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Sustained increase in adult neurogenesis in the rat hippocampal dentate gyrus after transient brain ischemia

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ABSTRACT

It is known that the number of newly generated neurons is increased in the young and adult rodent subventricular zone (SVZ) and dentate gyrus (DG) after transient brain ischemia. However, it remains unclear whether increase in neurogenesis in the adult DG induced by ischemic stroke is transient or sustained. We here reported that from 2 weeks to 6 months after transient middle cerebral artery occlusion (MCAO), there were more doublecortin positive (DCX+) cells in the ipsilateral compared to the sham-control and contralateral DG of the adult rat. After the S-phase marker 5-bromo-2′-deoxyuridine (BrdU) was injected 2 days after MCAO to label newly generated cells, a large number of BrdU-labeled neuroblasts differentiated into mature granular neurons. These BrdU-labeled neurons survived for at least 6 months. When BrdU was injected 6 weeks after injury, there were still more newly generated neuroblasts differentiated into mature neurons in the ipsilateral DG. Altogether, our data indicate that transient brain ischemia initiates a prolonged increase in neurogenesis and promotes the normal development of the newly generated neurons in the adult DG.

Neurogenesis occurs in two distinct brain areas throughout life in almost all mammals, the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the hippocampal DG [12]. Neuroblasts derived from the SVZ neural stem cells migrate tangentially to the olfactory bulb, where they differentiate into local interneurons. Hippocampal neural stem cells in the SGZ give rise to immature neurons, which migrate for a short distance to the granule cell layer (GCL) and differentiate into granular neurons that project axons to the CA3 region [14]. Neurogenesis in the hippocampus has been correlated with learning and memory, addiction, depression, epilepsy and schizophrenia [3].

The persistence of neurogenesis throughout life raises the possibility that neural stem cells mount an intrinsic regenerative response to replace neurons lost after stroke or other brain injury. Indeed, brain ischemia promotes the generation of new neurons derived from neural progenitor cells in the adjacent SVZ of the adult brain [8,19]. Previous studies have demonstrated that transient brain ischemic injury stimulates neurogenesis in the DG of adult and aged rodents [9].

The present study aims to address whether increase in neurogenesis induced by ischemic stroke in the adult rat DG is transient or long-lasting. We investigated neurogenesis by BrdU labeling and immunohistochemistry for cell type-specific markers in the DG during acute recovery (2 weeks and 6 weeks) and delayed recovery (6 months) from focal cerebral ischemic injury by using a widely used rat middle cerebral artery occlusion (MCAO) model [10].

Adult male Sprague–Dawley rats (240–250 g and about 8 weeks old at the time of surgery) were purchased from Shanghai Laboratory Animal Center, Chinese Academy of Sciences, China. The authors certify that all the experimental protocols used in the present study were in compliance with the NIH's Guide for the Care and Use of Laboratory Animals (1996) and institutional guidelines.

Rats were anesthetized with intra-peritoneal (i.p.) injections of chloral hydrate (360 mg/kg, body weight) and MCAO was induced by the intraluminal filament technique as previously described [1,8,10]. Briefly, the right common carotid artery (CCA) and external carotid artery were permanently ligated, and the MCA was occluded by a nylon monofilament inserted through the CCA. After

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2 h of occlusion, the filament was withdrawn. Sham-operated rats were treated identically except the MCA were not occluded after the nylon monofilament was inserted.

BrdU is an exogenous marker of DNA synthesis and is widely used in neurogenesis and neural stem cell research [16]. In the first experiment, BrdU (Sigma, St. Louis, MO, USA, 50 mg/kg in saline i.p.) was given twice daily at the interval of 8 h for 5 consecutive days from the third day after stroke. Rats were perfused 2 weeks, 6 weeks or 6 months after ischemia. In the second experiment, rats received twice daily injections of BrdU (50 mg/kg in saline i.p.) for five consecutive days after 6 weeks recovery from MCAO and rats were sacrificed 2 or 6 weeks after the last BrdU injection.

Animals were deeply anesthetized with chloral hydrate (400 mg/kg, body weight, i.p.) prior to intracardiac perfusion with 0.9% saline followed by 4% paraformaldehyde. Brains were post-fixed with 4% paraformaldehyde overnight and then cryoprotected at 4 °C for at least 24 h in 30% sucrose. The brain samples were frozen in embedding medium (O.C.T., Sakura Finetek, Torrance, CA, USA). Free-floating sections through the entire hippocampal axis were collected in 40 μm thickness in 6-well plates and were sampled 240 μm apart.

Immunohistochemistry staining was performed on 40 μm free floating sections in 24-well tissue culture plates. Sections for BrdU staining were pretreated with 2 N HCl for 1 h at room temperature to denature DNA. Sections were then blocked for 1 h in TBS with 0.3% Triton X-100 and 10% donkey serum. Primary antibodies were incubated for 24 h at 4 °C.

The primary antibodies were used at the following concentrations: goat polyclonal anti-DCX (Santa Cruz, CA), 1:200; mouse monoclonal anti-neuronal nuclei (Millipore, Temecula, CA), 1:400; rat monoclonal anti-BrdU (Accurate Chemical, Westbury, NY), 1:30; rabbit polyclonal anti-GFAP (Dako, Carpinteria, CA), 1:500; rabbit polyclonal anti-Ki67 (Vector Laboratories, CA), 1:500 and mouse monoclonal anti-PV (Millipore), 1:500. Secondary antibodies against appropriate species were incubated for 2 h at RT (all from Jackson, 1:200). All secondary antibody combinations were carefully examined to ensure that there was no cross-talk between fluorescent dyes or cross-reactivity between secondary antibodies. Fluorescently stained sections were coverslipped with Gel/Mount (Biomeda, Foster City, CA, USA). Omission of primary antibodies eliminated staining. Streptavidin and diaminobenzidine (DAB) were used to visualize the reaction product for bright-field staining sections.

Fluorescently immunolabeled sections were analyzed on a Olympus FV1000 confocal laser scanning microscope using the following filter sets with the indicated wavelengths (in nm) for the excitation laser line and emission filters: Cy2, 488/(505/530); Cy3, 543/(560–615). Confocal Z sectioning was performed at 0.8 μm intervals using a 60× (NA = 1.42) oil-immersion objective for single and double scanning. Images were acquired and a Z-stack was reconstructed using the Olympus software, cropped, adjusted and optimized in Photoshop 9.0. Images of enzyme histochemistry labeled sections and some fluorescently immune-labeled sections were acquired using an Olympus BX 51 microscope.

For cell quantification we used a traditional and well established method as described in detail previously [8,17,19,20]. Nonblinded quantification was performed on cells with fully colocalized relevant cell markers. BrdU labeled cells in bright-field were counted using 40× objective. Five to seven 40 μm sections per rat, spaced 240 μm apart were counted. Since 1/6 of the total number of sections was counted, we multiplied our counts by 6 to get total BrdU+ cells per DG. Cell counts for BrdU+/DCX+, BrdU+/NeuN+, DCX+/Ki67+, BrdU+/GFAP+ and BrdU+/PV+ cells were obtained in four 40 μm sections at 480 μm intervals in the DG using 60× objec-

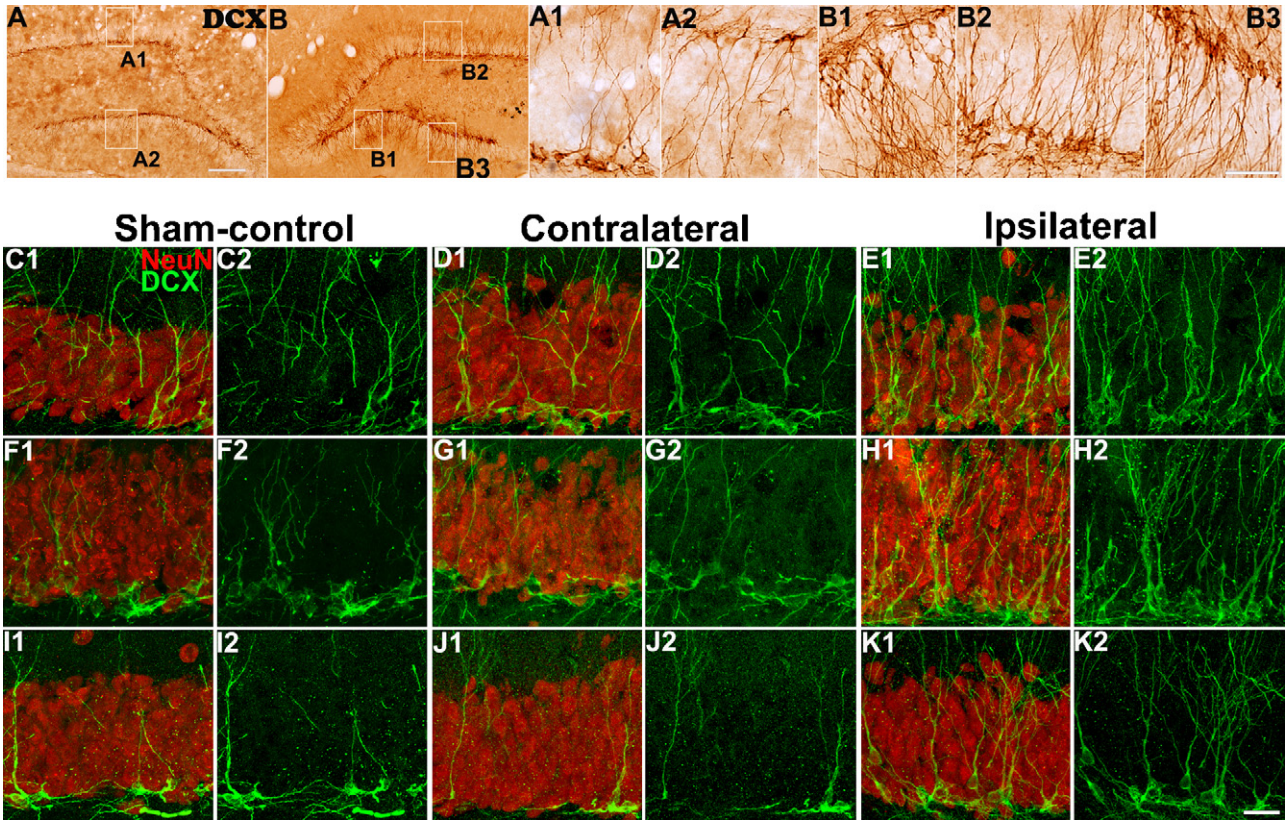

Fig. 1. More neuroblasts and immature neurons in the DG after MCAO. DCX+ cells are more abundant in the ipsilateral (B) compared to contralateral (A) DG 2 weeks after stroke. A1 and A2, higher magnification of the boxed areas in (A); B1–B3, higher magnifications of the boxed areas in (B). The number of DCX+ cells is significantly increased 2 weeks (C1–E2), 6 weeks (F1–H2) and 6 months (I1–K2) after stroke in the ipsilateral DG. Scale bars: A–B, 100 μm (in A); A1–B3, 50 μm (in B3); C1–K2, 20 μm (in K2).

tive. The total number of cells per DG was calculated by multiplying our counts by 12. The accuracy of counting double-positive cells using epifluorescence microscopy was determined by analysis of all cells in one random section, using a confocal laser-scanning microscope.

All statistical analysis was conducted in STATISTICA (StatSoft, USA). Data were analyzed using Student's *t*-test. A probability level of less than 0.05 was accepted as statistical significance. Degrees of statistical significance are presented as $^{*}P < 0.05$. Data in the text and figures are expressed as means ± SEM.

In the present study, neurological deficits, such as failure to extend left forepaw fully and circling to the left, were found 24–72 h after the onset of occlusion. Two hours of MCAO in adult rats resulted in large infarct involving the ipsilateral striatum, neocortex and hippocampus. Among the vast majority of results reported here, we selected brains that sustained moderate hippocampus injury (extensive loss of brain tissue with infarction). Brains without visible cell lose in hippocampus were excluded from analysis.

In order to investigate whether cerebral ischemia could stimulate neurogenesis in the DG, we first examined the location and amounts of DCX+ cells by immunostaining. DCX is a microtubule-associated protein primarily expressed by immature neurons and neuronally committed precursors, so it is widely used as a marker of adult neurogenesis [2]. Within the sham-control, contralateral and damaged DG, DCX+ cells formed clusters and located at the base of the granular zone. Some of these cells resembled neuroblast morphologies with processes paralleled to granule cell layer. Some extended processes through the entire granule cell layer and reached further into the molecular layer. Compared to the sham-control and contralateral, a readily visible increase in DCX immunostaining was observed in the ipsilateral DG after 2 weeks of recovery from MCAO (Fig. 1A and B). This result is consistent with previous observations that transient cerebral ischemia promotes neurogenesis in the DG [9,13].

In the sham-control and contralateral DG of aged animals examined (6 months recovery from MCAO), the amount of DCX+ cells greatly decreased (Fig. 1I). This result is consistent with previous reports that neurogenesis in the DG drastically reduced in the aged compared to the younger animals [15].

Quantitative data showed that there were always more DCX+ cells in the ipsilateral compared to sham-control and contralateral DG at 2 weeks (Fig. 1C–E, 231 ± 15% increase in the ipsilateral com-

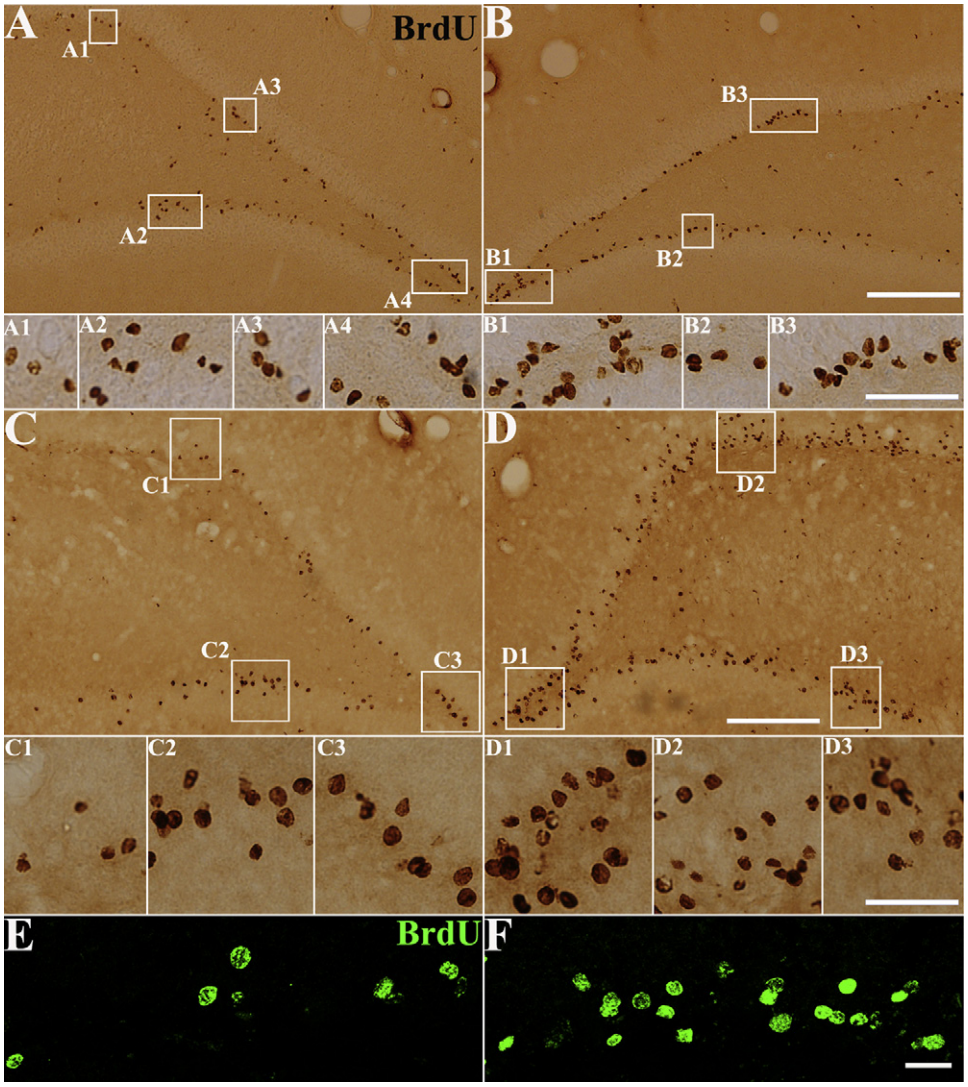

Fig. 2. Increase in cell survival in the rat DG following MCAO. Representative examples of BrdU labeling cells in the contralateral and ipsilateral DG 2 weeks (A, B), 6 weeks (C, D) and 6 months (E, F) after MCAO. A1–A4, higher magnification of the boxed areas in (A); B1–B3, higher magnification of the boxed areas in (B); C1–C3, higher magnification of the boxed areas in (C); D1–D3, higher magnification of the boxed areas in (D). Scale bars: A–B, 200 μm (in B); A1–B3, 50 μm (in B3); C–D, 200 μm (in D); C1–D3, 50 μm (in D3); E–F, 20 μm (in F).

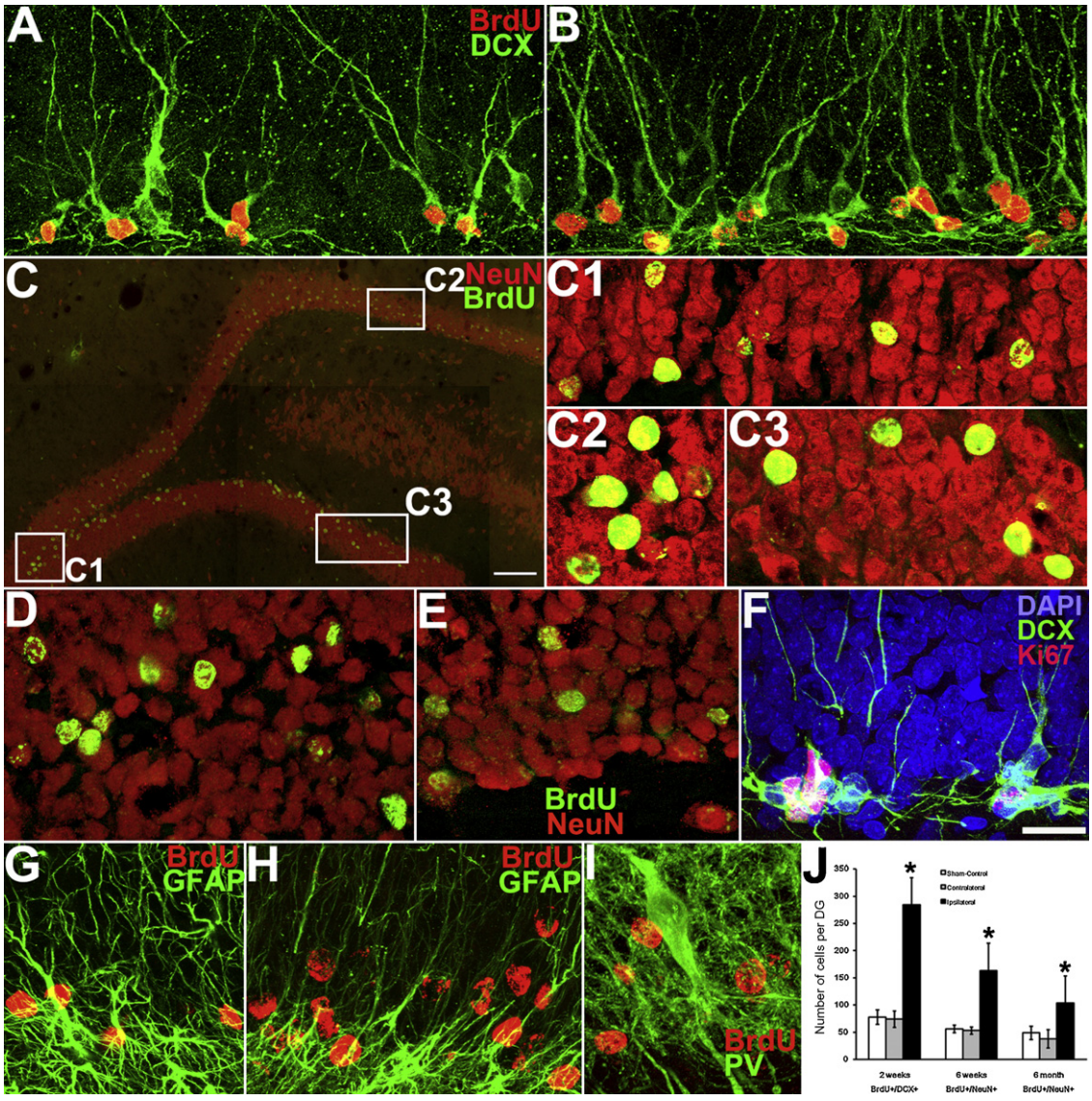

Fig. 3. Increase in newly born neuroblasts and mature neurons in the DG after MCAO. More BrdU+/DCX+ cells in the ipsilateral (B) compared to contralateral (A) DG 2 weeks after MCAO. BrdU+/NeuN+ cells are more abundant in the ipsilateral DG 6 weeks (C, C1–C3) and 6 months (D–E) after stroke. More DCX+/Ki67+ cells were observed in the ipsilateral DG (F). No BrdU+/GFAP+ cells were found in the ipsilateral DG 2 weeks (G) and 6 weeks (H) after stroke. BrdU+/PV+ cells were not found in the ipsilateral DG 6 weeks after ischemia (I). Quantification data were shown in (J). *$P < 0.05$. Scale bars: 20 μm in all images except in (C), 100 μm.

pared to sham-control DG, $P < 0.05$, Student's *t*-test, $n = 4$ rats), 6 weeks (Fig. 1F–H, 188 ± 16% increase in the ipsilateral compared to sham-control DG, $P < 0.05$, Student's *t*-test, $n = 5$ rats) and 6 months (Fig. 1I–K, 200 ± 12% increase in the ipsilateral compared to sham-control DG, $P < 0.05$, Student's *t*-test, $n = 5$ rats) of recovery from MCAO. We observed more DCX+/Ki67+ cells in the ipsilateral DG (Fig. 3F, 288 ± 12 *vs* 72 ± 8 cells in the ipsilateral *vs* sham-control DG, $P < 0.05$, Student's *t*-test, $n = 4$ rats). There was no significant difference in the contralateral DG between sham-control and operated groups. In addition, more DCX+/NeuN+ cells were also detected in the ipsilateral DG at 2 weeks, 6 weeks and 6 months of recovery from MCAO (Fig. 1).

Two weeks after stroke, BrdU+ cells were arranged along the border between the GCL and the hilus region with clusters of irregularly shaped nuclei (Fig. 2A and B). After 6 weeks and 6 months recovery, BrdU+ cells were large and round in appearance and the labeled cells had migrated into the granule cell layer and could be found through the whole layer (Fig. 2C–F).

Quantification analysis revealed that the number of BrdU+ cells in the ipsilateral was significantly higher (about 3-fold increase) than that of the contralateral DG (681.7 ± 85.2 *vs* 1485 ± 180.4 cells in the contralateral *vs* ipsilateral DG in 2 weeks group, $P < 0.05$, Student's *t*-test, $n = 5$ rats; 491.3 ± 41.6 *vs* 1307.3 ± 170.1 cells in the contralateral *vs* ipsilateral DG in 6 weeks group, $P < 0.05$, Student's *t*-test, $n = 4$ rats; 347.3± 97.5 *vs* 957.6 ± 85.5 cells in the contralateral *vs* ipsilateral DG in 6 months group, $P < 0.05$, Student's *t*-test, $n = 4$ rats). In the sham-control groups, the number of BrdU-positive cells was not significantly different between the contralateral and the ipsilateral DG neither at 2 weeks, 6 weeks or 6 months after surgery (data not shown). These data suggested that after transient brain ischemia, more cells are generated in the ipsilateral DG, and most of the newly born cells could survive at least 6 months.

To examine the neuronal identity of these newly generated cells shortly after ischemia, brain sections were subjected to double immunofluorescent labeling for BrdU and different markers. Two weeks after MCAO, more BrdU+/DCX+ cells were observed in the ipsilateral DG compared to the contralateral and sham-control DG (Fig. 3A, B and J; 78 ± 13 *vs* 284 ± 28 cells in the controlateral *vs* ipsilateral DG, $P < 0.05$ by Student's *t*-test, $n = 5$ rats), indicating that

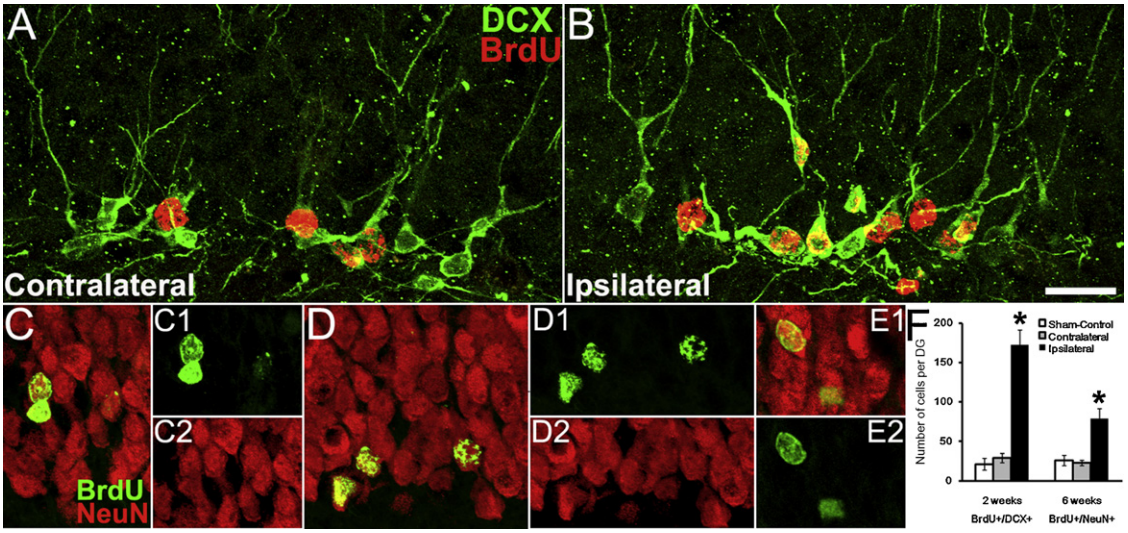

Fig. 4. Sustained increase in neurogenesis after longer recovery from MCAO. BrdU was injected intraperitoneally twice daily for five consecutive days at 6 weeks after stroke and BrdU was combined with immunostaining for neuronal markers at 2 or 6 weeks after last BrdU injection. BrdU+/DCX+ cells (A and B) and BrdU+/NeuN+ cells (C–E2) are more abundant in the ipsilateral DG. Quantification data were shown in (F). $^*P < 0.05$. Scale bar: 20 μm (in B).

those DCX+ cells observed in the ipsilateral DG are indeed newly generated immature neurons.

To determine whether these newly generated neuroblasts (BrdU+/DCX+ cells) differentiate into mature neurons, BrdU/NeuN double immunostaining was performed after 6 weeks and 6 months recovery from MCAO. Six weeks after ischemia, BrdU+/NeuN+ cells were located individually throughout the SGZ and the inner portion of the granule cell layer (Fig. 3C). Again, more BrdU+/NeuN+ cells were found in the ipsilateral compared to the contralateral DG (Fig. 3J, 53 ± 7 *vs* 163 ± 25 cells in the contralateral *vs* ipsilateral DG, $P < 0.05$ by Student's *t*-test, $n = 5$ rats). Similar phenomenon was observed in the 6 months groups in which BrdU+/NeuN+ cells were dispersed throughout the granule cell layer (Fig. 3D–F and J, 38 ± 12 *vs* 104 ± 16 cells in the contralateral *vs* ipsilateral DG, $P < 0.05$ by Student's *t*-test, $n = 5$ rats). These results indicated that newly generated neuroblasts differentiate into mature neurons after longer intervals of recovery from MCAO.

We also combined BrdU/GFAP and BrdU/parvalbumin (PV) double immunostaining. GFAP, typically found in the astrocytes, was not expressed by newborn cells in the sham-control, contralateral and ipsilateral DG after 2 and 6 weeks recovery from ischemia (Fig. 3G and H). We did not found any newly generated cells differentiated into PV+ interneurons too (Fig. 3I).

We also investigated the fate of newly generated cells after longer recovery from ischemic stroke. BrdU was injected for five consecutive days 6 weeks after MCAO. Two weeks after the last BrdU injection, more BrdU+/DCX+ cells were found in the ipsilateral than the contralateral DG (Fig. 4A and B). Six weeks after the last BrdU injection, similarly, more BrdU+/NeuN+ cells were found in the ipsilateral DG compared to the contralateral DG (Fig. 4C–E). This indicates that MCAO induces a persistent increase in adult neurogenesis in the rat hippocampal dentate gyrus.

In the present study, we demonstrate that moderate ischemia in adult rats stimulates robust and sustained proliferative response within hippocampal DG of the injured hemisphere. From 2 weeks to 6 months after stroke, more DCX-expressing cells were detected in the ipsilateral DG compared to the sham-control and contralateral DG. Using BrdU labeling methods, we found that more newly generated neuroblasts differentiated into mature DG granule cells and most of these granule cells survived at least 6 months. Moreover, we also showed that after longer recovery from MCAO, there were still more neuroblasts generated and differentiated into mature granule cells in the ipsilateral DG.

Studies have shown that stroke enhances cell proliferation and generation of neuroblasts in the SVZ [8,17,18] and DG [9,13]. In accordance with previous studies, our findings also show that robust production of new neurons in the DG occurs after stroke. However, our results are similar to, but different from previous studies that examine neurogenesis in the DG after adult stroke. Using ischemia models, previous studies reported the production of new neurons after ischemia, but the process of neurogenesis they observed was transient and lasted no longer than five weeks [5,9,13]. By contrast, we found a sustained increase in neurogenesis which lasted at least 6 months. The most plausible explanation for these discrepancies is that there are species differences in the neurogenic responses between mice and gerbils used in those studies, *vs* Sprague–Dawley rats in this study, as well as differences in ischemia models, which may also affect the outcomes [6].

In the normal DG, newly generated neuroblasts migrate and differentiate into mature neurons expressing NeuN in the granule cell layer [7]. Consistent with previous studies [5,6,9], our data showed that newly generated cells stimulated by ischemia gradually migrated into the granule cell layer and differentiated into mature neurons. Moreover, these neurons survived at least 6 months as has been shown by Liu et al. [9]. Conversely, BrdU+/GFAP+ cells or BrdU+/PV+ interneurons were not detected in the DG of ischemia hemisphere [5,9]. These results suggest that ischemia promotes normal development of newly generated neurons in the DG.

It has been shown that ischemia stimulates cell proliferation and survival in the DG [5]. However, it remains unclear about the survival, migration and differentiation of the cells generated after longer recovery from ischemia. Our data showed that there were still more DCX+, DCX+/NeuN+ and DCX+/Ki67+ cells in the ipsilateral DG after longer recovery from ischemia (6 weeks or 6 months). Since Ki67 is an endogenous marker of cell proliferation, the detection of DCX+/Ki67+ cells in the DG is a relatively strong indicator of persistent proliferation of DCX+ cells. Our results strongly suggest there is still an increase in neurogenesis after 6 months recovery from ischemia. This phenomenon is in agreement with continuous neurogenesis in the damaged neonatal and adult striatum [8,18,19] and damaged neocortex [17] from progenitors resided in the SVZ. This suggests that neural stem cells in different brain

areas (SVZ or SGZ) share similar response to brain injury. How does transient brain ischemia regulate adult neurogenesis over the long-term? Epigenetic modification of DNA, such as DNA demethylation and histone acetylation, may account for one of the reasons for these long-lasting effects [4]. A recent study has shown that Gadd45b links neuronal circuit activity to DNA demethylation in controlling key aspects of activity-dependent adult neurogenesis [11].

Our findings may have several important implications. For example, experimental studies to explore molecular mechanisms of neuronal replacement from endogenous neural stem cells in the damaged brain are not restricted to the acute post-ischemic phase but can be applied over an extended time. Understanding the molecular mechanism may have important therapeutic implications for treatment of neurological disorders and brain injury.

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