippocampal neuron layer in the mice that were analyzed 26 d after BrdU injection (Fig. 5*J,K*) ($n = 6$). Together, these results suggest that most BrdU-positive neurons only temporarily exist in the brain, but they eventually vanish without contributing to regeneration after cerebral hypoxia–ischemia

Abortive DNA synthesis is not an anomaly of the mouse CA1 hippocampal neurons

It was surprising that the Levine model induced far more BrdU and TUNEL double-labeled cells than the more commonly used MCAO model of stroke (for example, Katchanov et al., 2001). The stark contrast suggests that the combination of hypoxia and ischemia may be a potent trigger of cell cycle reentry and DNA synthesis. Alternatively, because the Levine model of stroke primarily injures the hippocampus, whereas the MCAO model mainly damages the striatum, the observed DNA synthesis by apoptotic cells might be a unique property of the mouse CA1 hippocampal neurons.

To distinguish between these two possibilities, we set out to test whether the combination of hypoxia and ischemia can induce cell cycle reentry in different types of neurons in rats. We found that the typical Levine procedure (unilateral occlusion of the common carotid artery followed by hypoxia) did not produce obvious brain lesions in rats, presumably because of the differences of the cerebrovascular supply between the two species (Fig. 6*A*) ($n = 6$). However, bilateral occlusion of the common carotid arteries followed by 30 min of hypoxia (7.5% oxygen) can induce hippocampal and cortical lesions in 80% of the rats (Fig. 6*B,D*) ($n = 15$). When a single dose of BrdU (50 mg/kg) was injected into the rats on the second day after hypoxia–ischemia, it labeled a large number of cells in the injured hippocampus and cerebral cortex (Fig. 6*C,E,F*). Furthermore, more BrdU-positive cells were located in the outer border than in the ischemic core (Fig. 6*D–F*). Confocal laser microscopic analysis showed that most BrdU-positive nuclei were segregated from the staining of an astroglial marker, GFAP (Fig. 6*G–I*), but colocalized with a neuron marker, NeuN (Fig. 6*J–L*), and TUNEL staining (Fig. 6*M–O*). Together, these results suggest that DNA synthesis by apoptotic neurons is not a unique property of the mouse CA1–hippocampal neurons but, rather, a general response to the combination of cerebral hypoxia and ischemia.

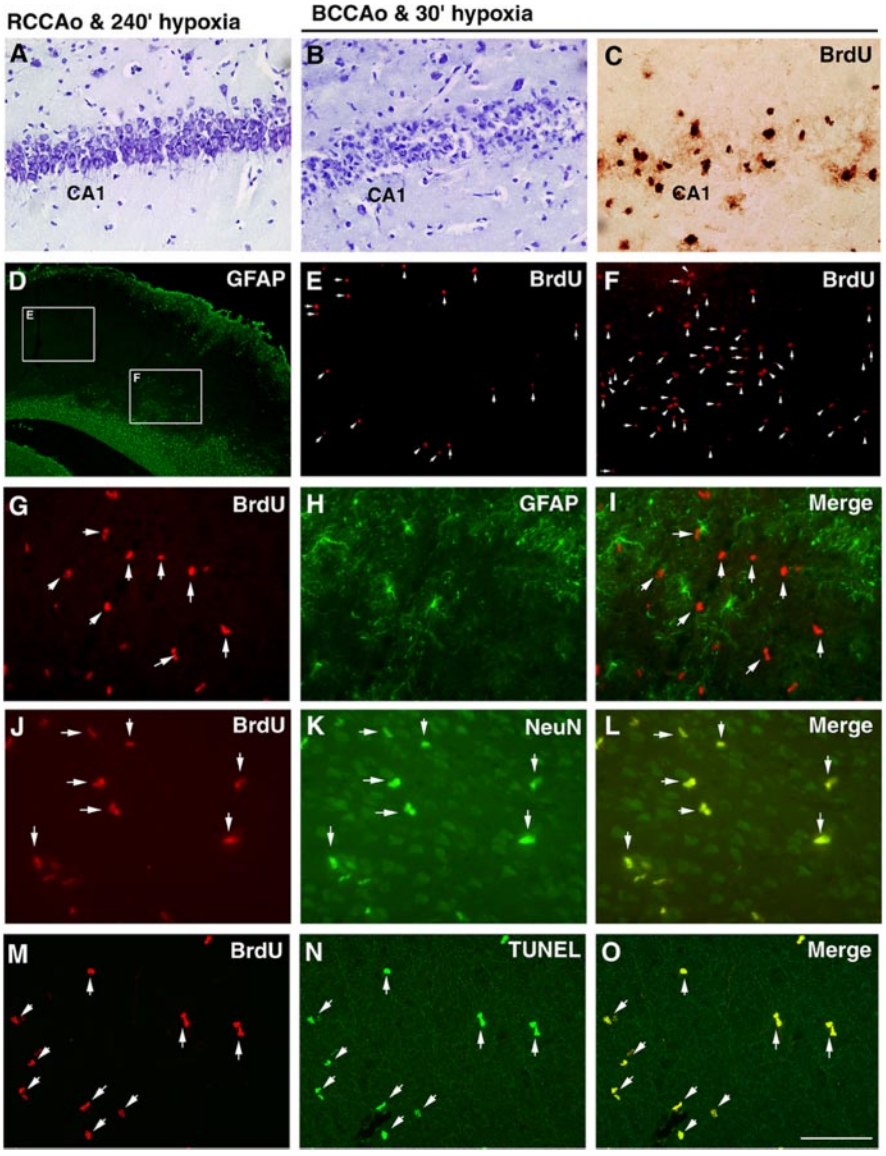

Figure 6. The combination of cerebral hypoxia and ischemia also induces cortical neurons to incorporate BrdU in rats. *A–C*, Occlusion of the right common carotid artery (RCCAo) followed by 4 hr of hypoxia does not produce detectable damage in the rat brain (*A*). In contrast, the occlusion of bilateral common carotid arteries (BCCAo) followed by 30 min of hypoxia causes neuronal damage (*B*) and BrdU labeling (*C*) in the CA1– hippocampal subfield. *D–F*, BrdU labeling was performed simultaneously with GFAP staining (*D*) to delineate the boundary of cerebral infarction. Many more BrdU-positive cells were found in the marginal zone (*F*) than in the core of infarction (*E*). *G–O*, Confocal microscopic imaging shows colocalization of BrdU and NeuN staining (*J–L*) and double labeling of BrdU and TUNEL (*M–O*) but segregated BrdU and GFAP staining (*G–I*) in the ischemic– hypoxic border zone in the cortex. Scale bar: *A, B, E, F*, 200 μm; *C, G–O*, 100 μm; *D*, 1 mm.

Discussion

Neurons in the adult brain are traditionally viewed as having permanently exited the cell cycle and entered a quiescent state (G_0-phase). However, recent evidence shows that mature neurons can reenter the cell cycle, which may be an important mechanism of cell death (Liu and Greene, 2001; Herrup and Arendt, 2002). Despite the recent progress in understanding the connection between aberrant cell cycle reentry and neuronal cell death, it remains uncertain what conditions favor the entry of dying neurons into the S-phase and apoptosis- and death-associated DNA synthesis. The current study shows that hypoxia and ischemia, along with adrenalectomy, are potent stimuli for dying, apoptotic cells to reenter the cell cycle. Kainate-induced status epilepticus, head trauma, and traditional middle cerebral artery occlusion stroke models, on the other hand, produce TUNEL-stained, apoptotic cells without inducing DNA synthesis. The data presented here are the first to show apoptotic cell death that may or

may not lead to abortive DNA synthesis depending on the type of injury or disease that had occurred previously.

Although the expression of cell cycle-associated proteins has been reported in almost every brain injury model, there are only a few reports showing unequivocal evidence of DNA synthesis by degenerating neurons *in vivo* (Al-Ubaidi et al., 1992; Yang et al., 2001; Klein et al., 2002; de Bruin et al., 2003). For example, the MCAO stroke model induces expression of CDK4, cyclin D1, cyclin B1, E2F1, phosphorylated RB, and a large number of TUNEL-positive cells (Osuga et al., 2000: Wen et al., 2004), but a similar study showed that only <1% of the TUNEL-positive nuclei after MCAO had incorporated BrdU (Katchanov et al., 2001). The small percentage of BrdU- and TUNEL-double-positive cells suggests that, using this particular model of focal ischemia, mature neurons very rarely pass the G_1/S-phase checkpoint, even though they reenter the cell cycle. Alternatively, the MCAO model may not activate the signaling pathways necessary for passing the G_1/S-phase checkpoint, or there may not be enough time for injured neurons to enter the S-phase before they disappear.

To begin to distinguish between these possibilities, we compared cell cycle reentry and DNA synthesis following the Levine model of stroke (Levine, 1960; Rice et al., 1981; Kuan et al., 2003), adrenalectomy, kainate-induced excitotoxicity, and acute head trauma. The data show that the majority of the apoptotic CA1 hippocampal neurons can reenter the cell cycle, pass the G_1/S-phase checkpoint, and resume DNA synthesis after hypoxia–ischemia. It is striking that the hypoxia–ischemia model of stroke used here strongly induces DNA synthesis in apoptotic neurons in contrast to the small percentage of TUNEL- and BrdU-double-positive cells in the MCAO model (Katchanov et al., 2001). Because cerebral hypoxia and ischemia also induce many BrdU- and TUNEL-double-positive cells in the rat cortex, the abortive DNA synthesis is unlikely a unique property of the mouse CA1 hippocampal neurons that are preferentially destroyed by the Levine model of stroke. Although it is unclear why the Levine model stimulates DNA synthesis in dying neurons, it is possible that hypoxia and ischemia produce more severe oxidative stress to induce DNA damage and activate cell cycle reentry (Shackelford et al., 2000; Kruman et al., 2004). However, we also show that adrenalectomy resulted in modest but definite numbers of TUNEL- and BrdU double-positive cells. This would suggest that the type of injury is not the only determining factor because adrenalectomy probably produces apoptotic granule cell neuronal cell death via loss of a trophic factor (Sloviter et al., 1989, 1993). By administering BrdU on day 2 and killing the mice on either day 7 or day 28 after cerebral hypoxia–ischemia, we showed that CA1 neurons persist in the damaged brain for at least 5 d after having incorporated BrdU and disappear by 28 d. Because most neurons disappear within 2–4 d after MCAO ischemia, it is possible that the prolonged neuronal cell death after hypoxia–ischemia provides sufficient time for induction of cell cycle genes necessary for new DNA synthesis in the dying neurons.

Taken together, we postulate that a critical factor that determines whether the apoptotic cells pass the G_1/S-phase checkpoint and synthesize new DNA is how long the apoptotic cells survive after the initial injury. The idea that protracted survival in lethally injured, apoptotic neurons leads to neurons that make new DNA is supported by the finding of DNA synthesis preceding cell death and polyploid neurons in Alzheimer's disease brains (Yang et al., 2001, 2003; Herrup and Arendt, 2002). Together, these data would support the possibility that injured neurons can undergo DNA synthesis once or even several times in parts of the genome as long as they do not die immediately after the initiating apoptotic injury or disease.

Whether degenerating neurons can resume DNA synthesis is also an important issue concerning the use of BrdU incorporation as a marker of neurogenesis in the adult brain. The incorporation of BrdU only signifies DNA synthesis without assurance that BrdU-incorporated cells complete the cell cycle. If dying neurons can reenter the S-phase to resume DNA synthesis, they may be mistaken for new neurons. Thus, the criteria for adult neurogenesis ideally should include evidence of DNA synthesis and neuronal maturation and absence of any evidence of apoptosis, as shown in the present study. However, it is not always possible to demonstrate colocalization of DNA synthesis and neuronal maturation and absence of apoptotic markers in occasions of only a few BrdU-incorporated cells scattered in the injured brain. Moreover, if damaged neurons can be rescued from death after synthesizing DNA by external factors, they may be interpreted as mature and newly born neurons based on the incorporation of BrdU. Therefore, it is prudent to evaluate in each model of brain injury whether there is neurogenesis, whether dying, apoptotic neurons synthesize DNA and incorporate BrdU, and whether a treatment rescues damaged neurons that have incorporated BrdU.

It should be emphasized that our results do not negate the studies of brain injury-induced proliferation of neural progenitors (Liu et al., 1998; Jin et al., 2001; Arvidsson et al., 2002; Nakatomi et al., 2002; Steindler and Pincus, 2002). Our results simply indicate that the incorporation of BrdU alone cannot be taken as sufficient evidence of neurogenesis in models of brain injury and that multiple markers using a variety of independent methods are needed to reach definite conclusions as to whether new neurons have been born in adult mammalian brain (Rakic, 2002; van Praag et al., 2002).

In conclusion, we have shown that cerebral hypoxia and ischemia are potent stimuli for death-associated DNA synthesis in postmitotic neurons. Future studies are needed to characterize the downstream signaling pathways that are induced to foster cell cycle reentry and G_1/S-phase transition. Finally, the cerebral hypoxia–ischemia model may also be useful for testing whether stimulation of cell cycle progression and/or inhibition of apoptosis may lead to regeneration of new neurons after stroke.

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