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# Cortical Neurogenesis in Adult Brains After Focal Cerebral Ischemia

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## 1. Introduction

Stroke ranks as the most common reason to disable adult patients and the second most common reason for mortality. Current treatment of ischemic stroke through thrombolysis or thrombectomy aims to initiate successful early reperfusion into the ischemic penumbral tissue. If it is promptly and properly performed, it may reverse the ischemic cascade and rescue the ischemic penumbra from being further recruited into infarct and thus improve neurological outcome. Once the infarct is formed, no treatment is currently available to enhance the post stroke brain repair. Under physiological conditions, neurons in the cerebral cortex are terminally differentiated shortly after birth. The phenomenon that a cavity usually forms in the post stroke adult brain elicits the histological postulation that adult brains do not have the capacity to generate new neurons in the cerebral cortex after pathological insults i.e., stroke. Nevertheless, neurological recovery of various degrees is commonly seen in stroke patients with clinical improvement that starts from a week after stroke onset and may last up to 18 months. The underlying mechanisms for this recovery are only sparsely understood, although many factors have been suggested such as deafferentiating, activity-dependent synaptic changes, altered membrane excitability, and outgrowth of axons and dendrites.

We have been interested in the post stroke brain repair through cell regeneration in the adult brains. One animal model being used for this purpose is the photothrombotic ring stroke in adult rats (Gu et al. 1999a; Hu et al. 1999; Hu et al. 2001; Wester et al. 1995) and mice (Jiang et al. 2006). This stroke model features a large anatomically predefined cortical penumbra (spatially confined by a ring-shaped ischemic locus) in the somatosensory cortex that undergoes critical hypoperfusion and subsequently spontaneous reperfusion (Gu et al. 1999a). Quick induction of immediate early genes in the penumbral cortex (Johansson et al. 2000) is followed by neuronal necrosis and apoptosis with progressively altered neuropil and nerve cell morphology that reach their maximum severity at 48 hours after stroke onset (Gu et al. 1999b; Hu et al. 2002). With a spontaneous reperfusion into the penumbral cortex starting at 72 hours, a remarkable morphologic restoration of the nerve cells starts, that evolves into a chiefly unremarkable cytologic appearance at 7 to 28 days after stroke induction (Gu et al. 1999b). To investigate the mechanism behind the dramatic

morphological recovery in the reperfused penumbral cortex, we examine the cell proliferation process in the post stroke cerebral cortex by in vivo delivery of a DNA duplication marker 5-bromodeoxy-uridine (BrdU) into the post stroke animals that is detected by single/double immunohistochemistry / immunofluorescence (Gu et al. 2000). The thymidine analogue BrdU is incorporated into cell DNA during the S-phase of a cell cycle, which can be detected immunohistochemically. BrdU cell labelling is currently a standard method to identify cell proliferation. Surprisingly, widespread BrdU-incorporated cells are consistently observed in the penumbral cortex at 48h and 72h after photothrombotic ring stroke. While the majority of these BrdU-immunopositive cells are proliferating astrocytes and macrophages, 3% to 6% of them are double-immunolabelled by BrdU and one of the neuron-specific marker Map-2 or beta-tubulin III at 7 and 100 days after stroke onset. Three dimension confocal analyses show colocalization of the neuron-specific marker NeuN and the BrdU in the same cells, suggesting stroke induced cortical neurogenesis in the adult cerebral cortex (Gu et al. 2000). To examine the generalizability of this novel finding, the rat model of reversible focal cerebral ischemia by middle cerebral artery (MCA) suture occlusion is also studied (Jiang et al. 2001). In this stroke model, reperfusion is induced mechanically through a withdrawal of the suture at 2h after MCA occlusion. Similarly with our previous findings, widespread BrdU single-immunopositive cells appear in the postischemic cerebral cortex, corpus callosum, striatum and dentate gyrus of the hippocampus ipsilateral to the ischemic infarct at 30 and 60 days after MCA occlusion. Approximately 6-10% of the BrdU positive cells are double immunopositive to one of the neuron markers Map-2, beta-tubulin III or NeuN in the penumbral cortex, indicating a long time survival of the newborn neurons in the post stroke cortex (Jiang et al. 2001).

To trace the origin of the post stroke newborn neurons in the photothrombotic ring stroke model in adult rats (Gu et al. 2009), BrdU is repeatedly injected. Brain sections are collected at different time points after stroke induction and examined by BrdU immunohistochemistry so that the initial spatial appearance of the proliferating cells and their possible migration inside the brains can be evaluated. To detect ongoing cell mitosis or cell death, the M-phase specific marker phosphorylated histone H<sub>3</sub> (Phos H<sub>3</sub>) and the spindle components  $\alpha$ -tubulin/ $\gamma$ -tubulin are examined by double immunofluorescence with the DNA duplication marker BrdU or nuclear apoptosis marker TUNEL. Cell type is ascertained by double immunolabeling with the neuronal markers Map-2ab/ $\beta$ -tubulin III and NeuN/Hu or the astrocyte marker GFAP. From 16h poststroke, BrdU-immunolabeled cells appear initially in the penumbral cortex. From 24h after stroke induction, Phos H<sub>3</sub> starts to be expressed in the penumbral cortex that is colocalized with BrdU in the same nuclei. Meanwhile, mitotic spindles immunolabeled by  $\alpha$ -tubulin/ $\gamma$ -tubulin appear inside the cortical cells containing BrdU-immunopositive nuclei. Unexpectedly, the markers of neuronal differentiation, Map-2ab/ $\beta$ -tubulin III/NeuN/Hu, are expressed in the Phos H<sub>3</sub>-immunolabeled cells, and NeuN is detected in some cells containing spindles. These data suggest that endogenous cells with neuronal immunolabeling may duplicate their nuclear DNA and commit cell mitosis to generate daughter neurons in the penumbral cortex, which contribute to the very early cortical neurogenesis in this stroke model in adult rats (Gu et al. 2009). This is in contrast with the reports of cortical neurogenesis after MCA occlusion where neural progenitor cells migrate from subventricular zone into post stroke cortex at 7-14 days after MCA occlusion that contributes to a late stage post stroke neurogenesis (Kreuzberg et al. 2010; Tsai et al. 2006). Therefore, stroke induced cortical neurogenesis may

originate either from remote progenitor cell migration or in situ cell division depending on different stroke models used and various phases studied.

The functional status of the observed post stroke neurogenesis is one of our major interests. To elucidate a conceivable functional capacity, neurotransmitter synthesis is studied in the post ischemic rat brains subjected to photothrombotic ring stroke and subsequent BrdU delivery (Gu et al. 2010). In order to detect a possible synthesis of neurotransmitters in the newborn cortical neurons, single/double/triple immunofluorescence cell labelling is performed with the neurotransmitter marker acetylcholine (Ach) and its substrate enzyme choline acetyltransferase (ChAT), the neurotransmitter marker GABA and the substrate enzyme glutamic acid decarboxylase (GAD) and BrdU. Among the BrdU-immunolabeled newborn cells at 48h, 5 days, 7 days, 30 days, 60 days and 90 days after stroke, some of these are doubly immunopositive to the cholinergic neuron-specific marker ChAT or GABAergic neuron-specific marker GAD. As analyzed by 3-D confocal microscopy, the neurotransmitters Ach and GABA are colocalized with BrdU in the same cortical cells. In order to confirm the neuronal identity of these neurotransmitter synthesizing newborn cells, NeuN, BrdU and GABA triple immunofluorescence is performed. Under 3-D confocal analyses, the BrdU-immunolabeled newborn cell which is synthesizing GABA is further triple-immunolabeled by NeuN. These data suggest that the newborn neurons are capable of synthesizing the neurotransmitters acetylcholine and GABA in the penumbral cortex (Gu et al. 2010), which is one of the fundamental requisites for these neurons to function during the poststroke recovery.

Stroke is the most common reason to handicap humans in the adult life and the second most common reason for clinical mortality. Post stroke patient care is one of the largest economical burdens in modern society. About 85% of clinical stroke is ischemic origin. A sudden occlusion or severe stenosis of a large or small cerebral artery leads to quick decrease of local cerebral blood flow in the brain tissue down to two critical penumbral thresholds, i.e., electrophysiological and membrane thresholds that differs the ischemic penumbra from ischemic core. If not promptly treated, ischemic penumbra is quickly recruited into the ischemic core and paninfarct of the brain tissue ensues. Aphasia, hemiplegia, and hemianopia are among the most common neurological deficits that handicap stroke patients. Intravenous thrombolysis within the 4.5 hours time window and thrombectomy within the 6 hours time window in MCA or vertebrobasilar stroke are the current treatment for acute ischemic stroke. Prompt recanalization may be achieved in some of the stroke patients that is associated with better clinical outcome. Once a cerebral infarct is formed, no specific treatment is currently available to enhance the poststroke brain repair.

Historically neurons were believed not to be regenerated in the adult brains (Ramón y Cajal 1913). In 1960s, Altman and Das reported neurogenesis in the hippocampus in post natal rats (Altman and Das 1965a). Through [<sup>3</sup>H]-thymidine DNA labelling and histology, they found that some cells that were [<sup>3</sup>H]-thymidine labelled in their nuclei had neuron morphological appearance in the postnatal hippocampus. Using the same DNA labelling technique, Altman reported further that neurogenesis persists in olfactory bulbs (Altman 1969). Proliferation and migration of neural stem cell from subventricular zone along a rostral migrating stream into olfactory bulbs was traced to be the cell resource for a life time continuous neurogenesis in the olfactory bulbs (Altman and Das 1965b). Proliferation of neural stem cells in the subgranular layer and their migration into granule layer contributed

to the continuous neurogenesis in the dentate gyrus of the hippocampus (Altman and Bayer 1990). In recent years neurogenesis in the dentate gyrus of hippocampus and olfactory bulbs has been extensively studied in different species including humans. Neurogenesis in these brain regions becomes enhanced or inhibited under different physiological and pathological conditions such as physical exercise, learning, stress, depression, drugs and stroke (Brown et al. 2003; Eriksson et al. 1998; Kempermann et al. 1997; Liu et al. 1998; Samuels and Hen. 2011; van Praag et al. 1999).

Ischemic stroke within the middle cerebral artery territory (MCA) is the most common form of stroke in humans. With a MCA stroke, tissue infarct occurs in part of cerebral cortex, striatum, or both. Aphasia, hemiplegia, apraxia, and hemineglect are among the most common neurological deficits to handicap the patients. Under physiological conditions, neurons in the cerebral cortex are terminally differentiated in the adult brains (Rakic 1985). Several weeks after ischemic stroke, a cavity usually appears in the infarct brain region, suggesting an inadequate capacity of the adult brain to compensate the cell loss through regeneration of neurons and glial cells. Nevertheless, spontaneous neurological improvement of various degrees occurs in the majority of poststroke patients. It starts from days after stroke, maximizes in the first 3 months, and may last up to 18 months (Hankey et al. 2007; Twitchell 1951). The pathophysiological mechanisms responsible for the poststroke spontaneous neurological improvement are sparsely understood. Emission of ischemic edema, spontaneous reperfusion of the ischemic penumbra, reversal of ischemic neuronal damage, sprouting of collateral axons and local dendrites, unmasking of potential neuronal pathways, induction of activity dependent synaptic activity, altered membrane excitability have been proposed to be the potential pathways.

In order to meet various purposes of experimental stroke studies, different animal models of focal cerebral ischemia have been established. One of the experimental stroke models being used is photothrombotic stroke in adult rodents. Hence, reproducible thrombosis can be induced photochemically in the cortex of adult rodents, wherein the ischemic lesion may be placed in any desirable location (Watson et al. 1985). In the photothrombotic "ring" version of this model, a large anatomically predefined cortical penumbra is induced in the somatosensory cortex of adult rats (Wester et al. 1995) within the ischemic annulus of the ring. The interior of the ring annulus is concentrically encroached by the radially expanding ring annulus, thus simulating penumbral stroke-in-evolution in an inherently reproducible fashion (Fig. 1). Microvascular platelet thrombi appear in the cortical lesion (Gu et al. 1999a; 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 (Fig. 1) (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, 24, and 48h after ischemia and then gradually recovers to 56 and 87% of baseline values at 72 and 96h (Gu et al. 1999c).

Dramatic changes in tissue morphology take place in the cortical penumbra (Fig. 2). Neuronal necrosis and apoptosis with a progressively altered neuropil prevail at 24–48h post ischemia (Gu et al. 1999b; Hu et al. 2002), reaching their maximum severity at 48h after stroke with most of the neurons exhibiting eosinophilia and pyknosis (Gu et al. 1999b). Meanwhile, VEGF-mediated angiogenesis is initiated in the same penumbral cortex to facilitate a late spontaneous reperfusion (Gu et al. 2001). At 72h after ischemia, a remarkable morphologic

restoration of the nerve cells in the penumbra cortex starts, which evolves into a chiefly unremarkable cytologic appearance at 7 to 28 days after stroke induction (Gu et al. 1999b).

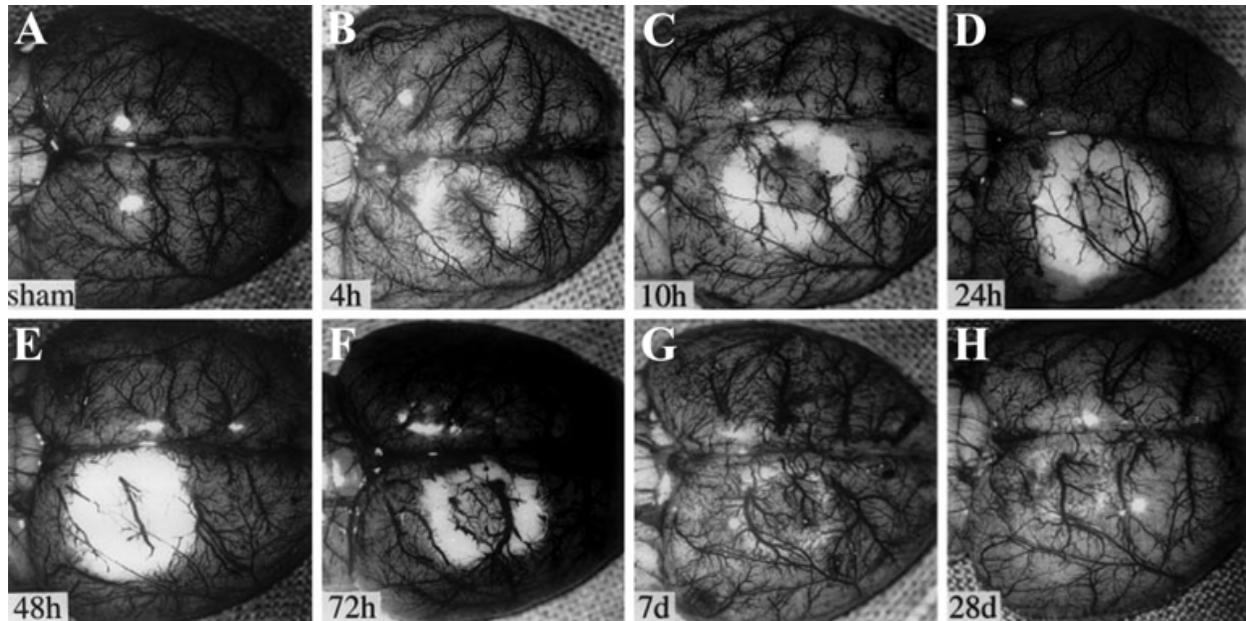


Fig. 1. Photograph of brain samples from post stroke (B-H) and sham-operated (A) rats that are transcidentally perfused with carbon-black. A ring-shaped cortical-perfusion deficit is consistently observed on the surface of somatosensory cortex at 4h post ischemia (B). It progressively increases at 10h (C), 24h (D), and reaches its maximum at 48h (E). Thus, the centrally located penumbral cortex enclosed by the ring-shaped ischemic locus looks pale, with a branch of the distal middle cerebral artery being narrowed, but not completely occluded. At 72h postischemia (F), the distal MCA with its small branches in the penumbral cortex becomes patent, representing reperfusion. It becomes more pronounced at 7 days (G) and 28 days (H) after stroke induction. (From Gu W, Jiang W, Wester P., *Exp Brain Res* 125:163-170, 1999. With permission from Springer-Verlag Heidelberg)

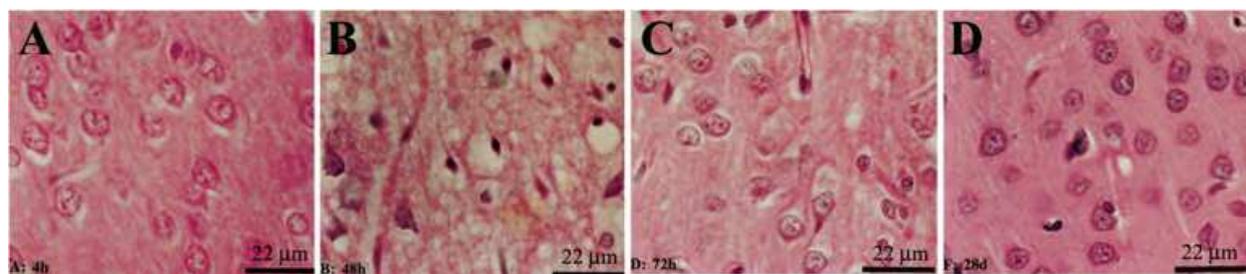


Fig. 2. Hematoxylin and Eosin (HE) staining of the coronal brain sections through the epicenter of the ring lesion showing tissue morphology in the central penumbral cortex. (A) At 4h post stroke induction, the neuropil is slightly pale and the nerve cells are mildly swollen. (B) At 48h after stroke, the majority of the nerve cells are eosinophilic in their cytoplasm and pyknotic and hyperchromatic in their nuclei. The neuropil is pale and edematous. (C) At 72h after stroke, dramatic recovery of tissue morphology is seen in the penumbral cortex, where only mild cytological changes, e.g., somal and nuclear swelling are observed. (D) At 28 days after ischemia, tissue morphology becomes unremarkable. (From Gu et al., *Exp Brain Res* 125:171-183, 1999. With permission from Springer-Verlag Heidelberg)

To explain this remarkable morphologic restoration, we assumed that cell proliferation might have occurred in the reperfused cortical penumbra in this stroke model (Gu et al. 2000). To test this hypothesis, the cell proliferation-specific marker 5-bromodeoxyuridine is delivered into the poststroke rats through repeated intraperitoneal injections (Gu et al. 2000). As a thymidine analogue, BrdU is incorporated into the cell nuclei when proliferating cells duplicate their DNA during the S-phase, which can be detected immunohistochemically (Gratzner 1982; Miller and Nowakowski 1988). After each injection, BrdU lasts for about two hours for cell uptake (Nowakowski et al. 1989). Repeated BrdU injection maximizes the chance for brain cells that proliferate at different times post stroke to incorporate BrdU into their nuclei. To minimize the possibility of any potential neurotoxic effect of BrdU to the ischemic penumbral tissue, a relative low dose of BrdU injections is used, i.e., 10 mg/kg at each delivery as compared with 75 to 120 mg/kg used elsewhere (Craig et al. 1999). To follow up a possible long-term survival of the newborn cells in the post stroke cortex, the animals are sacrificed either on the same day after the last BrdU delivery or on day 14 or on day 20 after the last BrdU injection.

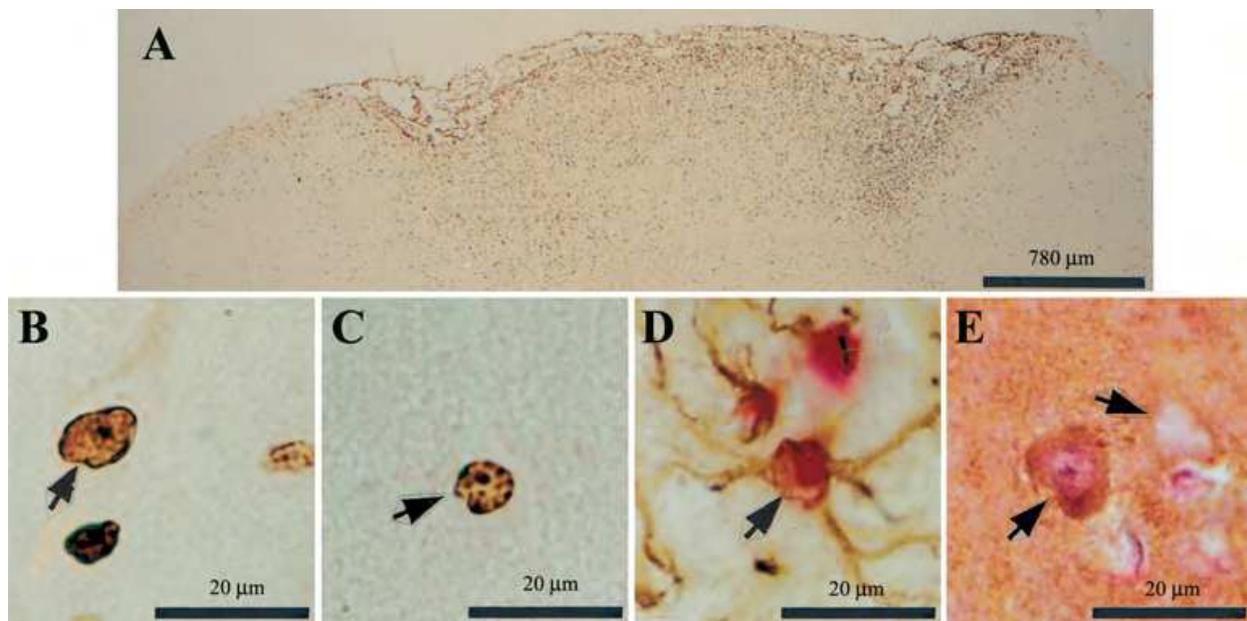


Fig. 3. BrdU immunohistochemistry in rat brains after photothrombotic ring stroke (A) BrdU single immunohistochemistry at 7 days after photothrombotic ring stroke. Widespread BrdU-immunolabeled cells (brown) are observed in the cerebral cortex near the two wedge-shaped infarct cores and the penumbral cerebral cortex that are lying between. (B) A BrdU-immunolabeled cell (arrow, brown) in the penumbral cortex exhibits a large round cell nucleus with a single nucleolus in the center at 7 days after stroke. (C) A BrdU-immunolabeled cell (arrow, brown) in the penumbral cortex exhibits a large round cell nucleus with a single nucleolus in the center at 100 days after stroke. (D) A newborn astrocyte in the penumbral cortex is BrdU-immunolabeled in the nucleus (arrow, red) and GFAP-immunopositive in the cytoplasm (brown) at 7 days post ischemia. (E) A newborn cell in cortical layer II in the penumbral cortex evinces a BrdU-immunolabeled cell nucleus (arrow left, red) and Map-2 immunopositive cytoplasm (brown) at 100 days after stroke induction. (From Gu W, Brannstrom T, Wester P., *J Cereb Blood Flow Metab* 20:1166-1173, 2000. With permission from Nature Publishing Group)

Detected through BrdU immunohistochemistry, widespread BrdU-incorporated cells are consistently observed in the penumbral cortex and the ischemic core at 7 days after stroke induction (Fig. 3A). At 100 days after ischemia, these cells are still seen, though the BrdU nuclear labeling is generally faded as compared with that observed at 7 days post ischemia. In the penumbral cortex, the majority of the BrdU incorporated cells at 7 days post ischemia represent glial cells ( $62 \pm 9.5\%$  of the total  $113 \pm 34$  BrdU single labeled cells counted per brain), macrophages ( $20 \pm 9.7\%$ ), and endothelial cells ( $12 \pm 3.7\%$ ). The corresponding proportions at 100 days after stroke are  $84 \pm 2.1\%$  for glial cells (out of a total  $82 \pm 30$  BrdU single-labeled cells counted per brain),  $3.2 \pm 3.9\%$  for macrophages, and  $8.8 \pm 5.1\%$  for endothelial cells. However, some of the BrdU-immunolabeled cell nuclei exhibit neuronal morphologic characteristics, that is, a large round nucleus with a single nucleolus in the center (Fig. 3B-C). To identify the cell lineage of these newborn cortical cells, the neuron-specific marker Map-2ab and the astrocyte specific marker GFAP are employed for double immunohistochemistry with BrdU (Fig. 3D-E). Among the dominant newborn astrocytes (Fig. 3D) in the post stroke penumbral cortex, we are surprised to see that some cells that are BrdU-immunolabeled in their nuclei are further Map-2-immunopositive in their cytoplasm (Fig. 3E), which suggests that these newborn cells are neurons (Gu et al. 2000). These cells are scattered randomly in cortical layers II-VI in the penumbral cortex and count for  $3.3 \pm 0.3\%$  of the total  $1405 \pm 108$  BrdU single-labeled cells per brain counted at 7 days, and  $5.8 \pm 1.4\%$  of the total  $745 \pm 95$  BrdU-positive cells counted per brain at 100 days after stroke induction.

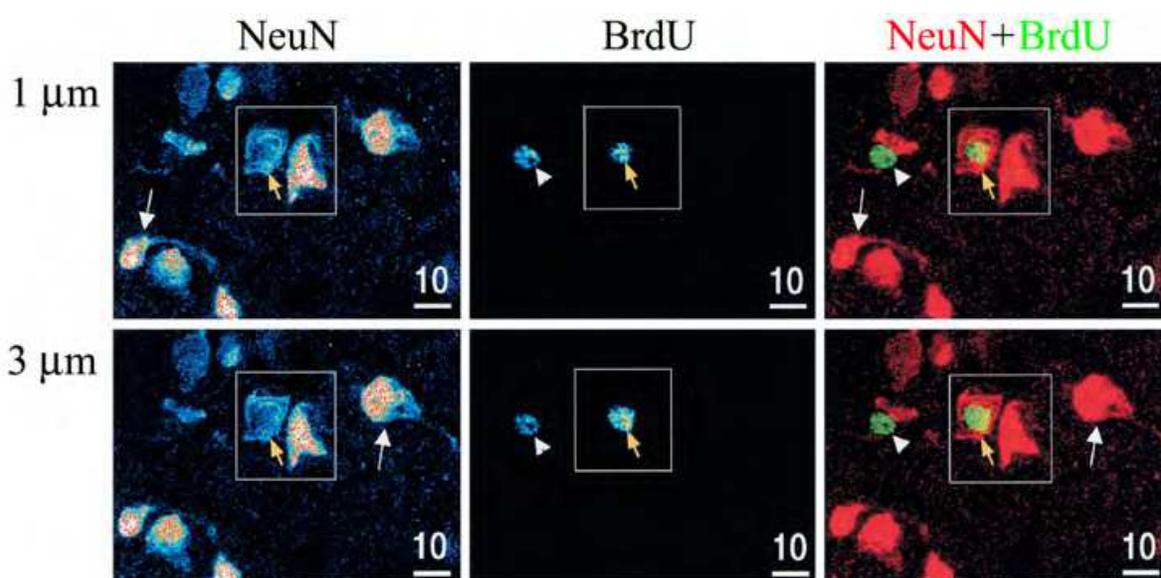


Fig. 4. 3D confocal analyses of NeuN and BrdU double immunofluorescence in the penumbral cortex at two adjacent Z-series planes at 30 days after photothrombotic ring stroke. The left column shows the signal intensity for NeuN, the middle column displays the signal intensity for BrdU, and the right column exhibits a merged image of the NeuN (red) and BrdU (green). The cell designated by the yellow arrows in square is both NeuN-immunopositive (left) and BrdU-immunopositive (middle), resulting in a yellow NeuN and BrdU double-immunopositive nucleus in the merged image (right). White arrowheads indicate a neighbouring NeuN-negative but BrdU-positive nonneuronal newborn cell. White arrows outside the square are pointed at two NeuN-immunopositive but BrdU-negative mature neurons. (From Gu W, Brannstrom T, Wester P., *J Cereb Blood Flow Metab* 20:1166-1173, 2000. With permission from Nature Publishing Group)

To confirm the co-localization of BrdU with a neuron-specific marker within the same cortical cells after stroke, three-dimensional confocal analyses of BrdU and NeuN double immunofluorescence is conducted (Fig. 4). At 3 days and 30 days after stroke induction, the neuron-specific marker NeuN is colocalized with BrdU in the same cortical cells under 3-D confocal analyses. These data suggest an occurrence of neurogenesis in the penumbral cerebral cortex in adult rats after photothrombotic ring stroke (Gu et al. 2000).

An obvious question raised about this finding is whether or not this phenomenon is stroke model specific. In other word, can cortical neurogenesis be found in any other stroke models? To explore the generality of poststroke cortical neurogenesis, cell regeneration is further explored in adult rats subjected to unilateral MCA suture occlusion (Jiang et al. 2001)- an animal model being widely used in stroke research (Longa et al. 1989; Memezawa et al. 1992). To induce the reperfusion, the intraluminal filament is withdrawn at 2 hours after MCA occlusion. BrdU is injected with a similar schedule as the previous study. Brain samples are collected at 30 days and 60 days after stroke induction.

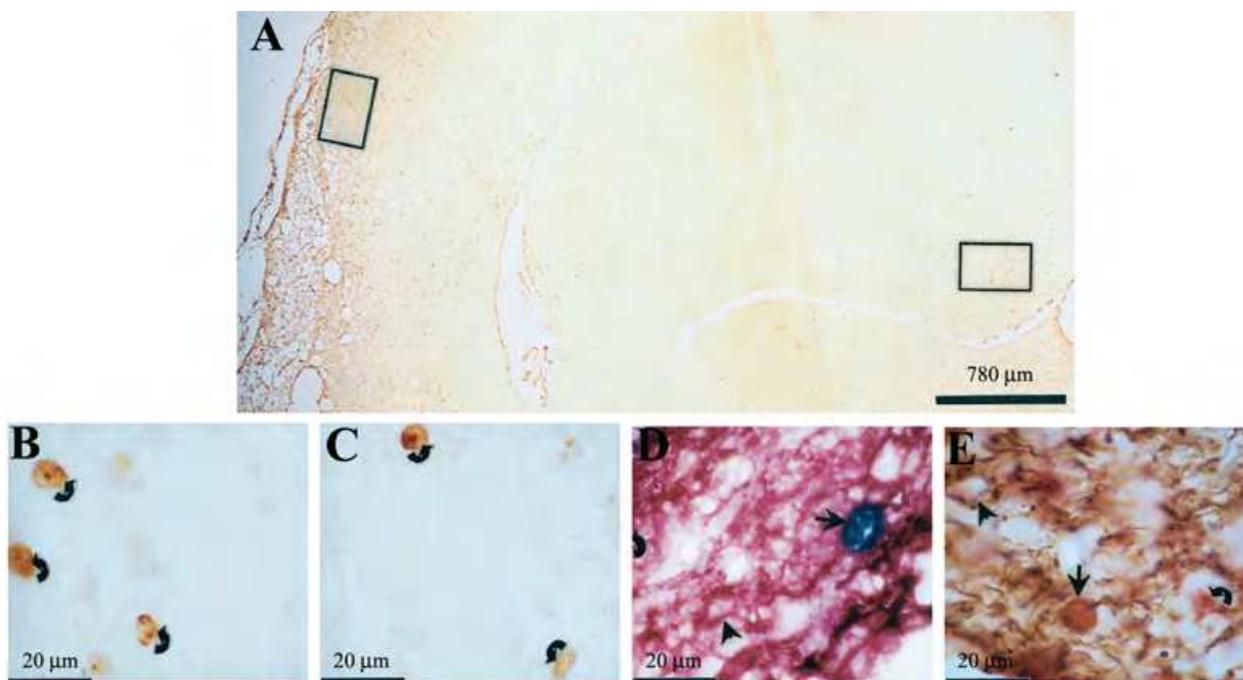


Fig. 5. BrdU immunohistochemistry in rat brains after MCA occlusion (A) BrdU single immunohistochemistry of coronal brain section at 30 days after stroke. Widespread BrdU-immunolabeled cells (brown) are observed in the ipsilateral cortex, with densest distribution in the boundary zone close to the pannecrotic region. BrdU-positive cells are also seen in the corpus callosum and the dentate gyrus of the hippocampus. (B) High magnification photograph of the periinfarct cortex from the left square in A, showing that some cells in the cortical layer II have a large round BrdU-immunolabeled cell nucleus with a single nucleolus in the center (arrow, brown). (C) High magnification photograph of the dentate gyrus of the hippocampus from the right square in A, showing that some cells are BrdU-immunopositive in their nuclei (brown, arrow). (D) BrdU and Map-2 double immunohistochemistry at 30 days after ischemia. In a cell in the cortical layer IV, the BrdU-immunolabeled nucleus (arrow, blue) is surrounded by a Map-2-immunopositive cytoplasm (purple) with dendritic processes. The arrowhead shows a Map-2-immunopositive mature neuron. (E) BrdU and  $\beta$ -tubulin III double

immunohistochemistry at 30 days after stroke. A cell in the cortical layer V has its BrdU-immunolabeled nucleus (arrow, red) surrounded by  $\beta$ -tubulin III-immunopositive cytoplasm (brown) with an extending neurite. (From Jiang et al., *Stroke* 32:1201-1207, 2001. With permission from Wolters Kluwer Health)

Widespread BrdU single-immunopositive cells are observed in the post ischemic cerebral cortex, striatum, corpus callosum, and dentate gyrus of the hippocampus ipsilateral to the ischemic infarct at 30 and 60 days after stroke induction (Fig. 5A). Some of them exhibit neuronal morphologic characteristics, i.e., a large round nucleus with a single nucleolus in the center (Fig. 5B). Three different neuron-specific markers, Map-2,  $\beta$ -tubulin III, and NeuN, are used in conjunction with BrdU to perform double-labeling immunohistochemistry. The cells doubly labelled by BrdU and Map-2 (Fig. 5D),  $\beta$ -tubulin III (Fig. 5E), or NeuN are randomly distributed through cortical layers II through VI, at higher density in the peri-infarct regions than in remote cortical regions. Some BrdU-immunopositive cells, doubly labelled by Map-2,  $\beta$ -tubulin III, or NeuN, are also found in the striatum close to the ischemic lesion. These cells varied in shapes and sizes and often have one or more recognizable Map-2- or  $\beta$ -tubulin III-immunopositive dendrites extending from their cell bodies. They count for approximately 6% to 10% of the total BrdU-immunopositive cells in the penumbral cortex. In 3-D confocal analysis, co-localization of BrdU and NeuN immunofluorescence is detected in the cortical cells at 30 days (Fig. 6) and 60 days after stroke induction. In these cells, the intense BrdU immunofluorescent signal in the cell nuclei is completely merged with the nuclear NeuN-immunopositive signal, from which the NeuN-immunopositive proximal dendrites are extended (Fig. 6). Therefore, neurogenesis occurs also in the penumbral cortex and striatum in adult rats after MCA occlusion (Jiang et al. 2001). In agreement with this study, cortical neurogenesis has been reported by several studies in MCA stroke in adult rats and mice (Chen et al. 2004; Jiang et al. 2001; Jin et al. 2003; Kreuzberg et al. 2010; Leker et al. 2007; Ohab et al. 2006; Tsai et al. 2006).

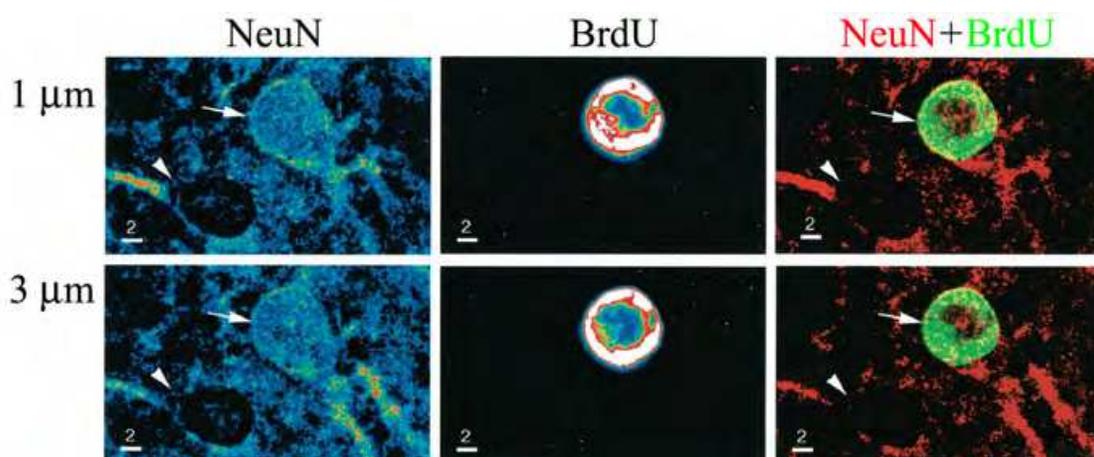


Fig. 6. 3D confocal analyses of NeuN and BrdU double immunofluorescence of cortical cells at two adjacent Z-series planes at 30 days after MCA occlusion. The left column shows the signal intensity for NeuN, the middle column displays the signal intensity for BrdU, and the right column exhibits a merged image of the NeuN and BrdU immunolabeling. In the right column, NeuN (red) and BrdU (green) are co-localized in the same nucleus (arrow) that results in a yellow appearance in the nucleus, which is surrounded by the NeuN cytoplasmic labelling with a neurite-like extension. (From Jiang et al., *Stroke* 32:1201-1207, 2001. With permission from Wolters Kluwer Health)

The reliability of using BrdU nuclear incorporation as the final judgement of adult neurogenesis, in contrast to DNA repair, is questioned especially in the poststroke brain tissue (Kuhn and Cooper-Kuhn 2007; Nowakowski and Hayes 2000). To address this debate, the cellular origin of the progenitor cells that divide and give birth to poststroke newborn cortical neurons must be clarified. In adult mice after MCA stroke, neural stem cells are traced to proliferate and migrate from subventricular zone into the post stroke penumbral cortex. It takes 7-14 days for the stem cells to arrive at the peri-infarct cortex where they differentiate into cortical neurons expressing the neuron-specific marker NeuN (Kreuzberg et al. 2010; Ohab et al. 2006). In contrast, newborn neurons are already identified in the reperfused penumbral cortex at 72 hour after photothrombotic ring stroke in rats (Gu et al. 2000). This time frame is far beyond the migrating speed that SVZ stem cells could make. Therefore we postulate that the newborn cortical neurons may have a cortical origin in this cortical stroke model (Gu et al. 2000).

The crucial point to prove this hypothesis is to determine whether or not cell division has occurred in the poststroke brain tissue, and if it does, when, where and who start to divide. To achieve this purpose, the S-phase marker BrdU is injected intraperitoneally every 4h after stroke induction up to 72h after stroke, then two times daily, and is ended at poststroke day 7. The brain samples are collected at 4, 10, 16, 24, 48, and 72h and 7 and 14 days after stroke induction for analysis (Gu et al. 2009). To explore a possible cell mitosis which is theoretically anticipated during the poststroke cortical neurogenesis, cell mitosis specific marker phosphorylated histone H<sub>3</sub> (Phos H<sub>3</sub>) (Hans and Dimitrov 2001; Hendzel et al. 1997) is investigated through immunohistochemistry/immunofluorescence in the brain sections. During a cell cycle, massive phosphorylation of the nuclear protein histone H<sub>3</sub> takes place immediately after the proliferating cells complete their DNA duplication in order to initiate the cell mitosis (Hans and Dimitrov 2001; Hendzel et al. 1997; Van Hooser et al. 1998), and the phosphorylated histone H<sub>3</sub> becomes quickly dephosphorylated after cell division (Hendzel et al. 1998). Therefore the Phos H<sub>3</sub> detects specifically mitotic cell nuclei. To visualize mitotic spindles, spindle components  $\alpha$ -tubulin (Wittmann et al. 2001) and  $\gamma$ -tubulin (Lajoie-Mazenc et al. 1994) are detected by immunocytochemistry and immunofluorescence. TUNEL labelling is used to detect DNA damage.

In 4h and 10h poststroke rats, a few BrdU-immunolabeled cells are randomly scattered in the brain sections. At 16h poststroke, BrdU-immunolabeled cells are consistently observed in the penumbral cortex. When consecutive sagittal brain sections are examined at this time, a few BrdU-immunolabeled cells are observed on the tangential migrating pathway from the SVZ toward the olfactory bulbs, but not toward the penumbral cortex. At 24h, 48h, 72h, 7 days, and 14 days poststroke, the number of BrdU-immunolabeled cells increases gradually in the penumbral cortex. From at 24h poststroke, Phos H<sub>3</sub> starts to be detected in the same cell nuclei doubly immunolabeled by BrdU (Fig. 7B). Meanwhile,  $\alpha$ -tubulin and  $\gamma$ -tubulin immunolabeled mitotic spindles are observed inside the cortical cells containing BrdU- and Phos H<sub>3</sub>-immunolabeled cell nuclei (Fig. 7C-D). Some of these cells exhibit anaphase or telophase morphology (Fig. 8B-C, Fig. 8F), indicating the completion of cell division. The cell density of Phos H<sub>3</sub>-immunopositive cells reaches its maximum at 48h and 72h poststroke and then declines at 7 days. These mitotic cells are dispersed among but distinctly separated from TUNEL-labelled cells (Fig. 7E). To identify a possible cell lineage of the mitotic cells, the neuronal marker Map-2ab,  $\beta$ -tubulin III, NeuN, and Hu and the astrocyte marker GFAP

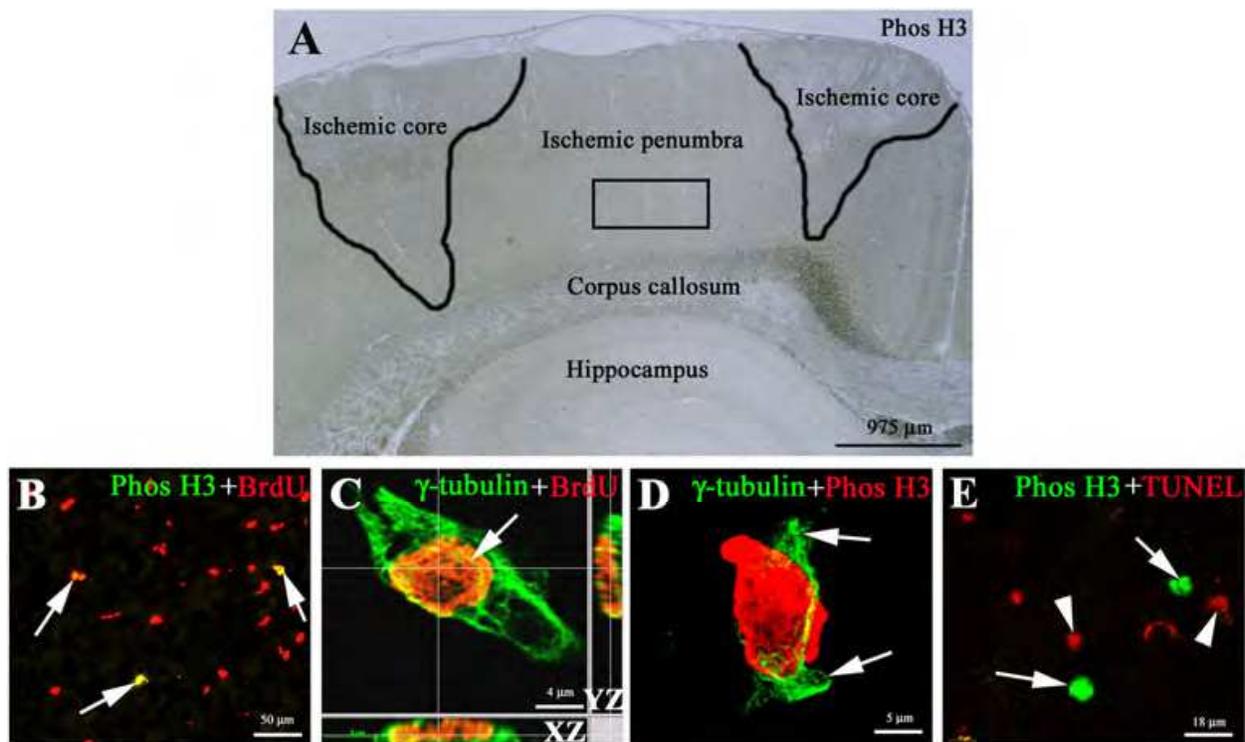


Fig. 7. (A) Low-magnification photograph of Phos H<sub>3</sub> single immunohistochemistry in a coronal brain section through the ischemic lesion at 48h after photothrombotic ring stroke. Two wedge-shaped cortical lesions corresponding to the annular ischemic core are demarcated in the cortex, between which lies the ischemic penumbra. Phos H<sub>3</sub>-immunopositive cells (brown) are observed in the penumbral cortex, ipsilateral corpus callosum, and subgranule layer of the hippocampus. (B) Confocal analyses of Phos H<sub>3</sub> and BrdU double immunofluorescence in the penumbral cortex at 48h post stroke. Numerous cells are BrdU-immunolabeled (red) in the cortical penumbra. Among which, three cells are doubly immunolabeled by Phos H<sub>3</sub> (green) in this field, which yields a yellow appearance in the BrdU and Phos H<sub>3</sub> double-immunopositive nuclei (arrows). (C) 3D confocal analyses of BrdU and γ-tubulin double immunofluorescence in a cortical cell at 24h poststroke. The γ-tubulin (green) appears as a spindle that is co-localized 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). (D) Maximal projection confocal image of Phos H<sub>3</sub> and γ-tubulin double immunofluorescence in the penumbral cortex at 48h post stroke. In a cell from cortical layer II, the Phos H<sub>3</sub>-immunopositive nucleus (red) is superimposed on a γ-tubulin-immunolabeled spindle (green) with the pole bodies at the opposite ends (arrows) and the linking microtubules stretching in between. (E) Confocal microscopy of TUNEL and Phos H<sub>3</sub> double immunofluorescence in the penumbral cortex at 48h after stroke. The Phos H<sub>3</sub>-immunolabeled cells (arrows, green) are separate from the TUNEL-positive cells (arrowheads, red). (From Gu et al., *Stem Cell Res* 2:68-77, 2009. With permission from Elsevier)

are used for double immunohistochemistry and immunofluorescence. Some Phos H<sub>3</sub>-immunolabeled cell nuclei are enveloped by the cytoplasm immunopositive to the neuron-specific markers β-tubulin III and Map-2 (Fig. 8A-C), or the astrocyte marker GFAP. Under 3D confocal analysis, NeuN or Hu is co-localized with Phos H<sub>3</sub> (Fig. 8D), α-tubulin- or γ-tubulin (Fig. 8E-F) in the same cells.

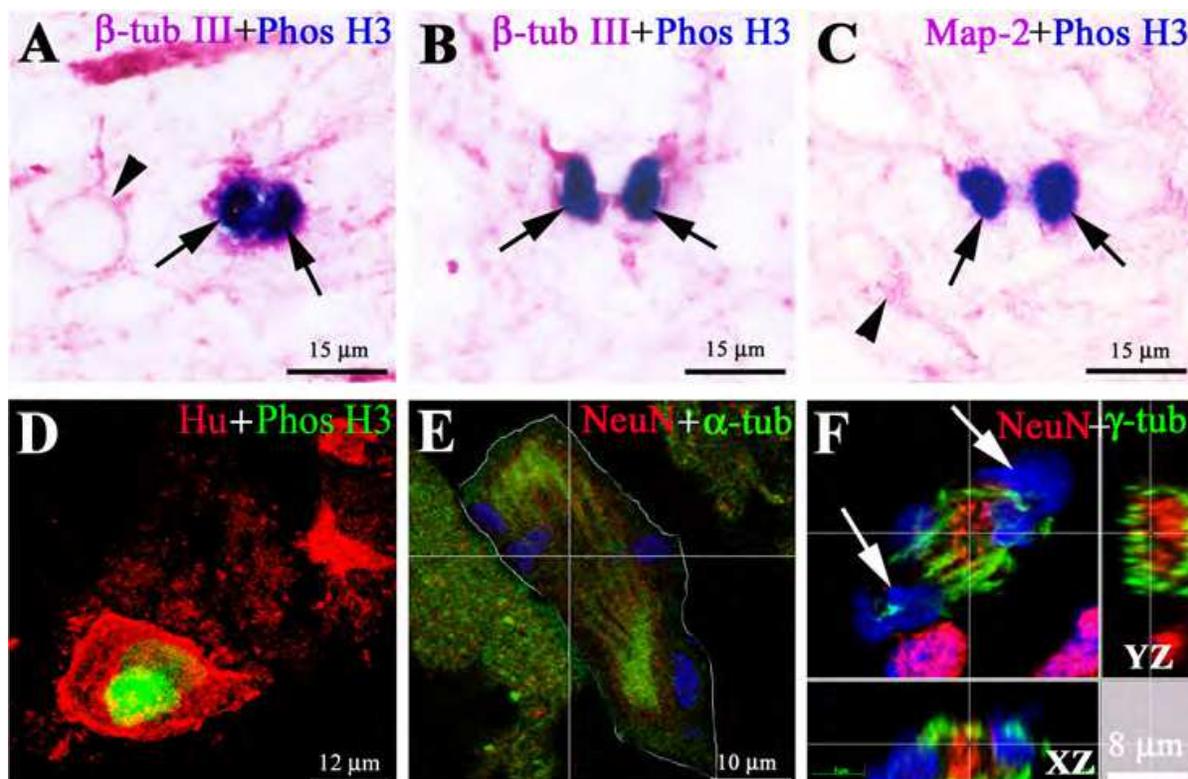


Fig. 8. (A) Phos H<sub>3</sub> and  $\beta$ -tubulin III double immunohistochemistry in the penumbral cortex at 48h after stroke. In a cortical cell, the Phos H<sub>3</sub>-immunolabeled nucleus appears in a partially duplicated shape (arrows, blue), which is surrounded by  $\beta$ -tubulin III-immunolabeled cytoplasm (purple). The arrowhead points to a cortical neuron that is singly immunolabeled by  $\beta$ -tubulin III. (B) Phos H<sub>3</sub> and  $\beta$ -tubulin III double immunohistochemistry in the penumbral cortex at 48h after stroke. A pair of Phos H<sub>3</sub> (arrows, blue) and  $\beta$ -tubulin III (purple) double-immunolabeled cells is nearly separated but still slightly connected through  $\beta$ -tubulin III-immunolabeled cytoplasm. (C) Phos H<sub>3</sub> and Map-2 double immunohistochemistry in the penumbral cortex at 48h after stroke. A pair of Phos H<sub>3</sub> (blue) and Map-2 (purple) double-positive cells (arrows) is splitting while slightly connected through Map-2-immunolabeled cytoplasm (purple). The arrowhead points to a cortical neuron singly immunolabeled by Map-2. (D) Projection confocal image of Phos H<sub>3</sub> and Hu double immunofluorescence in the penumbral cortex at 24h after stroke. A large cortical cell that is Hu-immunolabeled in the cytoplasm (arrow, red) contains a Phos H<sub>3</sub>-immunopositive nucleus in a duplicated shape (green). (E) Confocal microscopy of  $\alpha$ -tubulin, NeuN and DAPI triple immunofluorescence in the penumbral cortex at 24h post stroke. In a large cortical cell in layer III (delineated),  $\alpha$ -tubulin (green) appears as a bipolar spindle while the nuclear DNA is transformed into chromosomes (blue) randomly scattered inside the NeuN-immunopositive cytosol (red). (F) 3D confocal image of NeuN,  $\gamma$ -tubulin and DAPI triple immunofluorescence in layer III of the cortical penumbra at 48 h poststroke. In a cortical cell containing a  $\gamma$ -tubulin-immunolabeled spindle (green), the cell DNA (arrows, blue) is pulled toward its opposite ends while NeuN immunoreactivity (red) is detected at the central part of the cytosol. (From Gu et al., *Stem Cell Res* 2:68-77, 2009. With permission from Elsevier)

The initial appearance of the BrdU-immunolabeled cells within the penumbral cortex rather than in other parts of the brains suggests that the cells starting to proliferate belong to

endogenous cortical cells. Their initial entrance into S-phase occurs at 16h poststroke. Their transition from S-phase cells into M-phase, as hallmarked by the nuclear Phos H<sub>3</sub> expression (Fig. 7B) and cytoplasmic spindle formation (Fig. 7C), occurs at 24h poststroke. The length of the S-phase is therefore estimated about 8h and the length of the whole cell cycle is about 8–10h. This is in agreement with the cell cycle calculation performed in normal mouse brains (Nowakowski et al. 1989). The concurrence whereas distinct separation of these mitotic cells from TUNEL labelling suggests that cell regeneration concurs with but differs distinctly from DNA damage and cell death in the same penumbral cerebral cortex after the photothrombotic ring stroke (Fig. 7E). These data provide morphological evidence to support our claim that the sustained BrdU-incorporated neurons in the poststroke penumbral cortex represent neurogenesis (Gu et al., 2000; Gu et al., 2009) rather than DNA repair (Kuhn and Cooper-Kuhn 2007; Nowakowski and Hayes 2000).

It is not clear why differentiated neuronal markers are expressed in cells in metaphase during the poststroke cortical neurogenesis (Fig. 8E-F). In cell culture, differentiated neuron markers are expressed in newborn neurons immediate after their birth from the mother cell, i.e., after cytokinesis (Svendsen et al. 1995). A possible explanation of this phenomenon is that in response to ischemic stroke the endogenous progenitor cells may undergo cell division with a quickened cell differentiation. In agreement with this explanation, neural progenitor cells isolated from the poststroke penumbral cerebral cortex are cultured to generate neurons expressing differentiated neuronal markers (Shimada et al. 2010). An alternative possibility is that after ischemic insult somatic cortical neurons may reprogram themselves and thus function as pluripotent stem cells so that they start to divide and give birth to newborn neurons while they keep their neuronal identity throughout cell division. Future studies are needed to address on this issue.

Whether or not the adult cortical neurogenesis may contribute to the poststroke neurological improvement is one of our major interests. For the newborn neurons to function in the poststroke cerebral cortex, they must fulfil many basic criteria. For example, they must be able to establish synaptic connections with their surrounding neurons, to synthesize various neurotransmitters and receptors, and in response to afferent stimuli to generate action potentials and to release the corresponding neurotransmitters in order to activate their target receptors. Proper neurotransmitter deactivation system is also needed in order to terminate the action. To explore the function potential of poststroke cortical neurogenesis, we start with an examination on the possible biosynthesis of the excitatory neurotransmitter acetylcholine (ACh) and its substrate enzyme choline acetyltransferase (ChAT) and the inhibitory neurotransmitter  $\gamma$ -aminobutyric acid (GABA) and the corresponding substrate enzyme glutamic acid decarboxylase (GAD) in adult rats after photothrombotic ring stroke. To detect the newborn cells, BrdU is repeatedly delivered as mentioned previously. To detect neurotransmitter synthesis, brain sections are examined through double immunohistochemistry or immunofluorescence with BrdU and the neurotransmitter markers. To ascertain the cell identify, neuron-specific marker NeuN is used for triple immunofluorescence. With ACh, ChAT, GABA and GAD single immunohistochemistry, ACh, ChAT, GABA and GAD-immunolabeled neurons are frequently observed in the cerebral cortex outside the penumbral region. In contrast, all of the cells in the pannecrotic ischemic core and the majority of the cells inside the cortical penumbra are not immunolabeled by the neurotransmitters ACh and GABA. However,

some BrdU-immunolabeled cortical cells in the penumbral cortex are ChAT, ACh, GAD, or GABA-immunolabeled in their cytoplasm. These cells are randomly distributed in cortical layers II–VI in the penumbral cortex at 48h, 5 days, 7 days, 30 days, 60 days, and 90 days after stroke. Under 3-D confocal analyses, BrdU is colocalized with ACh (Fig. 9A) or GABA (Fig. 9B) in the same cells in the corresponding double immunofluorescence, in which the BrdU-immunolabelled cell nucleus is surrounded by ACh or GABA-immunolabelled cytoplasm. In the GABA, NeuN, and BrdU triple immunofluorescence (Fig. 9D-F), the neuron-specific marker NeuN is colocalized with the BrdU in the same nucleus (Fig. 9E), around which the GABA-immunoreactive cytoplasm is detected (Fig. 9F). This observation verifies the neuronal identity of these newborn cortical cells synthesizing corresponding neurotransmitters. In addition, it also helps to address on a theoretical concern that the presence of the neuronal marker NeuN inside the dividing cells might be a consequence of phagocytosis, i.e., the cells that look like dividing neurons are dividing macrophages that have taken neuronal markers. In the setting of focal cerebral ischemia the CBF threshold for neurotransmitter release (corresponding to 40–60% of the baseline CBF) is higher than that of the membrane threshold (corresponding to 20–40% of the baseline CBF) beyond which morphological damage of the ischemic tissue occurs (Hossmann 1994). Timewise, postischemic neurotransmitter release occurs earlier than cell apoptosis and necrosis through which ischemic cell death takes place. In the photothrombotic ring stroke the local CBF in the cortical penumbra drops to 34% of the baseline level at 2h after ischemia (Gu et al. 1999a). This CBF level is already well below the neurotransmitter threshold at which a nonspecific neurotransmitter release is triggered, while the tissue morphology remains intact (Gu et al. 1999b). Consequently, all of the neurons in the ischemic core and the majority of neurons inside the penumbral cortex are not immunolabeled by ACh or GABA at 24–48h (Gu et al. 2010). While some of the cortical cells in the penumbral cortex are DNA damaged in their nuclei, i.e., TUNEL-positive, they are distinctly separated from the dividing cells expressing the mitotic marker Phos H<sub>3</sub> (Fig. 7E). Thus, neurons lose their neurotransmitters at 2–16h after ischemia, and they die through apoptosis/necrosis at 24–48h after the photothrombotic ring stroke. Meanwhile, local cells in the penumbral cortex are induced to proliferate from at 16h after ischemia incorporating BrdU into their nuclei, and then they start to divide at 24–48h poststroke that give birth to daughter neurons expressing various neuronal markers and synthesizing neurotransmitters (Gu et al. 2009; Gu et al. 2000; Gu et al. 2010). It is not until from at 72h postischemia and later ED-1-immunopositive microglia start to appear in the postischemic cortex to clean up the dead cells through phagocytosis (Gu et al. 1999b). Therefore, cell division starts earlier than phagocytosis in the setting of photothrombotic ring stroke. Being a functional marker for living GABAergic neurons, the neurotransmitter GABA should never appear in the debris of dead neurons. The homogeneous colocalization of GABA and NeuN in the same cytoplasm including the proximal dendrites of the newborn neurons (Fig. 9F) speaks directly against the hypothesis that the NeuN immunoreactivity in the dividing cells comes from exogenous fragments of dead neurons. It is conceivable that one or several pieces of neuronal fragments may be engulfed by an activated microglia into its cytoplasm (Takahashi et al. 2005). In that case, it should be debris of dead neurons that becomes engulfed, not a whole living neuron exhibiting intact cell morphology including neurite-like extensions. In the newborn neurons, the neuronal marker NeuN labels both the BrdU-immunolabeled cell nucleus and the

perinuclear cytoplasm including the proximal dendrites in the newborn cortical neurons (Fig. 9E) (Gu et al. 2009; Gu et al. 2000; Gu et al. 2010). This NeuN cell labelling pattern agrees with what has been reported in normal adult neurons (Wolf et al. 1996). Therefore, the NeuN nuclear labelling itself speaks also against the exogenous NeuN hypothesis. It is because if a neuronal fragment is engulfed by a dividing microglia, it should barely be taken into the cytoplasm of the dividing microglia. It has no chance to integrate its immunoreactivity into the BrdU/Phos H<sub>3</sub>-immunopositive dividing nucleus of the phagocyte. Thus, the neuronal markers and neurotransmitters observed belong indeed to the same cells exhibiting dividing cell nuclei.

Therefore, the capability of neurotransmitter biosynthesis by these newborn neurons qualifies themselves for one of the fundamental prerequisites for their further function during the poststroke neurological and neuropsychological improvement.

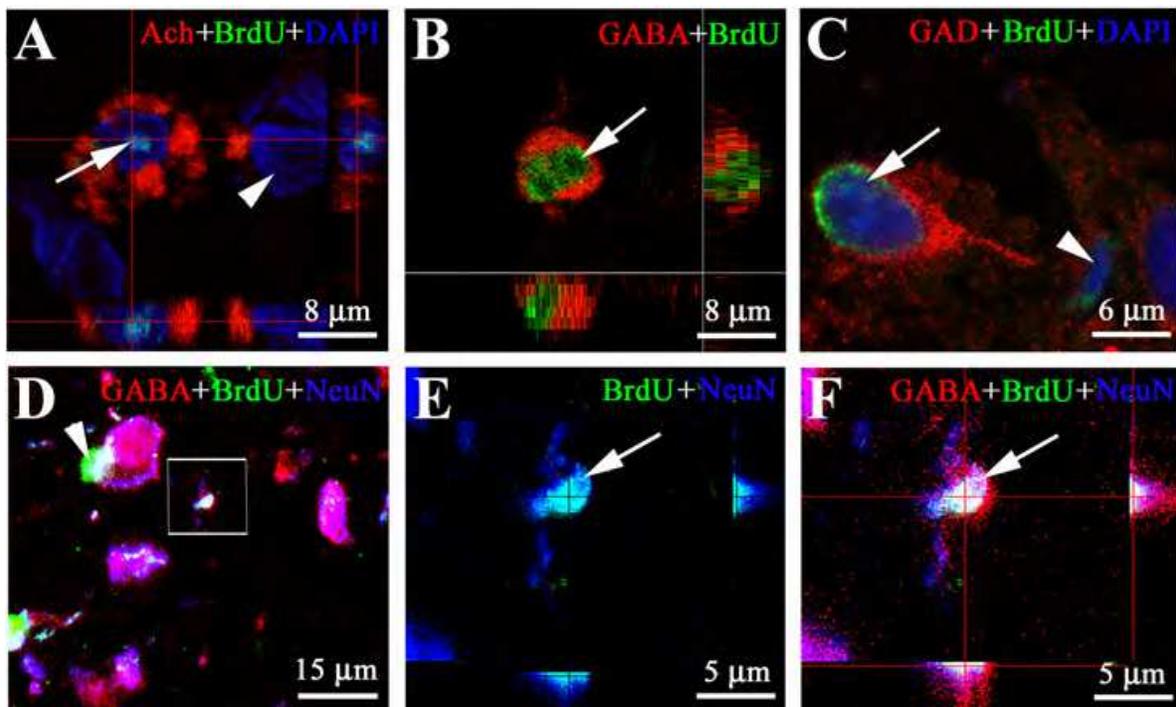


Fig. 9. (A) 3-D confocal analysis of Ach, BrdU, and DAPI triple immunofluorescence from layer IV of the cortical penumbra at 30 days after photothrombotic ring stroke. In a large cortical cell, the cell nucleus is BrdU-immunolabeled (green) and DAPI counterstained (arrow, blue), which is surrounded by Ach-immunopositive cytoplasm (red). Many non-cholinergic cells are seen in the same field (arrowhead, blue). (B) 3-D confocal analysis of GABA and BrdU double immunofluorescence in layer III of cortical penumbra at 48h after stroke. A cortical cell that is BrdU-immunolabeled in the nucleus (arrow, green) is GABA-immunopositive in the cytoplasm (red). (C) A section confocal image of GAD, BrdU, and DAPI triple immunofluorescence in layer III of penumbral cortex at 7 days after stroke. A cortical cell that contains a BrdU-immunolabeled (arrow, green) DAPI counterlabeled nucleus (arrow, blue) is GAD-immunopositive (red) in its cytoplasm. (D-F) GABA, BrdU and NeuN triple immunofluorescence (D) Projection image of GABA (red), BrdU (green), and NeuN (blue) triple immunofluorescence at low magnification. A cortical cell (framed) is triply immunolabeled by GABA (red), BrdU (green), and NeuN (blue). The arrowhead

points at a newborn nonneuronal cell singly immunolabeled by BrdU (green) superimposing on a GABAergic (red) NeuN-immunopositive (blue) cortical neuron. (E) BrdU and NeuN double-channel image of the cortical cell framed in panel D. The BrdU immunolabeling (arrow, green) is colocalized with NeuN (blue) in the same cell nucleus. (F) 3-D analysis of GABA, BrdU, and NeuN triple-channel image from the same scanning section as panel E. The GABA (red) is colocalized with NeuN (blue) and BrdU (green) in the same cell (arrow). (From Gu et al., *Stem Cell Res* 4:148-154, 2010. With permission from Elsevier)

In summary, neurogenesis occurs in the penumbral cerebral cortex in adult rodents after photothrombotic ring stroke and unilateral middle cerebral artery occlusion. Quick cell division within the penumbral cerebral cortex contributes to an early in situ cortical neurogenesis in the photothrombotic ring stroke model in rats. In contrast, neural stem cell migration from SVZ into the cortical penumbra provides a remote cell resource for a late stage cortical neurogenesis in the periinfarct cerebral cortex after middle cerebral artery occlusion. As early as at 48h after the photothrombotic ring stroke, the newborn cortical neurons start to synthesize the neurotransmitters GABA and acetylcholine exhibiting neurite-like extensions, which optimizes their further function in the post stroke recovery.

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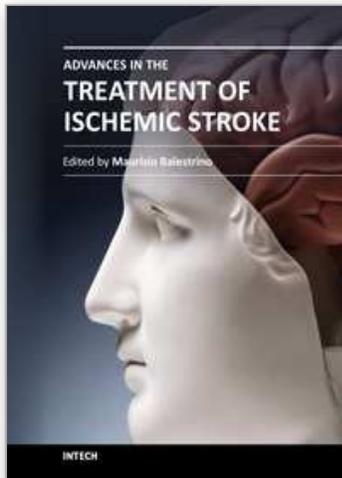
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In recent years research on ischemic stroke has developed powerful therapeutic tools. The novel frontiers of stem cells therapy and of hypothermia have been explored, and novel brain repair mechanisms have been discovered. Limits to intravenous thrombolysis have been advanced and powerful endovascular tools have been put at the clinicians' disposal. Surgical decompression in malignant stroke has significantly improved the prognosis of this often fatal condition. This book includes contributions from scientists active in this innovative research. Stroke physicians, students, nurses and technicians will hopefully use it as a tool of continuing medical education to update their knowledge in this rapidly changing field.

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