



Research paper

Neurogenesis changes and the fate of progenitor cells after subarachnoid hemorrhage in rats

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ABSTRACT

Background: Subarachnoid hemorrhage (SAH) is a devastating cerebrovascular disease that leads to poor outcomes. Neurogenesis, an essential recovery mechanism after brain injury, has not been fully elucidated after SAH.

Methods: A total of 122 SD rats were used in this study. For experiment one, the rats were randomly divided into six groups: sham and SAH with different time points (1,3,5,7,14 days) ($n = 12$ /group). An endovascular perforation method was conducted for SAH model. Rats were injected with 5-Bromo-2'-deoxyuridine (BrdU, 50 mg/kg) 24 h before euthanasia at different time points after SAH. The BrdU labeled cells were detected by immunohistochemistry; Doublecortin (DCX) and glial fibrillary acidic protein (GFAP) were measured by western blot and immunohistochemistry. For experiment two, rats were randomly divided into five groups: sham and SAH with different time points (1, 2, 4, 8 weeks) ($n = 6$ /group). Rats received BrdU (50 mg/kg) once daily for 7 days after the induction of SAH. Double immunofluorescence staining was used to verify proliferation, differentiation and migration of progenitor cells. Rotarod test and water maze used to test the neurobehavioral recovery.

Results: Our results showed that BrdU positive cells in hippocampus changed overtime after SAH. BrdU positive cells decreased as early as 1 day reaching lowest levels at 3 days after SAH, after which it gradually recovered. Similar change patterns were observed with DCX, which was reversed with GFAP. In addition, BrdU did not colocalize with cleaved caspase-3. The BrdU positive cells mainly differentiated into immature neurons for short-term fate, whereas they differentiated into mature neurons for long-term fate but not astrocytes, which facilitated neurobehavioral recovery after SAH.

Conclusion: Neurogenesis in the hippocampus changes overtime after SAH. The neuronal progenitor cells may play an essential role in the neurobehavioral recovery after brain injury induced by SAH, since short-term progenitors helped with the recovery of immature neurons in the hippocampus, whereas long-term progenitors differentiated into mature neurons.

1. Introduction

Subarachnoid hemorrhage (SAH) is a life-threatening cerebrovascular disease with high morbidity, mortality and disability that leads to poor outcomes and long-term complications (Nieuwkamp et al., 2009). Middle-aged patients are at highest risk of SAH associated with high fatality rates in all stroke subtypes. SAH is a major burden of disease to not only patients but also on our economy and society

(Lapchak and Zhang, 2017). Brain injury after SAH including early brain injury (EBI, occurs after SAH onset and lasted to 72 h) and delayed neurological deficits have been considered a major cause of death and disability. (Chen et al., 2014; Fujii et al., 2013).

Neurogenesis, characterized by proliferation, migration and differentiation of neural stem cells especially neuronal progenitor cells, occurs mainly in two regions: the subventricular zone (SVZ) of the lateral ventricles walls and the subgranular zone (SGZ) of the dentate gyrus

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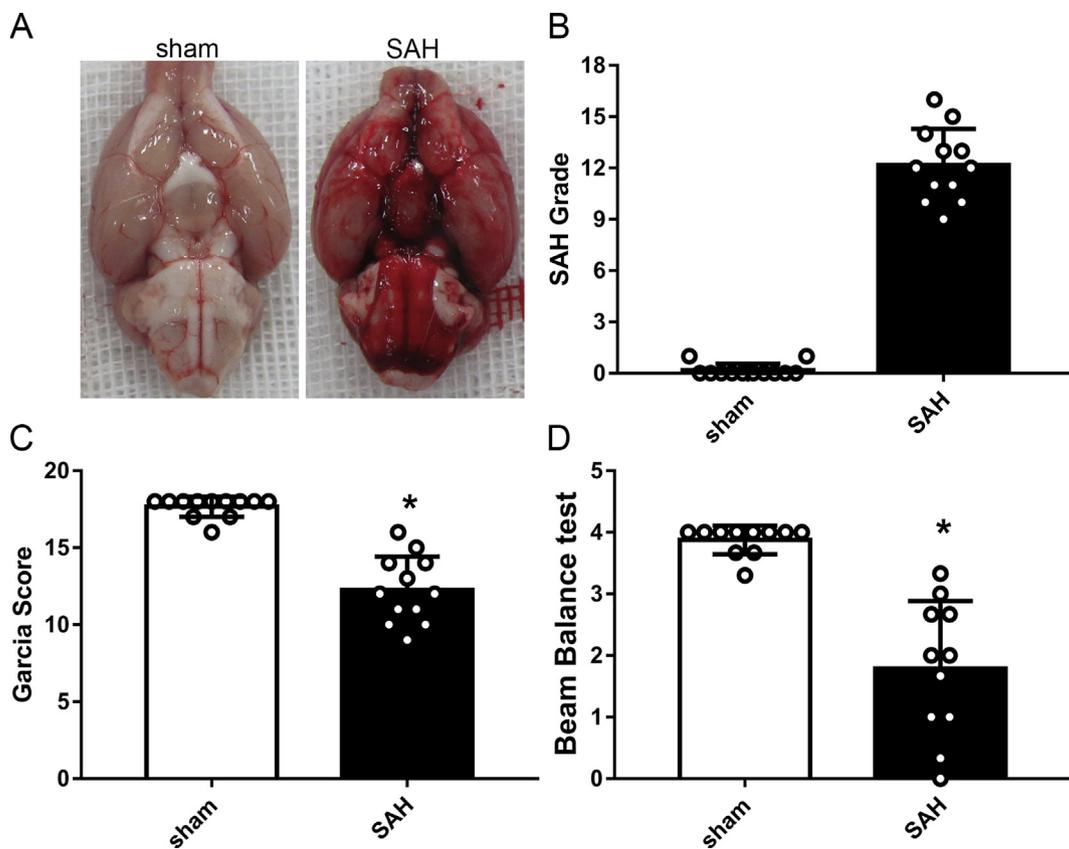


Fig. 1. SAH grade and neurological score for SAH 24 h group. A. Representative images of the brains from sham and SAH group at 24 h after operation. B. the SAH grade scores of the sham and 24 h SAH group. C. Modified Garcia and D. beam balance test of sham and SAH group at 24 h. Neurobehavioral score showed SAH group manifested significant neurological deficit compared to sham group. $n = 12$ per group. * $P < 0.05$ vs. sham group. Bars represent mean \pm SD.

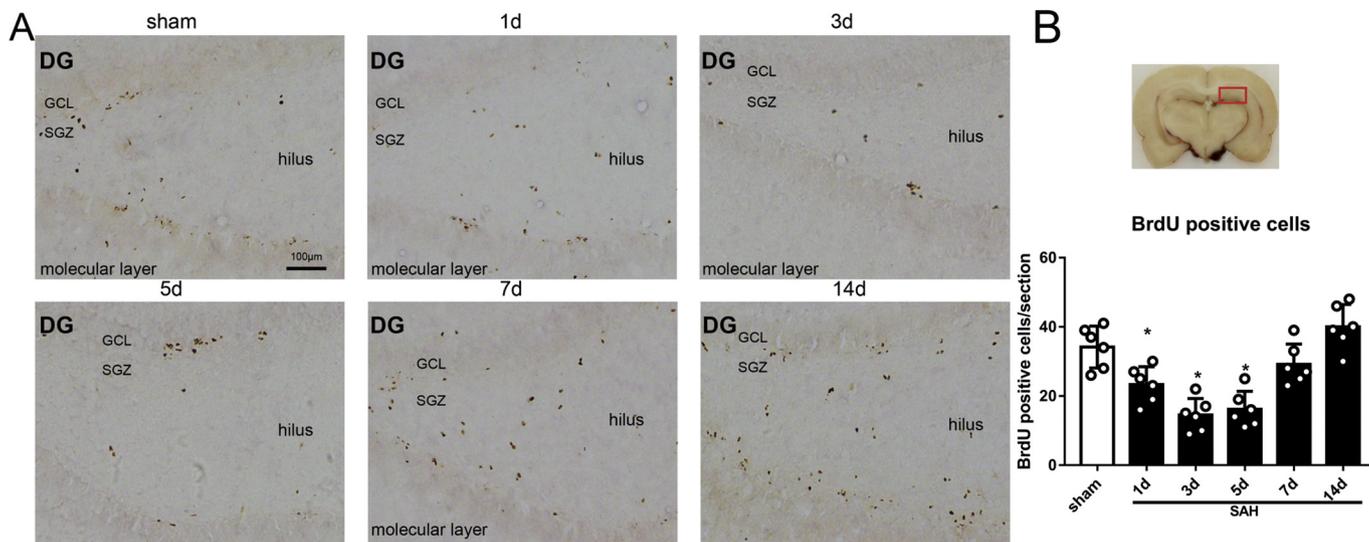


Fig. 2. BrdU labeling neurogenesis changes in left hippocampus after SAH. A. Immunohistochemistry staining of BrdU and B. quantitative analyses of BrdU positive cells change time course from the left hemisphere after SAH. $n = 6$ per group. * $P < 0.05$ vs. sham group. Bars represent mean \pm SD. BrdU, 5'-bromo-2'-deoxyuridine; GCL, granule cell line; SGZ, subgranular zone.

(DG) in the hippocampus (Gage, 2000). It has been reported that neurogenesis plays an essential role in the recovery process of many neurological diseases in vitro and in vivo, including cerebral ischemia (Adamczak et al., 2017; Chen et al., 2018; Jin et al., 2017; Song et al., 2017), traumatic brain injury (Carlson and Saatman, 2018; Choi et al., 2016; Hood et al., 2018) and SAH (Mino et al., 2003). These data

collectively demonstrated the possibility of recruiting latent regenerative potential cells to repair damaged tissue. However, the involvement of neurogenesis changes and short-term and long-term fate of progenitor cells in a rat model of SAH has not been fully elucidated. In the present study, we investigated temporal changes of neurogenesis in hippocampus and fate of progenitor cells in rats after SAH.

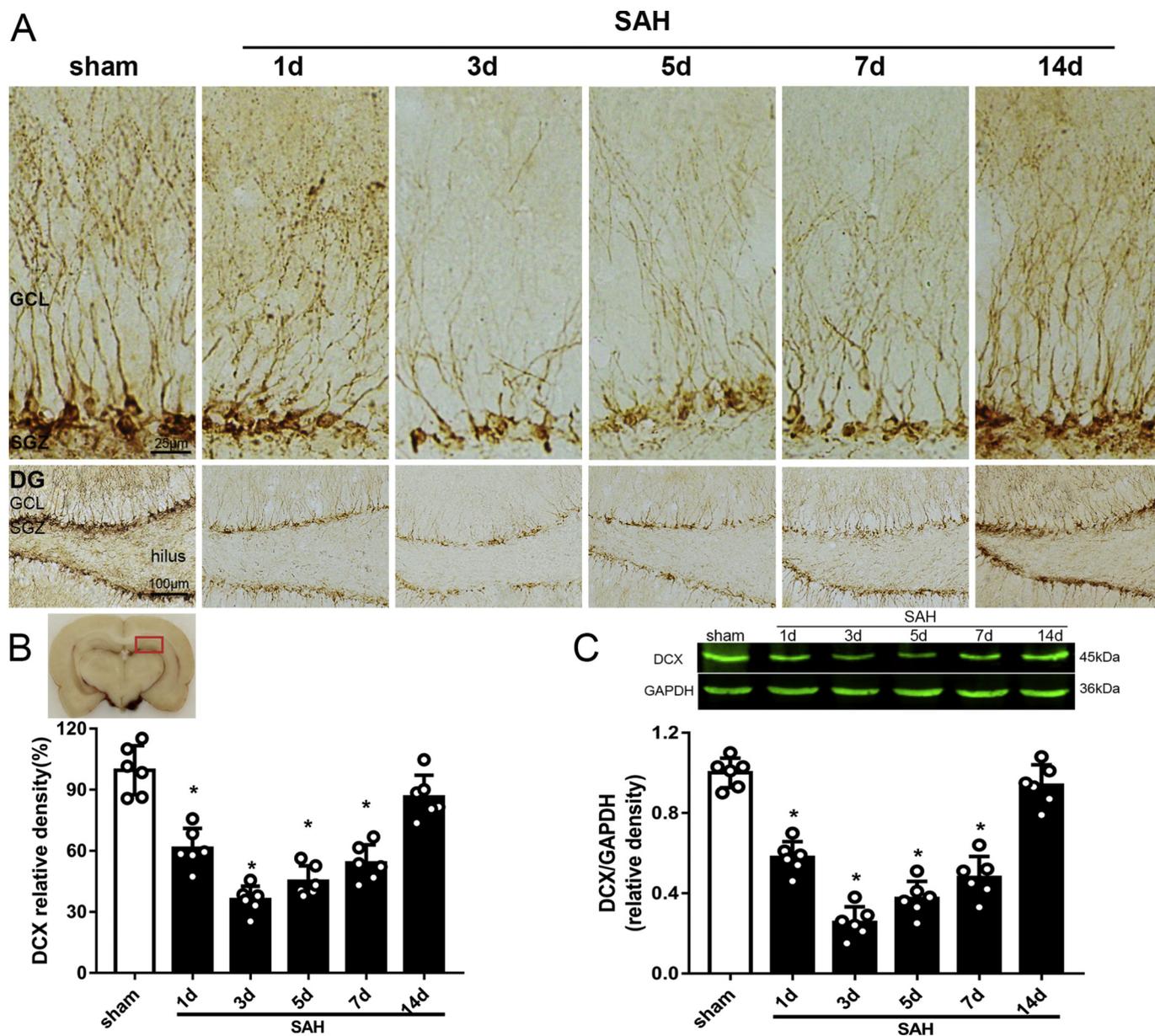


Fig. 3. DCX changes in left hippocampus after SAH. A. Immunohistochemistry staining of DCX and B. quantitative analyses of DCX change time course from the left hemisphere after SAH. n = 6 per group. C. Representative Western blot images and quantitative analyses of DCX time course from the left hemisphere after SAH. n = 6 per group. *P < 0.05 vs. sham group. Bars represent mean ± SD. DCX, doublecortin; GCL, granule cell line; SGZ, subgranular zone.

2. Methods and material

2.1. Animals and experimental SAH model

Adult male Sprague–Dawley rats (250 g–280 g) were purchased from the Animal Center of Central South University (Changsha, China). Rats were housed in a temperature (25 °C) and humidity-controlled room and with a 12-h light/dark cycle. Standard animal food and water ad libitum. All the experimental protocols were approved by the Ethics Committee of Central South University.

Experimental SAH model was conducted by a modified endovascular perforation method as previously described (Liu et al., 2014; Sugawara et al., 2008). Briefly, rats were anesthetized by intraperitoneal (i.p) injection with sodium pentobarbital (40 mg/kg). The left common carotid artery, external and internal carotid artery were carefully dissected, and a 4–0 monofilament nylon suture was inserted into the left internal carotid artery through the external carotid artery

stump until reach to the bifurcation of the anterior and middle cerebral artery where a resistance was felt, and then advanced 3 mm to perforate the bifurcation. Sham rats underwent identical procedures except the perforation.

2.2. Experimental design

Experimental designs are depicted in the Fig. S1. Experiment 1 – Short term study, seventy-two survival animals were divided randomly into six groups (n = 12/group): Sham, SAH (1, 3, 5, 7, 14 days (d)) groups. In addition, two SAH rats were injected with saline to act as a control, giving us a total of 74 rats in the experiment. The neurological function was measured by modified Garcia score and beam balance test at 24 h after surgery. Rats were euthanized at different time points after being anesthetized with sodium pentobarbital. Samples were collected for western blot (n = 6/group) and immunohistochemistry (n = 6/group).

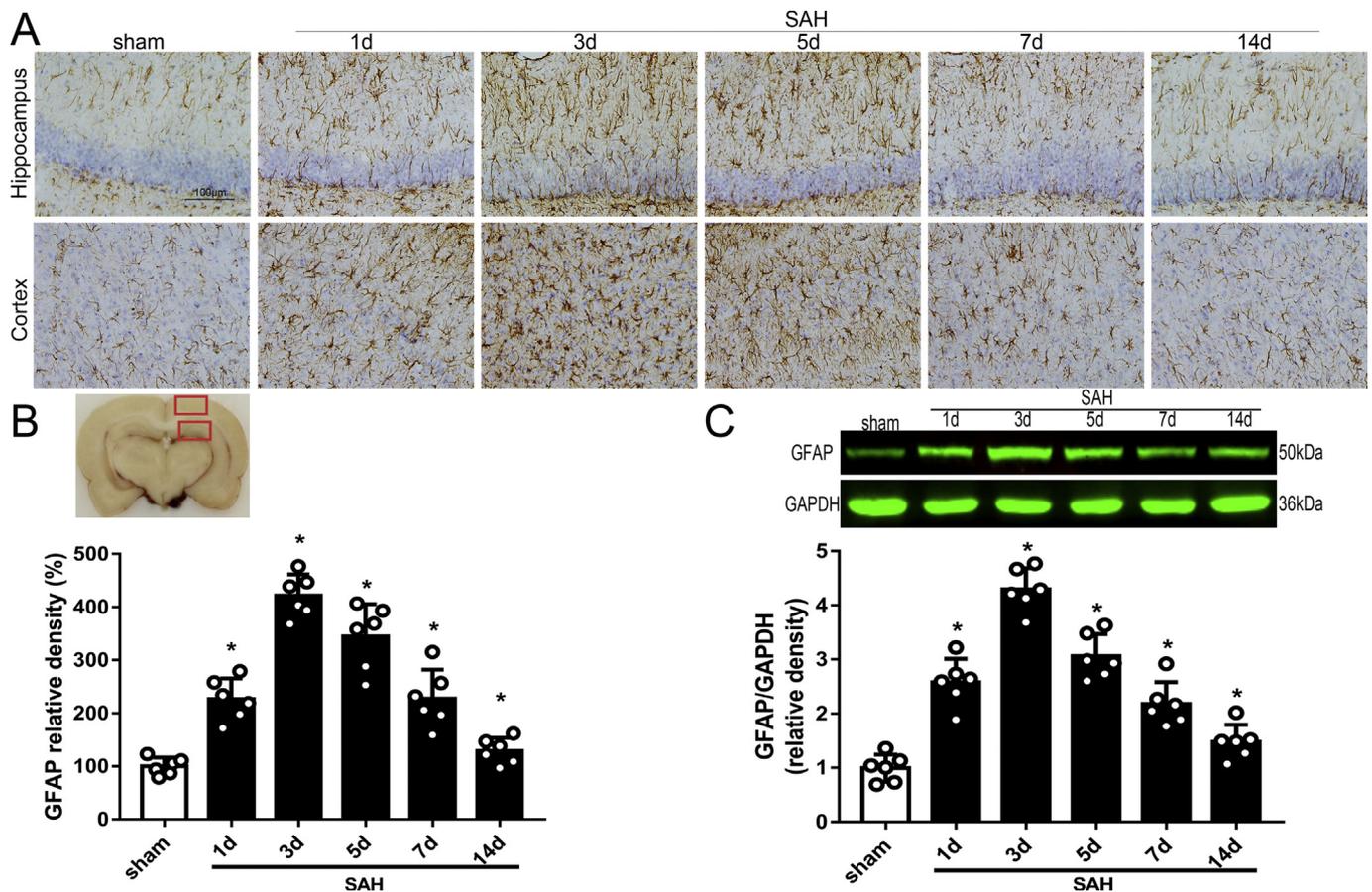


Fig. 4. GFAP changes in left hemisphere after SAH.

A. Immunohistochemistry staining of GFAP and **B.** quantitative analyses of GFAP change time course from the left hemisphere after SAH. $n = 6$ per group. **C.** Representative Western blot images and quantitative analyses of GFAP time course from the left hemisphere after SAH. $n = 6$ per group. * $P < 0.05$ vs. sham group. Bars represent mean \pm SD. GFAP, glial fibrillary acidic protein.

Experiment 2 – Long term study, thirty survival rats were divided randomly into five groups ($n = 6$ /group): Sham and SAH (1, 2, 4, 8 weeks (w) groups). Rotarod test were performed to measure the balance and sensorimotor coordinator abilities, and Morris water maze test was applied to measure the spatial cognition and memory after SAH. Brain samples were collected after perfusion by 4% paraformaldehyde for immunohistochemistry thereafter.

2.3. Detection of progenitor's proliferation after SAH

For experiment 1: Rats received i.p injections of 5'-bromo-2'-deoxyuridine (BrdU, 50 mg/kg dissolved in saline, Sigma Aldrich, USA) 24 h before euthanasia at different time points after SAH, to label newly generated cells. Another two SAH rats received saline injection with the same injection protocol as BrdU, to test the specificity of BrdU and its antibody ($n = 2$) (Fig. S2 A). For experiment 2: Rats received i.p injection of BrdU (50 mg/kg) once daily for 7 days after surgery. BrdU positive cells analysis was performed at 1, 2, 4, and 8 weeks after SAH. BrdU labeled nuclei were detected by immunohistochemistry.

2.4. SAH grading and short term neurological function

SAH grade, a parameter to evaluate the severity of SAH, was calculated based on a grading system as previously described (Sugawara et al., 2008). Briefly, the system was according to the blood score calculated from the six segments of basal cistern. The amount of subarachnoid blood clots defined as the following scores: score = 0, no subarachnoid blood; score = 1, minimal subarachnoid clots; score = 2,

moderate subarachnoid clots with recognizable arteries; and score = 3, blood clots without recognizable arteries. The total score from all six segments ranging from 0 to 18 were added up to find the total score. The neurological function was evaluated in all rats at 24 h after SAH induction using the modified Garcia scoring system and beam balance test as previously described (Sugawara et al., 2008; Xiao et al., 2018). The neurological deficit after SAH indicated the successes of experimental animal model. The assessment of SAH grading and neurological score were performed by independent researchers who were blind to the experiment.

2.5. Rotarod test and Water maze test

The Rotarod test was performed at the 1-, 2- and 3- weeks after induced SAH to assess the abilities of sensorimotor coordination and balance as previously described (Chen et al., 2011). Water maze test was performed at days 21–25 and 49–53 post -SAH to assess the spatial cognition and memory as previously shown (Sherchan et al., 2011; Yan et al., 2016).

2.6. Western blot analysis

Rats ($n = 6$ /group) were euthanized at 1d, 3d, 5d, 7d and 14d respectively after SAH induction or 24 h after sham operation. A whole cell protein extraction was performed on the left hemisphere that was obtained. Briefly, the samples were homogenized and centrifuged ($12,000 \times g$, 20 min at 4°C). Supernatants were collected followed by a protein concentrations detection using a BCA kit (Beyotime, China).

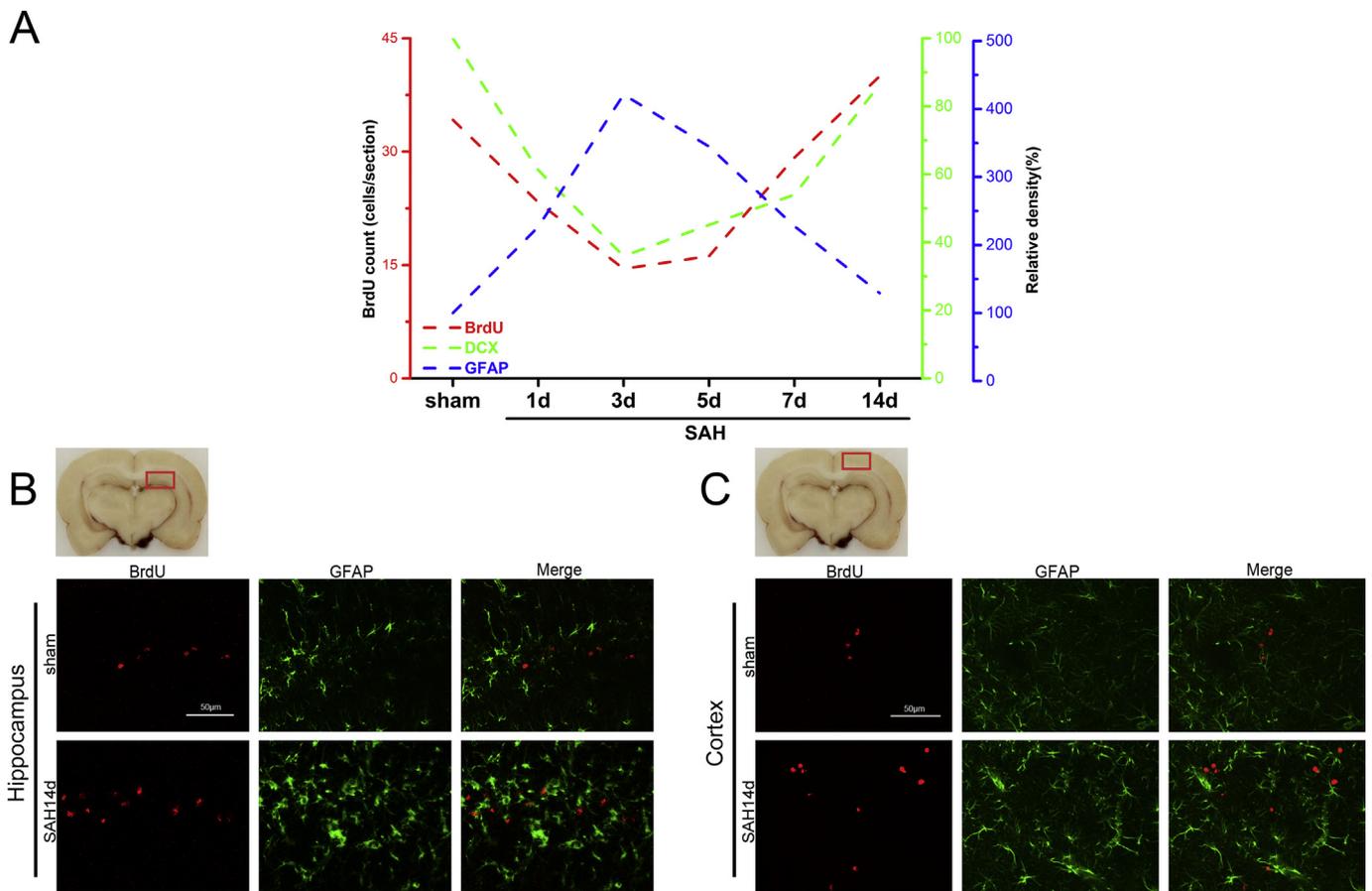


Fig. 5. Change patterns of BrdU with GFAP and DCX.

A. Line graph of BrdU positive cells in hippocampus change overtime together with GFAP and DCX. B.C. Double immunofluorescence staining for BrdU (red) with activated astrocytes (GFAP, green) in the left hippocampus and cortex after SAH. n = 6 per group. BrdU, 5'-bromo-2'-deoxyuridine; DCX, doublecortin; GFAP, glial fibrillary acidic protein. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

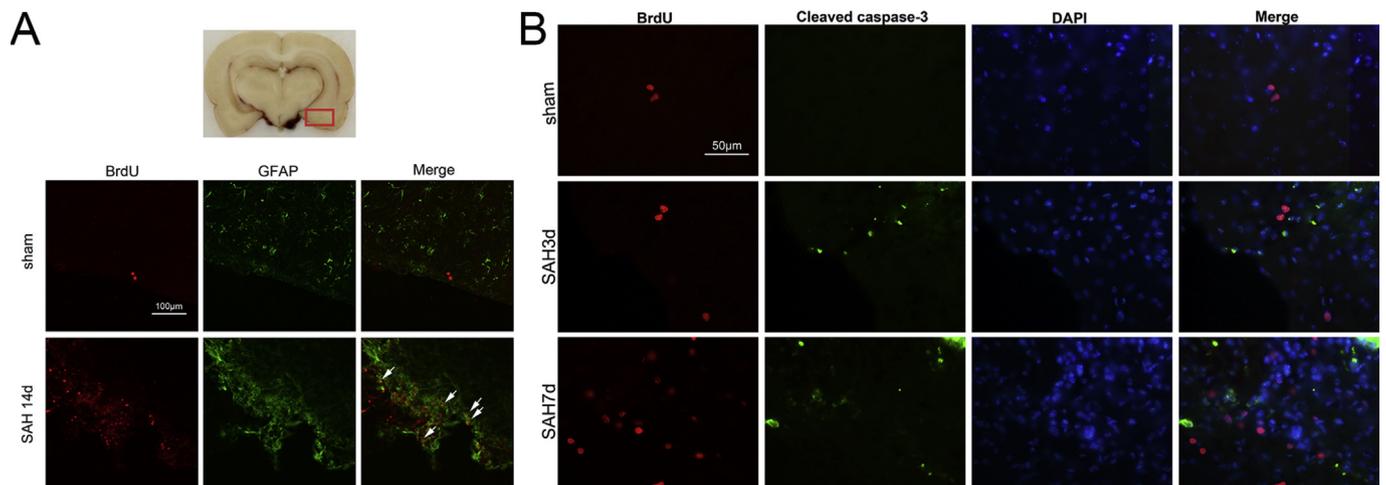


Fig. 6. BrdU positive cells increased on perforation side after SAH but not suffered with apoptosis.

A. Double immunofluorescence staining for BrdU with GFAP in the basal cortex with glial scar formation on perforation side of sham and SAH. C. Double immunofluorescence staining for BrdU (red) with cleaved caspase-3 (green). n = 6 per group. BrdU, 5'-bromo-2'-deoxyuridine.

The protein samples were loaded and separated by 10% sodium dodecyl sulfate–polyacrylamide (SDS-PAGE) gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, USA). After blocking with 5% nonfat milk in TBS at room temperature for 2 h, membranes were incubated with the following primary antibodies overnight at 4 °C: DCX (1:1000, Abcam, Cambridge, MA, USA), GFAP (1:1000, Abcam, Cambridge, MA, USA) and GAPDH (1:2000,

Proteintech, China). After incubation, the membranes were washed with TBST and incubated with appropriate secondary antibodies conjugated to Alexa Fluor® 790 (diluted 1:10000) for 1 h at room temperature. The signal was detected and quantified by the Odyssey infrared imaging system (LI-COR) (Odyssey®-CLX, LI-COR Biosciences, USA).

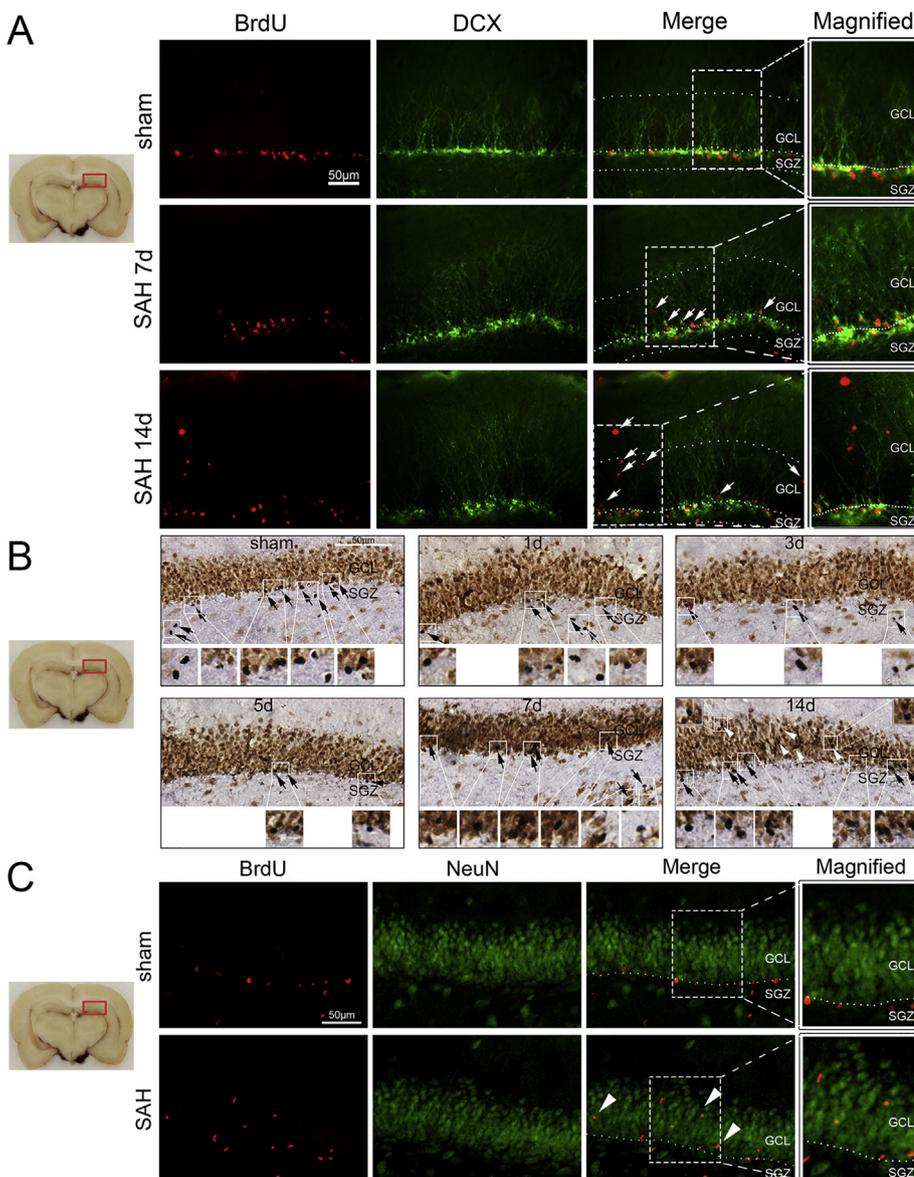


Fig. 7. Progenitor cells short term fate after SAH. **A.** Double immunofluorescence staining for BrdU (red) with DCX (green) in the left hippocampus after SAH. White arrowhead show BrdU positive cells migrate into GCL. **B.** immunohistochemistry staining for BrdU (black) and NeuN (brown) in different time point. Black arrows show BrdU positive cells in SGZ, white arrowhead show BrdU positive cells migrate into GCL. **C.** Double immunofluorescence staining for BrdU (red) with NeuN (green) in the left hippocampus at 14 days after SAH. White arrowhead show BrdU positive cells migrate in to GCL. $n = 6$ per group. BrdU, 5'-bromo-2'-deoxyuridine; DCX, doublecortin; NeuN, neuronal nucleus. GCL, granule cell line; SGZ, subgranular zone. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.7. Immunohistochemistry

Rats were euthanized with sodium pentobarbital and transcardially perfused with cold saline, followed by 4% paraformaldehyde. Brains were collected, afterwards post-fixed in 4% paraformaldehyde and cryoprotected in 30% sucrose. 30- μ m coronal brain sections were cut using a cryostat (Leica CM3050S, Buffalo Grove, USA). Each animal sections were subdivided for immunohistochemistry. First, the sections were rinsed 3 times with PBS, then free-floating treated with 3% H_2O_2 in PBS for 30 min. Then, (1) Sections were processed to immunohistochemical detection with BrdU labeled nuclei. DNA was denatured to expose the antigen by pre-treating sections with 50% formamide/ $2 \times$ SSC at 65 °C for 1 h, incubated in 2 N HCl at room temperature for 30 min. Sections were incubated in 5% normal horse serum in PBS with 0.1% Triton X-100 for 1 h and incubated with rat anti-BrdU antibody (1: 1000, Abcam, Cambridge, MA,USA); (2) Sections were blocked with 5% normal horse serum in 0.1% PBS-Triton X-100 for 1 h, followed by incubation with the primary antibodies anti-DCX (1:1000, Abcam, Cambridge, MA,USA), anti GFAP (1:1000, Abcam, Cambridge, MA, USA), anti-NeuN (1:1000, Millipore, USA) at 4 °C overnight. After rinsed with PBS 3 times, the sections were incubated with biotinylated secondary antibody at 1:400 for 1 h followed

by ABC reagents (1:400; Vector Laboratories, Burlingame, CA, USA) for 1 h, with the immunoreactivity visualized in 0.003% H_2O_2 and 0.05% 3, 3'-diaminobenzidine(DAB) or nickel enhanced DAB.

2.8. Immunofluorescence

For double immunofluorescence staining, in order to detect the BrdU labeled nuclei, sections were pre-treated with 50% formamide/ $2 \times$ SSC at 65 °C for 1 h, following acid hydrolysis using 2 N HCl in 0.1% PBS-Triton X-100 for 30 min at 37 °C to denature the DNA. After rinsed 3 times with PBS, the sections were pretreated in PBST containing 5% donkey serum for 1 h, then incubated overnight at 4 °C with the rat anti BrdU (1:1000, Abcam, Cambridge, MA,USA) together with one of the following:(1) rabbit anti NeuN (1:1000, Millipore, USA); (2) goat anti DCX (1:1000, Abcam, Cambridge, MA,USA);(3) rabbit anti GFAP (1:1000, Abcam, Cambridge, MA, USA); (4) rabbit anti cleaved caspase-3 (1:500, Cell Signaling Technology, Beverly, USA). Appropriate second antibodies including donkey anti-rat, anti-rabbit or anti-goat IgGs (1:200, Invitrogen, Carlsbad, CA, USA) conjugated with Alexa Fluor® 488 and Alexa Fluor® 594 were applied to visualize the immunofluorescence. The images were caught by Olympus BX51 microscope (CellSens Standard, Olympus Corporation, Japan).

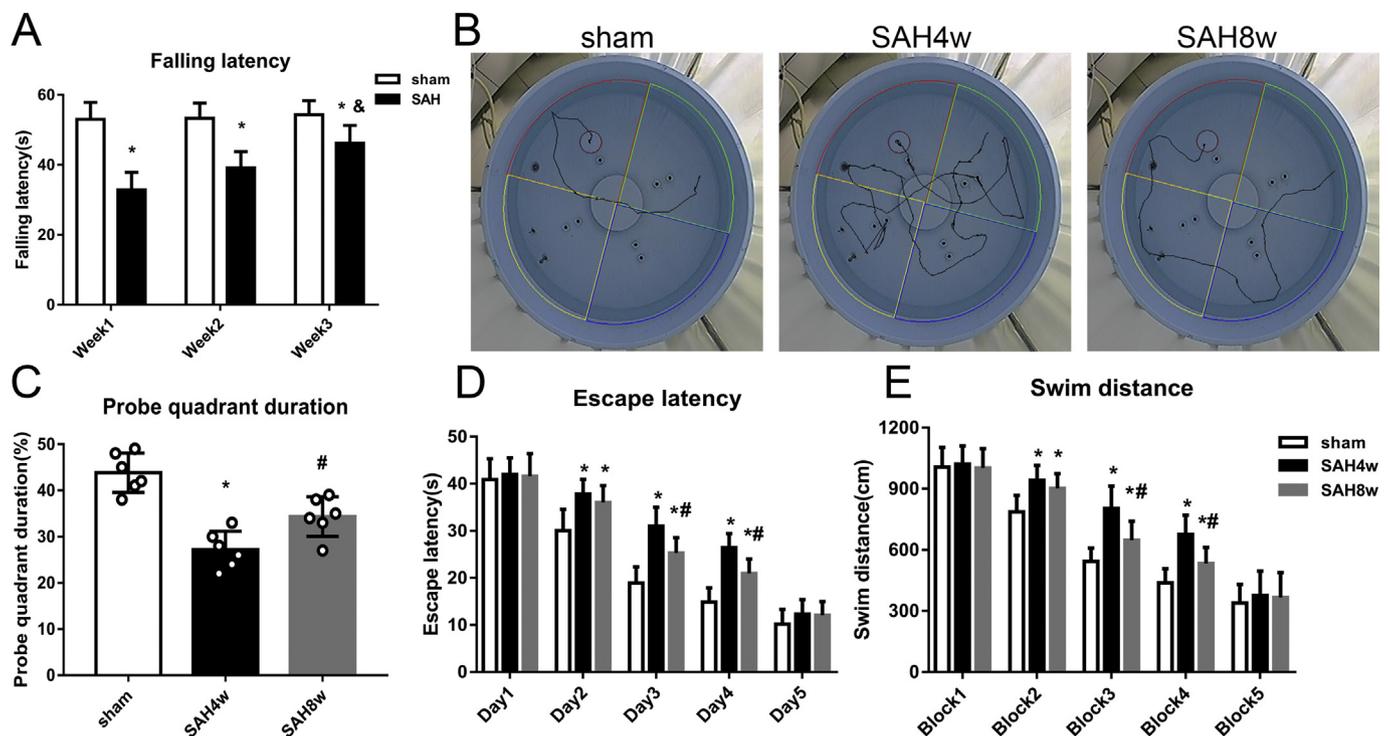


Fig. 8. Long term neurobehavior after SAH.

A. Rotarod test of sham and SAH group after 1, 2 and 3 weeks. B. Representative trace image of water maze test. C. Probe quadrant duration of water maze test. D. Escape latency and E. swim distance of water maze test. $n = 6$ per group. * $P < 0.05$, vs. sham group; & $P < 0.05$, vs. SAH week1; # $P < 0.05$, vs. SAH4w. Bars represent mean \pm SD.

2.9. Quantification and statistical analysis

The number of BrdU-positive cells in the hippocampal sections, specifically in DG, was counted in each three coronal sections (spaced 300 μ m apart) per animal in a high power field on Olympus BX51 microscope. Results were recorded as the average number of BrdU-positive cells per section. The densities of the DCX and GFAP were counted in three coronal sections (spaced 300 μ m apart) per brain in profiles regions of interest (ROI), that is DG of hippocampus for DCX and hippocampus and cortex for GFAP. Densities were measured by Image J software, with specific densities calculated by background subtraction. Results were recorded as the average relative density to sham per ROI. All data were graphed by GraphPad Prism 7 (San Diego, CA, USA) if applicable. The data were expressed as means \pm SD. One-way analysis of variance (ANOVA) followed by a Tukey's multiple-comparisons post hoc test was used to compare means of different groups in this study. The statistical analysis was carried out using SPSS 16.0 software (SPSS Inc., Chicago, Illinois, USA). Statistical significance was defined as $P < 0.05$.

3. Results

3.1. SAH grade and neurological score

A total of 122 rats were used in this study, which were divided into 18 sham operated rats and 104 SAH operated rats. The mortality was 17.3% (18 of 104) and 0 (0 of 18) in SAH and sham groups, respectively. Blood clots, which were mainly distributed around the Circle of Willis and ventral brain stem, were scored for the 24 h group (Fig. 1A). SAH grades were measured at 24 h, the average grade was 12 in SAH rats (Fig. 1B). SAH groups showed no statistical significance in SAH grade. The mortality and the physiological parameters (mean arterial blood pressure, heart rate, temperature, blood gas analysis) were measured (Fig. S1). Rats subjected to SAH performed significantly

worse compared to sham, as seen from the Garcia score and Beam balance test at 24 h after SAH ($P < .05$, Fig. 1C, D). Neurological deficit measured at 24 h between SAH groups showed no statistical significance.

3.2. Temporal patterns of 5-Bromo-2'-deoxyuridine (BrdU) positive cells detected in left hippocampus after SAH

Immunohistochemical (IHC) staining of BrdU demonstrated a significant amount of BrdU positive cells in the dentate gyrus (DG) of hippocampus, (Fig. 2A). The number of BrdU positive cells in DG was significantly decreased as early as 1 day after SAH and reached the lowest level at 3 days compared to sham group; thereafter, the BrdU positive cells recovered to their baseline level at 7 days after SAH (Fig. 2B).

3.3. Temporal patterns of doublecortin (DCX) detected in left hippocampus after SAH

Immunohistochemistry staining of immature neuron/neuronal precursor marker DCX showed that DCX positive cells are mainly localized in the DG, and that the dendritic morphology in the SAH group changed overtime. The cell body and dendritic injury after SAH occurred as early as 1 day and peaked at 3 days, then gradually recovered after 14 days (Fig. 3A). In addition, the dendritic branching complexity was decreased after SAH when compared to sham group, and then recovered to baseline at 14 days. Quantitative analyses of density showed that DCX level decreased as early as 1 day after SAH and reached its lowest level \approx 3 days and then gradually recovered to baseline at 14 days after SAH. Western blot was performed to confirm the DCX protein level at 1, 3, 5, 7, and 14 days in the left hemisphere after SAH. Consistently, western blot results showed the same pattern changes of DCX as IHC ($P < 0.05$; Fig. 3C).

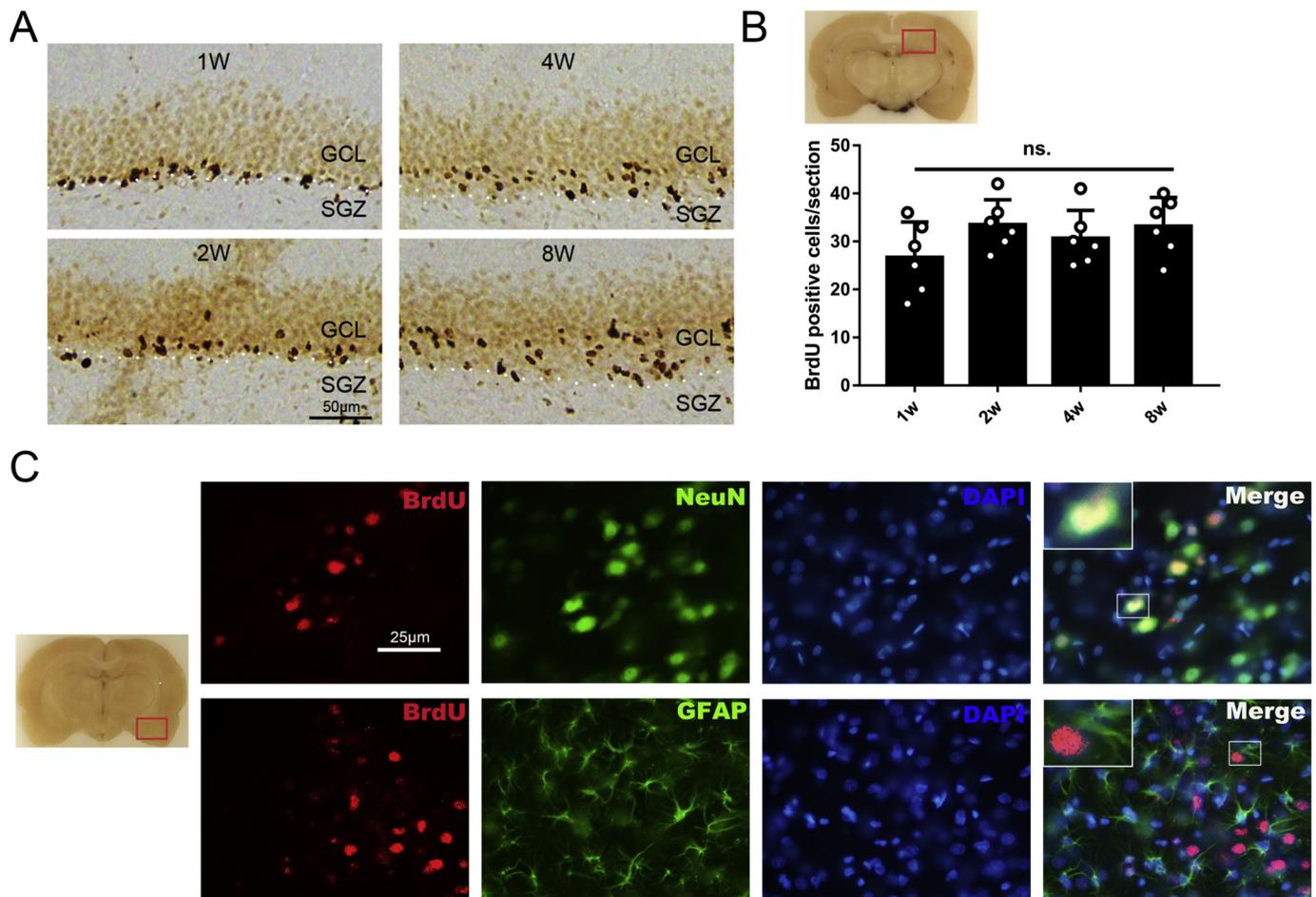


Fig. 9. BrdU positive cells long term fate after SAH.

A. Representative images and B. quantitative analyses of BrdU positive cells from DG of hippocampus at 1, 2, 4 and 8 weeks after SAH. $n = 6$ per group. C. Double immunofluorescence staining for BrdU (red) with neuron (NeuN, green) and astrocyte (GFAP, green) in the left basal cortex. $n = 6$ per group. ns, no significant difference. Bars represent mean \pm SD. BrdU, 5'-bromo-2'-deoxyuridine; GCL, granule cell line; SGZ, subgranular zone; NeuN, neuronal nucleus; GFAP, glial fibrillary acidic protein. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.4. Temporal patterns of glial fibrillary acidic protein (GFAP) in left cortex and hippocampus after SAH

The expression of activated astrocytes marker GFAP at 1, 3, 5, 7, and 14 days in the left cortex and hippocampus after SAH was detected by immunohistochemistry and western blot. Immunohistochemistry staining results showed that GFAP level increased as early as 1 day after SAH and peaked ≈ 3 days and then gradually recovered to baseline after SAH (Fig. 4 A, B). Our western blot results show the same changes of GFAP as IHC ($P < 0.05$; Fig. 4C).

3.5. The relation between BrdU positive cells with GFAP or DCX

The expression pattern of DCX showed a similar trend to that of BrdU labeled cells; whereas GFAP showed the opposite trend (Fig. 5A). Thus, we performed double immunofluorescence staining to test whether BrdU positive cells activated astrocyte proliferation. The results demonstrated that there were few BrdU positive cells co-labeled with GFAP in hippocampus (Fig. 5B) and cortex (Fig. 5C).

3.6. Number of BrdU positive cells increased in the astroglial scar on the left basal cortex after SAH; and the BrdU positive cells seemly not undergo apoptosis

Co-labeling with BrdU and GFAP positive cells were not found in sham, however, they can be detected in astroglial scar on the basal

cortex after SAH (Fig. 6A). We used double immunofluorescence of BrdU and cleaved caspase-3 to test if BrdU positive cells underwent apoptosis after SAH. Our results showed that both cleaved caspase-3 and BrdU can be detected in the rats' brain at 3 days and 7 days after SAH, however, no co-localization was found (Fig. 6B). Our results indicated that the BrdU positive cells did not undergo apoptosis, which indicated that the reduction of neurogenesis after SAH is not due to apoptotic progenitor cells.

3.7. The short-term fate of BrdU positive progenitor cells in the hippocampus after SAH

Progenitor cells short-term function includes proliferation and migration. The proliferation of progenitor cells in SGZ was verified by co-labeling with BrdU and DCX. Our results demonstrated that BrdU positive progenitor cells that were co-labeled with DCX as early as 7 days after SAH, proliferated and differentiated into immature neurons/neuronal precursors in the hippocampus (Fig. 7A). To test the migration of BrdU positive cells at different time points, we used the BrdU (nickel enhanced DAB staining) and NeuN (DAB staining) co-labeling method. Results showed that BrdU positive cells initially decreased and then recovered at 7 days after SAH (Fig. 7B). Meanwhile, BrdU positive cells migrated to the granule cell line (GCL) overtime, the result showed that BrdU positive cells migrated from SGZ to GCL at 14 days after SAH (Fig. 7B). Some of these cells also expressed the mature neuronal marker NeuN, which is indicative of differentiation into mature neurons

at 14 days after SAH in the hippocampus. Similar results were observed when we used double-immunofluorescence staining with BrdU and NeuN. BrdU positive cells migrated to GCL at 14 days after SAH and co-localized with NeuN (Fig. 7C). Taken together, these findings indicated that progenitor cells, in SGZ, function to proliferate and then migrate to the GCL after SAH.

3.8. Long-term neurobehavioral outcomes after SAH

To test the long-term effects after SAH, neurological function was assessed by using the Rotarod test and water maze test. These tests effectively cover a wide range of functions and are a good indicator of neurological deficits. In the Rotarod test, the falling latency is significantly shorter in SAH group when compared with the sham group in 1 week intervals for 3 weeks after SAH; but the SAH rats' performance improved significantly at 3 weeks compared to 1 week ($P < 0.05$; Fig. 8A). In the spatial maze test, the SAH group swam a longer distance, as well as had a longer escape latency to find the platform compared to sham group at 4 weeks. However, this was improved at 8 weeks after SAH ($P < 0.05$; Fig. 8B, D, E). In the probe trials of the water maze test, when the platform was removed, the SAH group spent less time in the target quadrant compared to the sham group at 4 weeks, while the SAH group significantly improved the duration spent in the probe quadrant at 8 weeks ($P < 0.05$; Fig. 8C).

3.9. The long-term fate of BrdU positive progenitor cells after SAH

Our immunohistochemistry results demonstrated that BrdU positive progenitor cells migrated to GCL at 2 weeks after SAH (Fig. 9A). There was no significant difference of progenitor cells in the hippocampus at 2, 4, and 8 weeks after SAH ($P > 0.05$; Fig. 9B). Co-labeling with BrdU and NeuN or GFAP were used to verify whether the progenitor cells migrated and differentiated into mature neurons or astrocytes. The results showed that the majority of BrdU positive cells, co-labeled with NeuN, became mature neurons at 4 and 8 weeks after SAH. However, there were very few BrdU positive cells co-labeled with GFAP, even 8 weeks after SAH (Fig. 9C). These findings indicated that the long-term fate of progenitor cells is migration and differentiation into neurons, but not astrocytes.

4. Discussion

In summary, our findings demonstrated that the amount of BrdU labeled progenitor cells changed overtime after SAH, and similar change patterns were observed with the immature neurons, which was reversed with the activated astrocytes. Neurogenesis decreased at 1 day reaching the lowest levels at 3 days after SAH, but then gradually recovered. In addition, we elucidated that the short-term fate of progenitor cells after SAH was to proliferate and migrate from SGZ to GCL in hippocampus, which was accompanied by an increase in DCX expression. The progenitor cells' long-term fate was to differentiate into mature neurons. Furthermore, neurogenesis after SAH was accompanied with neurobehavioral recovery.

Neurogenesis plays an essential role in the pathophysiology of acute brain injury, such as ischemic stroke (Chen et al., 2018; Jin et al., 2017) and traumatic brain injury (Carlson and Saatman, 2018; Choi et al., 2016; Hood et al., 2018), as well as neurodegenerative diseases, such as Alzheimer's disease (Bartolome et al., 2018; Coronel et al., 2018) and Parkinson's disease (L'Episcopo et al., 2018). The degree of neurogenesis is mainly determined by the neuronal progenitors' function, including proliferation, followed by migration and differentiation into mature cells (Iwai et al., 2001; Iwai et al., 2002; Sato et al., 2001). The neural progenitor cells have been detected in the adult human brain following SAH by specimen study (Sgubin et al., 2007). In the present study we elucidated changes in neurogenesis and the fate of progenitor cells in the process of recovery after SAH using a rat model.

In the present study, the neurogenesis temporal pattern showed that BrdU positive cells decreased 1 day after SAH, reached the lowest level at 3 days after SAH, and then gradually recovered. The immature neurons/neuronal precursors, as indicated by the DCX marker, showed the same time course as BrdU positive cells. However, the activated astrocytes, indicated by the GFAP marker, increased after SAH and peaked at 3 days and then decreased. Thus, BrdU positive cells and DCX showed similar temporal changes. On the contrary, the changes observed were reversed with GFAP.

Previous studies showed proliferated progenitors can differentiate into astrocytes, contributing to the astroglial scar formation after brain injury such as traumatic brain injury and ischemic stroke (Kernie et al., 2001; Komitova et al., 2006) (Mohn and Koob, 2015; Sadelli et al., 2017). Therefore, we speculated whether progenitors differentiate into astrocytes after SAH. Interestingly, co-localization of BrdU and GFAP in hippocampus and cortex was found to be minimal, except in the astroglial scar after SAH. These results indicated that the majority of BrdU positive progenitor cells did not differentiate into activated astrocytes after SAH. A recent study indicated that A1 activated astrocyte, induced by activated microglia, is neurotoxic and responsible for cell death after acute brain injury (Liddelow et al., 2017). In addition, astrocytes can decrease neurogenesis during virus-induced memory dysfunction (Garber et al., 2018). Consistently with previous reports, our results showed that the temporal change of neurogenesis is reversed with GFAP after SAH. This raises the possibility that A1 astrocytes may activate and decrease neurogenesis after SAH. Therefore, A1 astrocyte activation after SAH and their effects on neurogenesis should be given further consideration.

Apoptosis has been demonstrated to be a main cause of EBI that contributes to neurological deficits after SAH. In addition, neural stem cells (NSC) can be regulated by apoptosis (Ryu et al., 2016). It was previously reported that NSCs underwent apoptosis as seen from cleaved caspase-3 expression levels while under stimulation (Yu et al., 2008). On the contrary, our results showed that BrdU positive progenitors did not undergo apoptosis, since we did not observe any co-labeling between BrdU and cleaved caspase-3 in cells after SAH. This result indicated that the decreased neurogenesis after SAH was not due to progenitor apoptosis.

Neurogenesis plays an essential role in the recovery process of various kinds of brain injuries. Our results indicated that the short-term fate of BrdU positive cells was to differentiate into neuronal precursors that was marked by DCX, a unique microtubule-associated protein expressed at the early stage of differentiation and migration (Ayanlaja et al., 2017), meaning that these cells facilitate immature neurons/neuronal precursors' recovery. This can be explained as immature neurons may recover from an early time point. In addition, BrdU positive progenitors were found to migrate from SGZ to GCL in the DG. Furthermore, our results showed that BrdU positive cells differentiated into mature cells in the GCL and therefore did not express DCX at 14 days after SAH, as DCX is not expressed by mature neurons. To further support our results, we showed that BrdU positive cells co-localized with NeuN in the GCL at 14 days after SAH (Fig. 7B and C). Similarly, the results shown in Fig. 9C and Fig. S4 indicated that the long-term fate of BrdU progenitor cells is to differentiate into mature neurons. These neurogenesis changes were accompanied with improved balance and sensorimotor coordination abilities as seen from our Rotarod test. Previous studies demonstrated that memory is positively associated with neurogenesis in the hippocampus (Moon et al., 2016; Voss et al., 2013). Consistently, the water maze test showed that spatial cognition and memory function was improved after SAH and correlated with neurogenesis. Consistent with previous reports, BrdU positive progenitors migrated and differentiated into mature neurons which was seen as early as 14 days after SAH, with very few differentiating into astrocytes even at 8 weeks after SAH (Mino et al., 2003; Tian et al., 2017). Taken together, our results showed neurogenesis function (proliferation, migration and differentiation) and the fate of progenitors

in this process, are accompanied with neurobehavioral improvement after SAH.

These findings are clearly distinct from other acute brain injury diseases, such as cerebral ischemia and traumatic brain injury. However, the exact mechanism as to why there is a decrease in neurogenesis following SAH has not been elucidated in the present study. EBI, occurring within 72 h after SAH is considered the main cause of neurological deficit, which includes apoptosis and inflammation (Fujii et al., 2013). There are some connections between our results and EBI: First, progenitors may be damaged after initial SAH and aggravated by EBI, which can explain why we observed low levels of BrdU positive cells at 72 h after SAH. Secondly, the inflammatory response, a main cause of EBI, is up-regulated after SAH, which has been reported to reduce neurogenesis (Chen et al., 2013; Ryan et al., 2013). Moreover, microthrombi formation and microcirculation dysfunction after SAH alter the micro-environments around progenitor cells which affects its survival, as neurogenesis is usually accompanied by angiogenesis. BrdU was widely used to label cycling cells in the S phase. The efficiency of BrdU labeling vary among different cell types because exogenous BrdU are incorporated into DNA through the salvage pathway. Thus, repeated injections were applied in our experiment, as it can partially overcome this inefficient labeling (Rocha et al., 1990). Although there has been previous reports on BrdU toxicity (Rocha et al., 1990; Tough and Sprent, 1994), in our study, we found that there were no negative effects on health after repeated intraperitoneal injections, which were performed once daily for 7 days.

One limitation to our study is that we did not test the cell cycle period of the neuronal progenitor cells. Previous studies have shown that ischemic stroke reduced the cell cycle of neuronal progenitor cells by decreasing the G1 phase, however, it does not influence the S phase and G2 + M phase (Zhang et al., 2006; Zhang et al., 2008). The cell cycle after SAH needs to be explored in the future because it is possible that the cell cycle length affects the degree of neurogenesis. The pathophysiology of neurogenesis is complicated. The exact mechanism of neurogenesis changes after SAH need to be further explored.

5. Conclusion

The present study suggests that neurogenesis, labeled by BrdU, decreased after SAH insult and then gradually recovered by progenitor cells' proliferation, migration and differentiation. The fate of the progenitors in this process are accompanied with neurobehavioral recovery. This study may provide valuable information on neurogenesis after SAH, which may aid in the development of future therapeutic strategies.

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Conflict of interest

The authors declare no conflict of interest.

Ethical approval

All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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