SHORT REPORT

Neurotransmitter synthesis in poststroke cortical neurogenesis in adult rats

Weigang Gu a,b,⁎, Chao Gu a, Wei Jiang a, Per Wester a

a Umeå Stroke Center, Department of Public Health and Clinical Medicine, Medicine, University of Umeå, S-901 87 Umeå, Sweden
b Department of Clinical Neuroscience and Neurology, University of Umeå, S-901 87 Umeå, Sweden

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Abstract Neurogenesis occurs in the cerebral cortex of adult rats after focal cerebral ischemia. Whether or not the newborn neurons could synthesize neurotransmitters is unknown. To elucidate such a possibility, a photothrombotic ring stroke model with spontaneous reperfusion was induced in adult male Wistar rats. The DNA duplication marker BrdU was repeatedly injected, and the rats were sacrificed at various times after stroke. To detect BrdU nuclear incorporation and various neurotransmitters, brain sections were processed for single/double immunocytochemistry and single/double/triple immunofluorescence. Stereological cell counting was performed to assess the final cell populations. At 48 h, 5 days, 7 days, 30 days, 60 days and 90 days after stroke, numerous cells were BrdU-immunolabeled in the penumbral cortex. Some of these were doubly immunopositive to the cholinergic neuron-specific marker ChAT or GABAergic neuron-specific marker GAD. As analyzed by 3-D confocal microscopy, the neurotransmitters acetylcholine and GABA were colocalized with BrdU in the same cortical cells. In addition, GABA was colocalized with the neuron-specific marker Neu N in the BrdU triple-immunolabeled cortical cells. This study suggests that the newborn neurons are capable of synthesizing the neurotransmitters acetylcholine and GABA in the penumbral cortex, which is one of the fundamental requisites for these neurons to function in the poststroke recovery. © 2009 Elsevier B.V. All rights reserved.

Introduction

Ischemic stroke ranks at the top as far as disabling patients in their adult life. The outcome of ischemic stroke depends largely on the severity and duration of the local cerebral blood flow reduction. Early and even late spontaneous reperfusion is associated with a favorable neurological outcome after ischemic stroke in humans (von Kummer et al., 1995). After ischemic stroke, neurological improve-

Abbreviations: BrdU, 5-bromodeoxyuridine; Ach, acetylcholine; ChAT, choline acetyl transferase; GABA, gamma-aminobutyric acid; GAD, glutamic acid decarboxylase.

⁎ Corresponding author. Umeå Stroke Center, University of Umeå, S-901 87 Umeå, Sweden. Fax: +4690 785 2563.

E-mail address: weigang.gu@neuro.umu.se (W. Gu).

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i.e., a piece of somatosensory cortex, is concentrically encroached by a radially expanding annular ischemic core, thus simulating stroke-in-evolution. Microvascular platelet thrombi appear in the cortical lesion (Gu et al., 1999b; Wester et al., 1995), reminiscent of clinical thromboembolic stroke. Delayed, but consistent, spontaneous reperfusion of the cortical penumbra can be presaged in the "ring" model by manipulation of the irradiating laser beam intensity (Gu et al., 1999a). Consequently, local cerebral blood flow (CBF) in the penumbra decreases to 59–34–26–33% of baseline values at 1–2–24–48 h after stroke induction, and then gradually recovers to 56–87% of baseline values at 72–96 h (Gu et al., 1999c). Quick induction of immediate early genes (Johansson et al., 2000) is followed by a prevalence of neuronal necrosis and apoptosis at 24–48 h postischemia in the penumbral cortex (Gu et al., 2009, 1999b; Hu et al., 2002). Early angiogenesis and the presence of blood elements within the newly formed vessels (Gu et al., 2001) may suggest functional angiogenesis (Gu et al., 1999b). Concurrent with the apoptotic and necrotic cell death in the penumbral cortex, local cells are induced to proliferate and divide, generating newborn neurons (Gu et al., 2009), which contribute to the poststroke cortical neurogenesis (Gu et al., 2000). Neurite-like structures are readily immunolabeled by various classical neuron markers in the newborn neurons that survive up to 3–18 months poststroke (Gu et al., 2000; Jiang et al., 2001). Nevertheless, neither the neuronal immunolabeling nor the morphological appearance provide any information about the functional potential of these newborn neurons. As the interest in endogenous poststroke neurogenesis grows, a decent study of its functional potential is warranted. One of the fundamental prerequisites of functional ability for poststroke neurogenesis is that the newborn neurons should be capable of synthesizing various neurotransmitters. The present study aims to detect the possible occurrence of neurotransmitter synthesis in newborn neurons in adult rat brains after photothrombotic ring stroke with spontaneous reperfusion.

Results

5-Bromodeoxyuridine (BrdU) single immunohistochemistry

Widespread BrdU-immunolabeled cells were observed in the postischemic cerebral cortex, corpus callosum, and dentate gyrus of the hippocampus ipsilateral to ischemic lesion at 48 h, 5 days, 7 days, 30 days, 60 days, and 90 days after stroke induction (Fig. 1A). Most of the BrdU-immunolabeled cells appeared in the cortical penumbra surrounded by the ring-shaped cortical lesion and its surrounding cortex, and less in the ring lesion area. These cells were distributed randomly through cortical layers I to VI. In the cortex contralateral to the ischemic lesion, a few BrdU-immunopositive cells were observed. In the sham-operated rats that received the same repeated BrdU injections as the ischemic groups, a few BrdU-immunolabeled cells were seen to be randomly scattered in the bilateral cortex. In the BrdU-negative controls, i.e., the rats that did not receive any BrdU injections, no BrdU-immunolabeled cells were observed in the brain sections.

Figure 1  (A) Low magnification photograph of BrdU single immunohistochemistry in the rat cerebral cortex at 5 days after the photothrombotic ring stroke. Two wedge-shaped cortical infarcts (ischemic core) representing ring-shaped ischemic lesion are demarcated, between which lays the cortical penumbra. Numerous BrdU-immunolabeled cells (in purple) appear in the penumbral cortex and in the cortex near the ring lesion. Scale bar: 532 μm. (B) High magnification photograph of Ach and GFAP double immunohistochemistry in the cortex at 7 days after stroke. The Ach-immunolabeled neurons (in brown, arrowhead) are separated from the GFAP-immunolabeled astrocytes (in black, arrowed). Scale bar: 11 μm. (C) High magnification photograph of ChAT and GFAP double immunohistochemistry in the cortex at 7 days after stroke. The ChAT-immunolabeled cortical neurons (in brown, arrowhead) are separated from the GFAP-immunolabeled astrocytes (in black, arrowed). Scale bar: 11 μm. (D) High magnification photograph of GAD and GFAP double immunohistochemistry in the cortex at 7 days after stroke. The GAD-immunolabeled cortical neurons (in brown, arrowhead) are separated from the GFAP-immunolabeled astrocytes (in black, arrowed). Scale bar: 11 μm.
Double immunohistochemistry of GFAP and neurotransmitter markers

In order to examine the specificity of neurotransmitter markers, double immunohistochemistry of the astrocyte marker GFAP with one of the neurotransmitter markers choline acetyltransferase (ChAT), acetylcholine (Ach), glutamic acid decarboxylase (GAD), and gamma-aminobutyric acid (GABA) was conducted. In the sham-operated and poststroke brains, ChAT, Ach, GAD, and GABA were analyzed by 3-D confocal microscopy. The colocalization of BrdU with one of the neurotransmitter markers confirms the neuronal identity of the newborn cells synthesizing GABA.

Double immunolabeling of BrdU and neurotransmitter markers

To detect BrdU and neurotransmitter markers in the same part of the cortex, the density of the Map-2ab single-immunopositive neurons was $104,500 \pm 30,900$ cells/mm$^3$ (M±SD).

Discussion

The most interesting finding of this study is the detection of the neurotransmitters acetylcholine and GABA in the newborn neurons in the penumbral cerebral cortex in adult rats after photoinfarct stroke. These data support further the previous finding that the newborn cells identified by various classic neuronal markers in the penumbral cerebral cortex are indeed neurons (Gu et al., 2000, 2009; Jiang et al., 2001; Jin et al., 2003; Tsai et al., 2006). In the previous studies of stroke-induced cortical neurogenesis (Gu et al., 2000; Jiang et al., 2001) and cortical cell division (Gu et al., 2009), the differentiated neuronal markers Neu N, Hu, Map-2ab, and β-tubulin III and the astrocyte marker GFAP are used for the determination of neuron and astrocyte cell identity. A theoretical concern with this methodology is that some of these neuronal markers might be expressed by other cell types rather than neurons in the postischemic penumbral cortex.

To screen a possible colocalization of BrdU with one of the neurotransmitter markers ChAT, Ach, GAD, and GABA in the cortical cells, the corresponding double immunohistochemistry experiments were performed. Many neurons outside the penumbral cortex were immunolabeled by these neurotransmitter markers. In contrast, the majority of the cells inside the penumbral cortex were not immunolabeled by Ach and GABA. In some cortical cells in which the cell nuclei were BrdU-immunolabeled, the cytoplasm was immunopositive to ChAT, Ach, GAD, or GABA. These cells were randomly distributed in cortical layers II–VI in the penumbral cortex at 48 h, 5 days, 7 days, 30 days, 60 days, and 90 days after stroke. In the pansensory ischemic core, such cells were not seen. In order to detect a real colocalization of BrdU with these neurotransmitters in the cortical cells, the corresponding double immunofluorescence labelings were analyzed by 3-D confocal microscopy. BrdU was colocalized with the ChAT (Figs. 2A and B), Ach (Figs. 2C–E), GAD (Fig. 2F), and GABA (Fig. 2G; Figs. 2H–K) in the same cortical cells at 48 h, 5 days, 7 days, 30 days, 60 days, and 90 days after stroke.

GABA, BrdU, and Neu N triple immunofluorescence

To confirm the neuronal identity of the GABA and BrdU double-immunolabeled cells in the penumbral cortex, GABA, BrdU, and Neu N triple immunofluorescence was performed. Neu N was colocalized with GABA in the same cell in which the cell nucleus was BrdU-triple-immunolabeled (Figs. 2H–K). Neurite-like extensions were sometimes seen in these cells (Figs. 2H–K).

Cell counting

The cell density of BrdU single-immunopositive cells in the ischemic cortex was $43,700 \pm 18,800$ cells/mm$^3$ (M±SD) at 48 h poststroke. It increased to $204,600 \pm 105,600$ cells/mm$^3$ (M±SD) at 7 days, and then decreased to $4600 \pm 1100$ cells/mm$^3$ (M±SD) at 30 days poststroke. At this time, the density of the BrdU and Ach double-immunopositive cells was $390 \pm 535$ cells/mm$^3$, which corresponds to about 8% of the total BrdU-immunopositive cells in the ischemic cortex. The density of BrdU and GABA double-immunopositive cells was $157 \pm 187$ cells/mm$^3$, which counts for about 3% of total BrdU-immunopositive cells in the ischemic cortex. In the same part of the cortex, the density of the Map-2ab single-immunopositive neurons was $104,500 \pm 30,900$ cells/mm$^3$ (M±SD).
The sustained appearance of Ach and GABA in the BrdU double-immunolabeled cells in the present study agrees with the notion that the BrdU immunolabeling represents cell regeneration in the setting of the photothrombotic ring stroke, in contrast to DNA damage (Kuan et al., 2004). This is because in the setting of focal cerebral ischemia the CBF

**Figure 2**  (A) Projection confocal image of ChAT (in red, Alexa Fluor 546), BrdU (in green, Alexa Fluor 488), and DAPI (in blue) immunofluorescence in the penumbral cortex at 60 days after stroke. (B) A section confocal image of ChAT and BrdU double immunofluorescence in a cell from cortical layer II (framed area in panel A). A BrdU-immunolabeled cell nucleus (in green, arrowed) is surrounded by ChAT-immunopositive cytoplasm (in red). (C) 3-D confocal analysis of Ach, BrdU, and DAPI triple-channel image from layer IV of the cortical penumbra at 30 days after stroke. In a large cortical cell, the cell nucleus (arrowed) that is counterlabeled by DAPI (in blue) is BrdU-immunolabeled (in green), which is surrounded by Ach-immunopositive cytoplasm (in red) in the 3-D confocal analysis. Several cells that are counterstained by DAPI (in blue, arrowhead) are not immunolabeled by Ach and BrdU in the same field. (D) 3-D confocal analysis of Ach and BrdU double-channel image from the same scanning plan as panel C. The BrdU immunolabeling in the cell nucleus (in green) is weak at 3 days after stroke, a phenomenon that is commonly observed in long-term neurogenesis studies (Gu et al., 2000; Jiang et al., 2001). (E) 3-D confocal analysis of Ach, BrdU, and DAPI triple-channel image from layer III of the cortical penumbra at 90 days after stroke. In a cortical cell, the BrdU-immunolabeled cell nucleus (in green, arrowed) is surrounded by Ach-immunopositive cytoplasm (in red) in 3-D analysis. The arrowhead is pointed at a cortical cell in the same field that is BrdU single-immunolabeled (in green) in the cell nucleus counterstained by DAPI (in blue). (F) A section confocal image of GAD, BrdU, and DAPI triple immunofluorescence in layer III of cortical penumbra at 7 days after stroke. A BrdU-immunolabeled cell nucleus (in green, arrowed) which is counterstained by DAPI (in blue) is surrounded by GAD-immunolabeled cytoplasm (in red). The arrowhead is pointed at a DAPI (in blue) counterstained cell nucleus singly immunopositive to BrdU (in green). (G) 3-D confocal analysis of GABA and BrdU double immunofluorescence in layer III of cortical penumbra at 48 h after stroke. In a cortical cell, the cell nucleus is BrdU-immunolabeled (in green, arrowed) that is surrounded by GABA-immunopositive cytoplasm (in red) in 3-D analysis. (H–K) GABA, BrdU, and Neu N triple immunofluorescence in layer IV of cortical penumbra at 72 h after stroke. (H) Projection image of GABA (in red, Alexa Fluor 546), BrdU (in green, Alexa Fluor 488), and Neu N (in blue, Alexa Fluor 405) triple immunofluorescence at low magnification. A cortical cell (framed) is triple immunolabeled by GABA (in red), BrdU (in green), and Neu N (in blue). The arrowed point at a BrdU single-immunolabeled (in green) cortical cell superimposing a GABA (in red) and Neu N (in blue) double-immunopositive neuron. (I) BrdU single-channel image of the cortical cell framed in panel H. The cell nucleus is BrdU-immunolabeled (in green, arrowed). (J) BrdU and Neu N double-channel image from the same scanning section as panel I. The BrdU immunolabeling (in green, arrowed) is colocalized with Neu N (in blue) in the same cell in 3-D confocal analysis. (K) GABA, BrdU, and Neu N triple-channel image from the same scanning section as panels I–J. The GABA (in red) is colocalized with Neu N (in blue) and BrdU (in green) in the same cell (arrowed) in 3-D analysis.
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 structural damage of the ischemic tissue occurs (Hossmann, 1994). Timewise, post-stroke neurotransmitter release occurs earlier than cell apoptosis and necrosis through which ischemic cell death takes place. As noted, in the photothrombotic ring stroke model the local CBF in the cortical penumbra drops to 34% of the baseline level at 2 h after ischemia (Gu et al., 1999a). This CBF level is already well below the neurotransmitter threshold at which neurotransmitter release is triggered, while the tissue morphology remains intact (Gu et al., 1999b). Consequently, as many neurons outside the cortical penumbra are Ach or GABA single immunopositive, the majority of cells inside the penumbral cortex are not Ach or GABA-immunolabeled at 24–48 h after stroke, which may suggest posts ischemic neurotransmitter leakage. Some of the cortical cells are TUNEL-positive, representing nuclear DNA damage (Gu et al., 2009). On the other hand, certain cells in the penumbral cortex are induced to proliferate. They incorporate the DNA duplication marker BrdU, express various mitosis-specific markers, and finally divide to generate newborn neurons (Gu et al., 2009). At this time, Ach and GABA are detected in some cortical cells that are BrdU doubly immunolabeled in their nuclei, rather than those that are TUNEL positive. Thus, cell regeneration concurs with ischemic cell death in the same cortical penumbra in the setting of photothrombotic ring stroke (Gu et al., 2009). A 4.7 times increment of BrdU single immunolabeling in the penumbral cortex is observed at 7 days as compared with that at 48 h posts ischemia. At 7 days after ischemia, most of the BrdU-immunolabeled cells in the ischemic penumbra represent newborn astrocytes, endothelial cells, and inflammatory cells (Gu et al., 1999b). At 30 days after stroke, 8.4% of the BrdU-immunolabeled cells in the penumbral cortex are BrdU and Ach double immunopositive, and 3.4% of the BrdU-immunolabeled cells are BrdU and GABA double immunopositive. These cells are survived at 90 days after stroke. If the BrdU nuclear incorporation in the neurons were resulted from DNA damage, i.e., abortive cell cycle reentry as seen in the hippocampus after cerebral hypoxia (Kuan et al., 2004), these neurons should have lost their neurotransmitters and then they should have become damaged in their nuclear DNA and thus should have been TUNEL positive, as in contrast to being Ach or GABA immuno-positive but TUNEL negative. Furthermore, they would have died shortly of cell apoptosis after stroke, instead of being able to survive up to 90 days after stroke. Therefore, the BrdU immunolabeling in the present study speaks for cell regeneration in the penumbral cortex instead of DNA damage. Interestingly, GABA is reported to be synthesized in new interneurons in neocortex and striatum in normal adult rats (Dayer et al., 2005).

It is conceivable that certain neurons being minimally damaged in their nuclear DNA, after posts ischemic neurotransmitter release, might incorporate a trivial amount of BrdU during their DNA repair. However, such a trivial BrdU incorporation seems hardly detectable by current immunolabeling techniques. To our knowledge, long-term survival of DNA-damaged neurons exhibiting simultaneous BrdU nuclear labeling and neurotransmitter synthesis has not been reported. Therefore, the cells in the present observation are most likely to represent cholinergic and GABAergic newborn neurons synthesizing the neurotransmitter Ach and GABA. Nevertheless, the synthesis of the Ach and GABA in the newborn neurons does not warrant a spontaneous recruitment of these cortical neurons into poststroke neurological recovery. The functional neurotransmitter release, the activation of their corresponding receptors, the postrelease deactivation, and the proper connection of the newborn neurons with surrounding cells are all important for the newborn neurons to function. These issues are beyond the scope of the present study and should be addressed in future studies.

### Experimental procedures

#### Stroke induction

Animal care and all experimental procedures were carried out in accordance with the European Communities Council Directive (86/609/EEC), and the experimental protocol was approved by the Ethics Committee for Animal Research at Umeå University. The photothrombotic ring stroke model with spontaneous reperfusion was induced in adult rats as previously described (Gu et al., 1999a; Wester et al., 1995). Briefly, adult male Wistar rats weighing 280–340 g were first anesthetized with 3.5% halothane in an induction chamber, and then maintained by 2% halothane and a mixture of 70% nitrous oxide with 30% oxygen delivered through a closely fitting face mask. Femoral arterial and venous polyethylene (PE-50) catheters were inserted for monitoring arterial blood pressure, blood gases, and glucose and administration of erythrosine B. The rats were intubated with PE-240 and mechanically ventilated (Ugo Basile small-animal respirator, Stoelting) with approximately 1% halothane in a 70:30 mixture of N2O:O2. Respiratory adjustments were made, when necessary, to ensure normal arterial blood gases as measured by the ABL 300 analyzer (Radiometer, Copenhagen, Denmark). Head temperature and rectal temperature were kept at 37.0–37.5 °C. The rats were mounted on a stereotaxic frame (David Kopf, Tujunga, CA, USA). The photothrombotic ring stroke model with spontaneous reperfusion was induced on the right parietal skull bone as previously described (Gu et al., 1999a; Wester et al., 1995). To label many proliferating cells while minimizing any potential cellular toxicity, the cell proliferation-specific marker 5-bromoodeoxyuridine (Sigma) was repeatedly administered intraperitoneally at low doses (10 mg/kg BrdU dissolved in saline for each injection) (Gratzner, 1982; Gu et al., 2000; Jiang et al., 2001). The BrdU administration was started immediately after stroke induction, two times daily, and ended at 48 h, 5 days, 7 days, and 10 days after stroke induction. The animals were sacrificed at 48 h, 5 days, 7 days, 30 days, 60 days, and 90 days after stroke (n = 4 in each group) or 7 days after sham operation (Gu et al., 2000; Jiang et al., 2001) by transcardial perfusion with 37 °C Histochoice tissue fixative or decapitation (Gu et al., 2000; Jiang et al., 2001). Brains were paraffin-embedded or quickly frozen at –80 °C.
Immunohistochemistry

Paraffin-embedded coronal brain sections (10 μm thick) through the lesioned cortex were immunostained according to the protocol recommended for using the Vector staining kit (Jiang et al., 2001). Brain sections were deparaffinized in xylene and dehydrated through graded ethanol series. Endogenous peroxidase was deactivated by 30 min incubation in freshly prepared 3% (v:v) H2O2:methanol. The sections were incubated in 2 M HCl in saline for 30 min to denature the DNA for BrdU detection. Antigen retrieval and deactivation of the endogenous alkaline phosphatase were achieved by 3 × 7 min of boiling in 0.1 M citrate buffer (pH 6.0) in a microwave oven with a 5-min cooling interval. Single-labeling immunohistochemistry was conducted with Elite Vectastain ABC-peroxidase-Vector-VIP (Vector Laboratories, Burlingame, CA, USA). Double immunohistochemistry was conducted by sequential detection of the first primary antibodies with Vectastain ABC-AP-Vector-NovaRed (Vector Laboratories), and then detection of the second primary antibodies with Elite Vectastain ABC-peroxidase-Vector-SG (Vector Laboratories). The primary antibodies used were DNA duplication marker mouse-anti-BrdU (Becton Dickinson, San Jose, CA, USA); astrocyte-specific marker mouse-anti-GFAP (DAKO, A7S, Glostrup, Denmark); rabbit-anti-ChAT (Chemicon, AB5042, Temecula, CA, USA), rabbit-anti-Ach (Chemicon, AB5922); rabbit-anti-GAD (Novus Biologicals, Inc., AB2348, Littleton, CO, USA), rabbit-anti-GABA (Chemicon, AB5016); the neuron-specific marker mouse anti-Map 2ab (Boehringer Mannheim, Indianapolis, IN, USA). To verify the specificity of the immunostaining, primary antibodies were omitted from the staining procedure in a set of control experiments. Naive animals without BrdU injections were used as anti-BrdU-negative controls (n=4) (Gu et al., 2000; Jiang et al., 2001).

Confocal microscopy

Double/triple immunofluorescent brain sections were scanned with a laser scanning confocal microscope (Nikon Microscope ECLIPSE E800, Japan). In order to eliminate the potential bleed-through between different fluorophores, a separate fluorophore detection procedure was employed in the scanning, i.e., one employing sequential laser excitation in each optical plane (Gu et al., 2000, 2009; Jiang et al., 2001). When one fluorophore was scanned, all other channels were closed (i.e., zeroed). Confocal parameters (e.g., pinhole sizes) were adjusted to minimize the thickness of the optical sections.

Stereological cell counting

Three coronal brain sections through the ischemic lesion were randomly selected from each brain at 48 h, 7 days, and 30 days poststroke (n=3 in each group) (Gu et al., 2000, 2009). To assess the final cell populations, brain sections from the 30-day poststroke group were immunostained for BrdU and Ach, or BrdU and GABA double immunolabeling, or for Map-2ab single immunolabeling and counted under a CAST-Grid system (Olympus, Albertslund, Denmark) (Gundersen et al., 1988). To get a dynamic overview on the poststroke cell proliferation, BrdU single immunostaining was also performed in the 48 h and 7-day poststroke groups and brain sections were counted under the CAST-Grid system. The region of interest was defined as the cortical region confined between two artificial delineations from the lateral and interior edges of the ischemic ring lesion down to the corpus callosum as described previously (Gu et al., 2009). Both the ischemic cortical ring lesion and the centrally located penumbra were thus included in the stereological cell counting.

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