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Letter

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Doublecortin-immunoreactive Neuroblasts in Each Layer of the Main Olfactory Bulb After Transient Cerebral Ischemia in Gerbils

Jung Hoon Choi[1,2], Ki-Yeon Yoo[1,2], Ok kyu Park[1], Choong Hyun Lee[1], In Koo Hwang[3], Hyung-Cheul Shin[2,4] and Moo-Ho Won[1,2*]

[1]*Department of Anatomy and Neurobiology, and Institute of Neurodegeneration and Neuroregeneration, College of Medicine, Hallym University, Chuncheon, Korea*
[2]*Institute of Natural Medicine, Hallym University, Chuncheon, Korea*
[3]*Department of Anatomy and Cell Biology, College of Veterinary Medicine and BK21 Program for Veterinary Science, Seoul National University, Seoul, Korea*
[4]*Department of Physiology, College of Medicine, Hallym University, Chuncheon, Korea*

Neurogenesis in the adult brain occurs continuously throughout life. The main olfactory bulb (MOB) is the first central relay of the olfactory system. We examined proliferation of newly generated cells in each layer of the gerbil MOB after 5 min of transient cerebral ischemia using doublecortin (DCX), a marker of neuronal progenitors. Many DCX immunoreactive neuroblasts were found in the all layers of the MOBs of control and ischemia groups. Ten to 15 days after ischemia/reperfusion, no difference in numbers of DCX immunoreactive neuroblasts was found in the MOB. Thirty days after ischemia/reperfusion, significant increase of DCX immunoreactive cells was observed in all layers of ischemic MOB. This result indicates that neuroblasts increase in the MOB from 30 days after transient cerebral ischemia in gerbils.

Key words: Doublecortin, main olfactory bulb, migration, transient ischemia

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Neurogenesis occurs during embryonic and early postnatal stages, and is typically completed at mature stage (Hollyday, 2001). There are distinct regions of active proliferation even in the adult mammalian brain, which are known to generate neurons continuously throughout life (Eriksson *et al.*, 1998; Schmetsdorf *et al.*, 2005). The main olfactory bulb (MOB) is the first central relay of the olfactory system (Fujita *et al.*, 2003; Pappas *et al.*, 2003). This paleocortex is the only known target of neural progenitors produced in the anterior part of the subventricular zone (SVZ) lining the lateral ventricles. The SVZ, which lines the lateral ventricles, gives rise to new interneurons that reach the MOB via the rostral migratory stream (RMS) (Fukushima *et al.*, 2002; Katakowski *et al.*, 2005; Chou *et al.*, 2006).

Doublecortin (DCX) gene encodes a 40-kDa microtubule-associated protein, which is specifically expressed in neuronal precursors in the developing and adult central nervous system (CNS) (Jin *et al.*, 2004). Due to its specific expression pattern, attentions have been drawn to DCX as a marker for neuronal precursors and neurogenesis (Karl *et al.*, 2005; Rao *et al.*, 2006; Winner *et al.*, 2006). In addition, DCX is frequently used as a marker of neuronal migration, because DCX is necessary for adult SVZ cells migration as well as embryonic radial migration (Deuel *et al.*, 2006; Koizumi *et al.*, 2006; Ocbina *et al.*, 2006).

Transient cerebral ischemia enhances cell proliferation in the hippocampal dentate gyrus (Iwai *et al.*, 2002) and the SVZ (Jin *et al.*, 2001; Zhang *et al.*, 2001). It was reported that the number of cells labeled with bromodeoxyuridine (BrdU), a marker for cell proliferation, increased with a delayed peak in the MOB after ischemic insult (Iwai *et al.*, 2003). However, we could not find reports that showed proliferation of neuroblasts in each layer of the ischemic MOB in detail. In the present study, therefore, we studied ischemia-induced changes of DCX-labeled cells in each layer of the MOB for a long time after 5 min of transient cerebral ischemia in gerbils.

Male Mongolian gerbils (*Meriones unguiculatus*) were obtained from the Experimental Animal Center at Hallym University (Chuncheon, Korea). Gerbils were used at 6 months of age (body weight, 65-75 g). The animals were housed in a conventional state under adequate temperature (23°C) and humidity (60%) control with a 12-h light/12-h dark cycle, and provided with free access to water and food. Procedures involving animals and their care confirmed with the guidelines, which are in compliance

*Corresponding author: Moo-Ho Won, Department of Anatomy, College of Medicine, Hallym University, 39 Hallymdaehakgil, Chuncheon, Gangweon 200-702, Korea
Tel: +82-33-248-2522
Fax: +82-33-256-1614
E-mail: mhwon@hallym.ac.kr

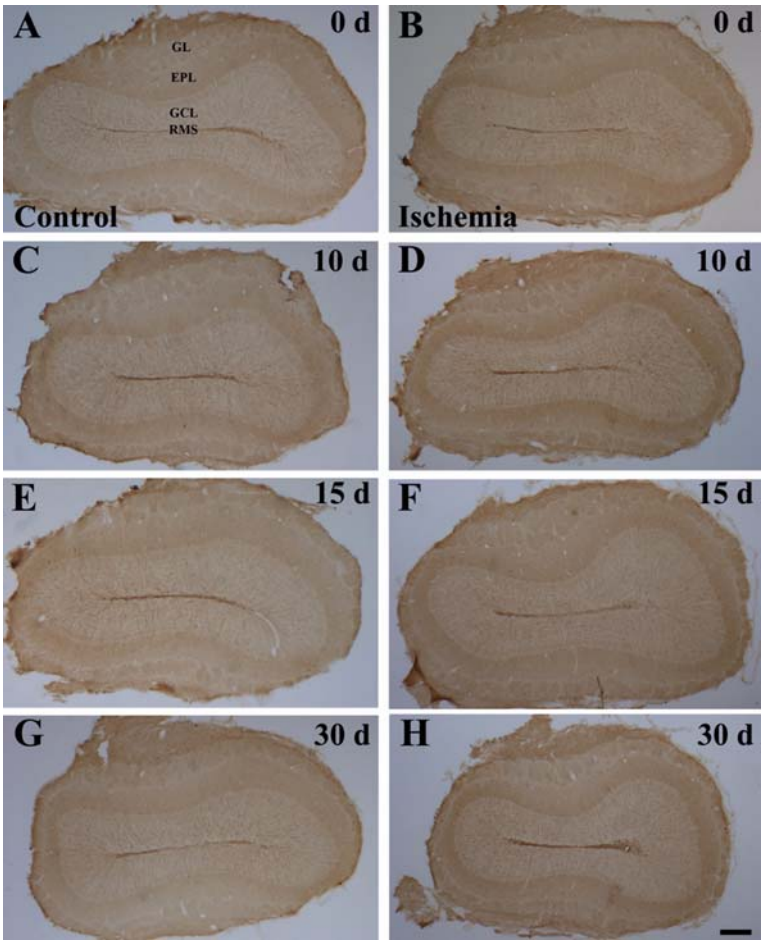

Figure 1. Low magnification of DCX immunoreactivity in the gerbil MOB of control (A, C, E and G) and ischemia-operated groups (B, D, F and H) after ischemia/reperfusion (I/R). DCX immunoreactive cells are distributed throughout the MOB. EPL, external plexiform layer; GCL, granule cell layer; GL, glomerular layer; RMS, rostral migratory stream. Bar=200 μm.

with current international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985, revised 1996), and they were approved by the Institutional Animal Care and Use Committee at Hallym's Medical Center.

The animals were anesthetized with a mixture of 2.5% isoflurane in 33% oxygen and 67% nitrous oxide. Thereafter, 5 min transient ischemia was induced via common carotid arteries occlusion according to our previous procedures (Hwang *et al.*, 2009). Sham-operated animals were subjected to the same surgical procedures except for arteries occlusion.

Sham- and ischemia-operated animals ($n=7$ at each time point) at designated times (0, 10, 15 and 30 days after reperfusion) were anesthetized with urethane, and perfused transcardially with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in PBS. Olfactory bulbs were removed, and cryoprotected by infiltration with 30% sucrose overnight. Thereafter frozen tissues were serially sectioned on a cryostat (Leica, Wetzlar, Germany) into 30-μm coronal sections, and they were then collected into six-well plates containing PBS.

For immunohistochemistry, the sections were sequentially treated with 0.3% hydrogen peroxide (H_2O_2) in PBS for 30 min and 10% normal rabbit serum in 0.05 M PBS for 30 min. They were then incubated with diluted goat anti-DCX antibody (1:50, SantaCruz Biotechnology, Santa Cruz, USA) overnight at room temperature and subsequently exposed to biotinylated rabbit anti-goat IgG and streptavidin peroxidase complex (1:200, Vector, Burlingame, USA). They were then visualized by staining with 3,3'-diaminobenzidine in 0.1 M Tris-HCl buffer (pH 7.2) and mounted on gelatin-coated slides. The sections were mounted in Canada Balsam (Kanto, Tokyo, Japan) following dehydration. A negative control test was carried out using pre-immune serum instead of primary antibody in order to establish the specificity of the immunostaining. The negative control resulted in the absence of immunoreactivity in all structures (data not shown).

For calculating numbers of DCX positive cells, 15 sections per animal were randomly selected. Ten counting squares (50 ×50 μm) for glomerular and granule cell layers, same sampling squares (100×100 μm) for external plexiform layer were used (West, 1993). Each studied field in each tissue was selected in the middle point of the medial MOB at the level of the rostrocaudal extent of −7.0 mm anterior to the Bregma based on the atlas

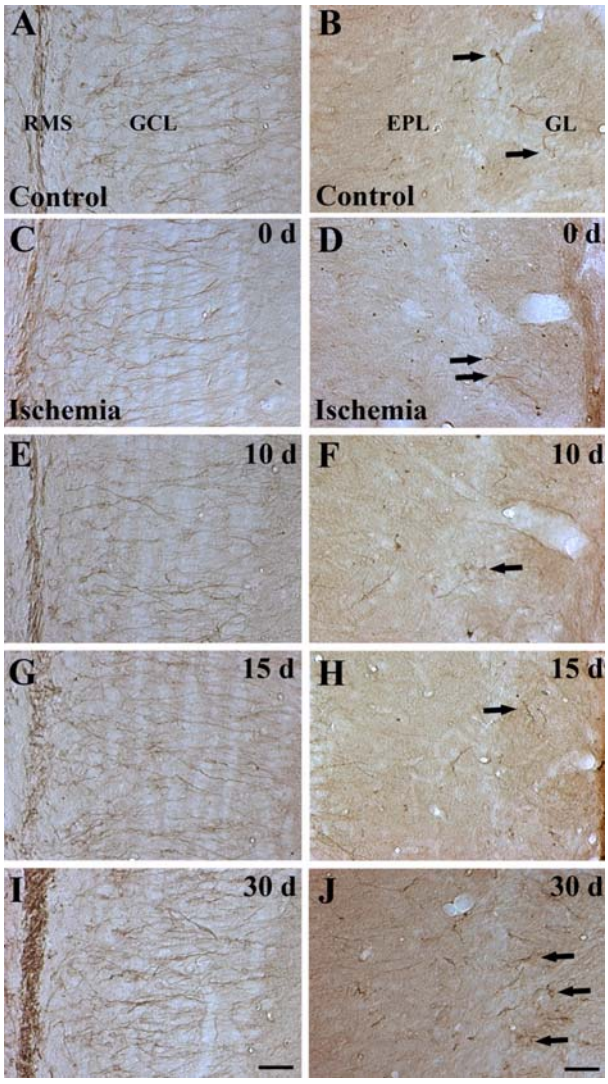

Figure 2. High magnification of DCX immunoreactivity in the glomerular (GL), external plexiform layers (EPL), granule cell layers (GCL) and rostral migratory stream (RMS) in the gerbil MOB of control (A and B) and ischemia-operated groups (C–J). Arrows indicate DCX immunoreactive neuronal precursors. Note the increase in DCX immunoreactive neuroblasts in all layers of MOB 30 days after I/R. Bar=50 μm.

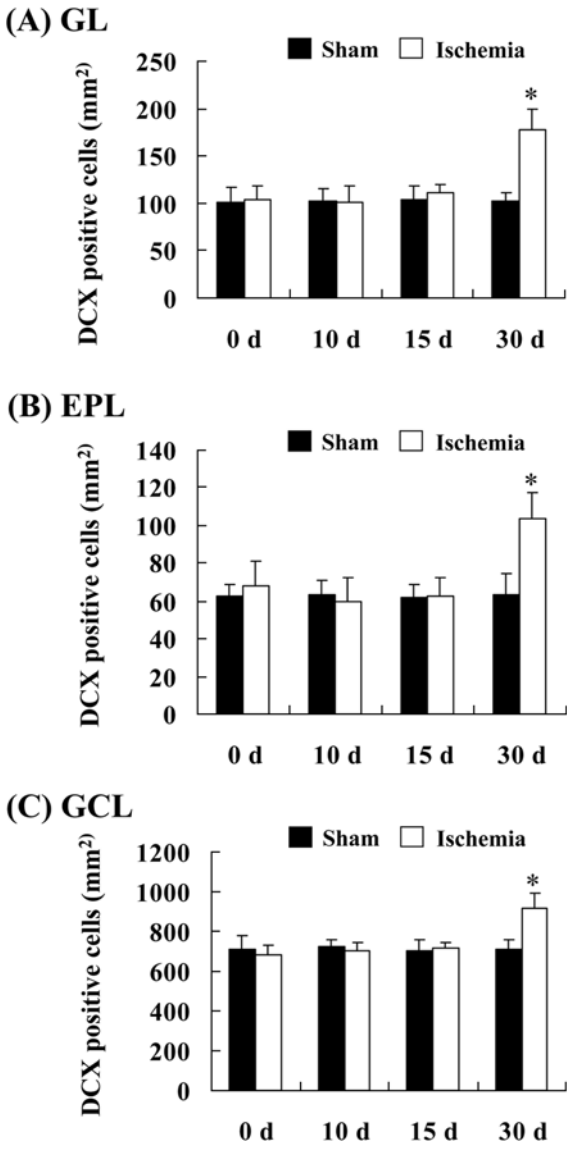

Figure 3. Changes in DCX immunoreactive neuroblasts in the GL (A), EPL (B) and GCL (C) after transient cerebral ischemia. (n=7 per group; *P<0.05, significantly different from the control-group). The bars indicate the means±SEM.

(Loskota *et al.*, 1974). Tissue images were obtained through an Axiophot Zeiss light microscope connected via CCD camera to a personal computer monitor. DCX positive cells were captured with an Applescanner, and the number of DCX positive cells was calculated using an image analyzing system equipped with a computer-based CCD camera (software: Optimas 6.5, CyberMetrics, Scottsdale, USA).

The inter-animal differences in each group, as well as the inter-experimental differences, were not statistically significant. Differences among the means were statistically analyzed by one-way analysis of variance followed by Duncan's new multiple range method to elucidate differences between all age groups. Statistical significance was considered at $P<0.05$.

Many DCX immunoreactive neuroblasts were detected in the granule cell layer (GCL) and rostral migratory stream (RMS) (Figures 1A and 2A), however a few DCX immunoreactive neuronal precursors were observed in the glomerular and external plexiform layers (GL and EPL) in the control group (Figures 1A and 2B). Distribution pattern and the number of DCX immunoreactive neuroblasts in each layer did not change during 30 days after ischemia/reperfusion (I/R) (Figures 1A, 1C, 1E, 1F and 3). Similar to the control group, DCX immunoreactive neuroblasts were mainly observed in the GCL and RMS (Figures 1B, 1D, 1F, 2C, 2E and 2G), and a few DCX immunoreactive neuroblasts were observed in the GL and EPL 0 to 15 days after I/R (Figures 2D, 2F and 2H). In these groups, the number of DCX

immunoreactive neuroblasts in each layer was not changed (Figures 3A, 3B and 3C). Thirty days after I/R, the number of DCX immunoreactive neuroblasts significantly increased in all layers of the MOB of ischemia-operated group compared to the control group (Figures 1H, 2I, 2J and 3C).

In the present study, we investigated changes in neuroblasts in the gerbil MOB after transient cerebral ischemia using DCX, a marker for neuroblasts. In control and ischemia groups, many DCX immunoreactive neuroblasts were observed in the RMS and GCL, but a few DCX immunoreactive neuroblasts were observed in the GL and EPL. This result is consistent with previous studies that recruited neurons in the MOB continue to arise postnatally from the SVZ of the lateral ventricles through the RMS which is a unique forebrain structure that provides a long-distance migratory route for the neural stem cells of the SVZ (Altman, 1969; Byrd and Brunjes, 2001; Fukushima *et al.*, 2002), and they were located in the deep GCL during the adult neurogenesis (Lemasson *et al.*, 2005).

It has been reported that neurogenesis increases in the SVZ after transient cerebral ischemia in the rat (Jin *et al.*, 2001; Zhang *et al.*, 2001) and gerbil (Iwai *et al.*, 2003). Newly generated cells in the SVZ migrate into the MOB through the RMS during the first two weeks (Pencea *et al.*, 2001; Ming and Song, 2005). These new cells then migrate radially to the outer cell layers and differentiate into interneurons (Carleton *et al.*, 2003). In this study, we found that no changes in numbers of DCX immunoreactive neuroblasts until 15 days after I/R, however increase of DCX immunoreactive neuroblasts was found in all layers of the MOB 30 days after I/R. This result is supported by the previous study that accumulation of newly generated cells significantly increased 30 days after ischemia in the gerbil MOB which was identified by BrdU labeling (Iwai *et al.*, 2003).

In conclusion, our results indicate that proliferation of neuroblasts in the MOB is enhanced by transient cerebral ischemia in the gerbil, showing that proliferation in the gerbil ischemic MOB significantly increased in all layers from 30 days post-ischemia.

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