Intracerebral hemorrhage (ICH) is associated with high rate of mortality and morbidity, but lacks effective therapies. Accumulating studies indicated that the hippocampal neurogenesis plays an essential role in the recovery of neurological function after ICH. The Notch1 signaling pathway shows important roles in neurogenesis. However, the effects of Notch1 on the recovery of neurological function after ICH remain unclear. Here, we used ICH mice model to investigate whether Notch1 signaling was involved in the hippocampal neurogenesis and the recovery of neurological function post-ICH. Our results showed that the rate of symmetric division pattern of hippocampal neural stem cells (NSCs) decreased significantly at 3 days after ICH. Meanwhile, the expression of Notch1 in the hippocampus also was reduced significantly. However, Notch1 activator treatment enhanced the expression of Notch1 and increased the number of Sox2+GFAP+ cells. Further, the rate of symmetric division pattern of NSCs also increased after Notch1 activator treatment in mice with ICH. Importantly, the number of DCX+ cells and BrdU+NeuN+ in hippocampus were increased on 28 days post-ICH as the Notch1 expression was upregulated. The motor function and spatial memory ability in post-ICH mice following Notch1 activator treatment also were improved. Taken together, our results suggested that Notch1 signaling could influence the recovery of long-term neurological function by regulating the proliferation and differentiation of the hippocampal NSCs in mice after ICH. Our study may provide ideas for the improvement of neurological function and spatial memory defects after ICH. NeuroReport 32: 489–497 Copyright © 2021 The Author(s). Published by Wolters Kluwer Health, Inc.

Keywords: intracerebral hemorrhage, neural stem cells, neurogenesis, Notch1, symmetric division

Introduction

Intracerebral hemorrhage (ICH)/intraparenchymal hemorrhage (IPH) is associated with high rate of mortality and morbidity [1]. Patients who suffer from ICH usually have long-term neurological impairments, but this condition lacks effective therapies. Therefore, new therapies need to be found to protect the damaged brain and promote neurological function recovery after ICH. Previous studies found that the functional recovery after ICH is mainly involved in neurogenesis, angiogenesis and synaptic plasticity [2,3]. Recently, accumulating evidences indicate that endogenous neurogenesis might play a potential role in functional recovery after ICH [4].

Adult neurogenesis occurs in two distinct brain areas, the subgranular zone (SGZ) of the hippocampal dentate gyrus and the subventricular zone (SVZ) of the lateral ventricles. The neural stem cells (NSCs) in the hippocampus could self-renew by proliferation and give rise to new neurons and astrocytes [5,6]. Impaired neurogenesis in the hippocampus has been correlated with many diseases, including dementia, depression, epilepsy, schizophrenia, etc. [7–9]. Substantial studies suggest that ICH could induce endogenous regeneration of NSCs and the generation of new neurons [10]. The newborn neurons could migrate into the damaged brain regions to replace neurons lost [11]. Thus, ICH-induced neurogenesis is critical for functional recovery after stroke. Promoting the endogenous regenerative mechanism may be a novel idea for post-ICH functional recovery.

Previous studies showed that Notch1 signaling pathway plays a key role in regulating adult neurogenesis, which controls cell fate acquisition and plays vital roles during maintenance, proliferation, and differentiation of NSCs [12]. In adult brain, the Notch1 mainly express in cells of SVZ and SGZ. The Notch1 signaling could be influenced by numerous factors, including aging, ischemic stroke, depression, etc. [13–15]. Importantly, previous studies have revealed that Notch1 signaling was essential for the hippocampal NSCs pool maintenance after epilepsy [16].
However, the effects of Notch1 signaling on hippocampal neurogenesis and NSCs pool after ICH were unknown.

In the present study, we intend to examine the effects of Notch1 signaling on hippocampal neurogenesis, the recovery of long-term neurological function and spatial memory after ICH. Our results suggested that Notch1 signaling influence the recovery of long-term neurological function and spatial memory by modulating the proliferation and differentiation of the hippocampal NSCs in mice after ICH.

**Methods**

**Ethics statement and animals**

All procedures involving animals were approved by the Animal Studies Committee of Xi’an Jiao Tong University and complied with the Guide for the Care and Use of Laboratory Animals (Guide; NRC 2011). In this study, adult male C57/BL6 mice were purchased from the Animal Center of the Xi’an Jiao Tong University (Xi’an, China). To avoiding the influence of estrogen on behaviors, 8-weeks-old mice were used in study and only male was used. The mice were provided food and water ad libitum, and were housed under a 12 h light and 12 h dark cycle. Light:dark cycle was regular. Mice were assigned to different groups randomly.

**Intracerebral hemorrhage models and 5-bromo-2-deoxyuridine treatment**

The detailed procedures used to construct the ICH model were established in our previous studies [17]. Mice were briefly anesthetized intraperitoneally with 4% phenobarbital sodium at a dose of 5 μl/g and were immobilized on the stereotaxic apparatus (RWD Life Science Co, Shenzhen, China). The autologous blood was obtained by tail artery. A scalp incision was made along the midline, and then the stereotactic manipulator arms were adjusted such that the 25-gauge needle was positioned 0.8 mm (anterior–posterior) and 2 mm (left, medial–lateral) from the bregma. At these coordinates, a 1-mm cranial burr hole was drilled, and then the needle tip was advanced 3.5 mm ventrally through the burr hole. All experimental mice received a total of 20 μl autologous blood injected into the caudate nucleus successively. The needle of microsyringe was held in place for 10 min after injection to avoid the blood back streaming. Only the needle of microsyringe was held in place for 10 min for mice in Sham group. The craniotomy was then sealed with bone wax, then the scalp was closed with sutures. Body temperature was maintained at 37°C throughout the procedure. The mice that died due to anesthesia and unsuccessful ICH models including asymptomatic and dead mice before euthanasia were excluded from this study. The inclusion criteria included mice with successful ICH models, and with symptoms caused by ICH. Mice with symptoms caused by ICH indicated that a successful ICH surgery was performed. To assess cell proliferation in the dentate gyrus, mice received 5-bromo-2-deoxyuridine (BrdU; Sigma-Aldrich, St Louis, MO, USA) every 24 h after ICH, for a total of three injections at a dose of 50 mg/kg, then sacrificed 24 h after the last BrdU injection (day 3 post-ICH).

**Administration of Notch signaling activator**

As previous study reported [13], mice were anesthetized intraperitoneally with 4% phenobarbital sodium at a dose of 5 μl/g and implanted with an osmotic minipump (Alzet 1003D, Alza Corporation, CA, USA) in the ipsilateral lateral ventricle after ICH performed. Briefly, the Notch1 activator was prepared as previous described [13], Notch1-activating antibody (Sigma-Aldrich, St Louis, MO, USA), 10 μg/ml, was dissolved in the artificial cerebrospinal fluid (Sigma-Aldrich, St Louis, MO, USA) at a 1:4 dilution. Then the cannula was placed into the Tleft lateral ventricle (bregma: 0.6 mm posterior, 1.1 mm left lateral and 3.0 mm deep). Each mouse was infused for 3 days with 0.10 μl/h of Notch1-activating antibody by continuous intracerebroventricular infusion. Only artificial cerebrospinal fluid was used for the vehicle of Notch activator in Sham and ICH + Vehicle groups.

**Behavior tests**

**Rotarod test**

Motor coordination was assessed using rotarod (RWD Life Science Co, Shenzhen, China) as previously described [18]. Twenty-eight days after ICH, the mice were prepared for rotarod test. Rotarod test consisted of three trials. For each trial, mice were placed on the rod at a speed of 10 rpm/s with 0.2 rpm/s² acceleration. Trials 1 and 2 were done back-to-back to prevent associations between falling and returning to the home cage. After 10 min of trial 2, trial 3 was done. The average time spent on the rod before falling for the best two trials is recorded.

**Beam walking**

As previous study reported [19], the beam was made of wood (6 mm wide and 120 mm in length) and 30 cm high above the ground. During the test session, the mice were placed on the beam at one end and allowed to walk to the other end where a home box was located. The number of foot faults for the right hindlimb was recorded over 50 steps counted in both directions on the beam was recorded.

**Morris water maze**

Spatial learning and memory was assessed using MWM (RWD Life Science Co, Shenzhen, China) as previously described [20]. The mice were trained on day 1 (day 23 post-ICH) before testing to eliminate the preferences of dimensions and the arrangement of the MWM in different experimental groups. The following 4 days (day 24–27 post-ICH), the mice were then trained to find a hidden platform to habituate the mice to handling and to
swimming, with four trials/day, 60 s per trial, and 20 s on the platform. On the last day (day 28 post-ICH), to assess the platform quadrant preference, a probe trial was conducted and the platform was removed. The time spent searching correct quadrant was recorded and numbers of entries into correct quadrant were measured.

**Tissue preparation**

At the designated time points (1 day, 3 days, 5 days and 7 days after ICH or sham group), the animals were prepared for analysis. For immunohistochemistry and immunofluorescence, the animals were perfused transcardially with 0.9% normal saline followed by 4% paraformaldehyde in 0.1 M PBS. The brains were embedded in paraffin and then immersed in 30% sucrose, cut into 25 μm coronal sections.

**Immunohistochemistry and immuno-fluorescence**

According to previous methods [17], for immunohistochemistry staining, the paraffin brain sections were first washed in 0.01 M PBS to remove the cryoprotectant solution. The sections were then incubated overnight at 4°C with the primary antibody goat anti-DCX (1:500, Abcam, UK), rabbit anti-NeuN (1:1000, Abcam, UK), rabbit anti-Sox2 (1:1000, Abcam, UK) and mouse anti-GFAP (1:500, Millipore, Temecula, CA, USA). The sections were incubated with a secondary antibody for 2 h at 37°C, followed by the avidin-biotin complex (Dako, Glostrup, Denmark) or counterstained with 4',6-diamidino-2-phenylindole (Sigma-Aldrich, St. Louis, MO, USA). The first three adjacent sections were used for antibody anti-DCX, the next three adjacent sections for antibody anti-NeuN and the last three adjacent sections for antibodies anti-Sox2.
and mouse anti-GFAP. These sections were adjacent and selected about at bregma −2.06 mm, and five animals per group were used for analysis. The average number of DCX+, SOX2+GFAP+ and BrdU+NeuN+ cells in these sections per mouse was recorded. For BrdU immuno-fluorescent staining, which was conducted as previous study reported [21], quantification of BrdU-positive cells in the whole hippocampus was performed by stereological cell counting method as previously described [21]. Serial 25-μm sections through the rostrocaudal extent of the dentate gyrus were selected at 10-section intervals for immunofluorescent staining and counterstaining with DAPI to mark nuclei in the dentate gyrus. The total sum of the BrdU+ cells in SGZ was recorded.

Cell alignment of BrdU-labeled cells was analyzed in order to evaluate the mode of cell division. Briefly, the vertical (asymmetrical) division mode is defined as daughter cells that are aligned vertically to the axis of the SGZ, while horizontal (symmetrical) division mode describes daughter cells that are aligned horizontally to the axis of the SGZ.

**Western blot**

According to our previous protocol [17], total protein extracts were prepared from hippocampus in ipsilateral hemispheres. The proteins were separated by standard SDS-PAGE and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, MA, USA) using a semidy electroblotting system (Transblot SD, Bio-Rad). The PVDF membranes were then incubated with a rabbit antibody against Notch1 (1:1000, Millipore, MA, USA), rabbit polyclonal anti-NICD (Notch intracellular domain) (1:500, Abcam, UK) and a mouse monoclonal anti-β-actin (1:2000, Cell CWBIO, Beijing, China) at 4°C overnight. The membranes were further incubated with horseradish peroxidase conjugated anti-rabbit, anti-mouse and anti-goat secondary antibodies (1:1000, Zhongshan Golden Bridge Inc., China) at 25°C for 1.5 h. Bound antibodies were visualized using a chemiluminescence detection system.

**Statistical analysis**

All of the data are presented as the mean ± SEM. Analysis was performed using SPSS 13.0 software. The t-test for independent samples was used to compare two groups, and comparisons among multiple groups were examined using one-way analysis of variance (ANOVA), followed by Tukey’s post hoc test to compare the difference between two group. Statistical test for MWM training (learning over 4 days) was conducted by two-way ANOVA with repeated measures. Differences were considered significant at a $P < 0.05$. 

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The effects of Notch1 activator treatment on Notch1 expression. (a, b) Representative bands and densitometric quantification of Notch1, the activated form of Notch1 expression in the hippocampus of ICH mice after Notch1 activator treatment. Data are presented as mean ± SEM (n = 5). **$P < 0.01$. ICH, intracerebral hemorrhage; NICD, Notch intracellular domain.
Results
Notch1 signaling decreased in the hippocampus after intracerebral hemorrhage

Notch1 signaling pathway could controls the cell fate and plays a key role in regulating adult neurogenesis [12,22]. Thus, to evaluate the effect of ICH on Notch1 signaling in the hippocampus, we performed western blotting to investigate the expression of Notch1 in hippocampus. We found that Notch1 expression was gradually decreased in the hippocampus at the first day, with a valley at 3 days post-ICH ($F_{4, 21} = 19.113, P < 0.001$) (Fig. 1a, b).

Intracerebral hemorrhage induced neural stem cells proliferation, but the rate of horizontal division decreased significantly in the subgranular zone

To investigate the effects of ICH on NSCs proliferation in the SGZ, we used the BrdU incorporation assay. In accordance with previous study result [23], we also found the BrdU positive cells in the SGZ increased significantly after ICH (Fig. 1c, d). There are two modes of cell division of NSCs, symmetric (horizontal aligned) and asymmetric (vertical aligned) cell division (Fig. 1e) [21,24], which are essential to hippocampal neurogenesis. Among symmetric cell division, which increases the

Effects of Notch1 activator treatment on proliferative NSCs on 3 days post-ICH in the hippocampus of mice. (a, b) BrdU-positive cells (red) in the SGZ for ICH + Vehicle and ICH + Notch1 groups. (c) Quantitative analysis the ratio of symmetric/asymmetric cell division of ICH + Vehicle and ICH + Notch1 groups on 3 days post-ICH. (d, e) The number of Sox2 (red) and GFAP (green) double-positive (Sox2+GFAP+) cells in the SGZ on 3 days after ICH for ICH + Vehicle and ICH + Notch1 groups. (f, g) The number of BrdU (red) and GFAP (green) double-positive (BrdU+GFAP+) cells in the SGZ on 3 days after ICH for ICH + Vehicle and ICH + Notch1 groups. Data are presented as mean ± SEM (n = 5). *P < 0.05, **P < 0.01. BrdU, 5-bromo-2-deoxyuridine; ICH, intracerebral hemorrhage; NSCs, neural stem cells; SGZ, subgranular zone.
Effects of Notch1 activator treatment on the hippocampal neurogenesis, neurological function and spatial memory of mice on 28 days post-ICH. (a, b) DCX-positive cells (brown) in the hippocampus on 28 days post-ICH for the Sham, ICH + Vehicle and ICH + Notch1 groups. After Notch1 treatment, DCX-positive cell number was significantly increased in the hippocampus of ICH mice on 28 days post-ICH. (c, d) The number of BrdU (red) and NeuN (green) double-positive (BrdU^+NeuN^+) cells in the SGZ on 28 days after ICH for the Sham, ICH + Vehicle and ICH + Notch1 groups. (e) Motor coordination was reduced by ICH, while Notch1 activator treatment rescued this deficit significantly. (f) On 28 days after ICH, the ICH mice showed significantly increased footfaults in the walking beam test. The treatment of Notch1 activator decreased the times of footfaults among ICH mice in finishing the task. (g) Spatial learning was assessed in a Morris water maze on 24–27 days post-ICH. On 25–27 days after the ICH injury, the escape latency in the ICH group significantly increased compared to the Sham group. While Notch1 activator administration alleviated the learning impairments significantly. Spatial memory deficit was assessed in a probe trail and the percentage of time spent in target quadrant (h) and number of entries into quadrant (i) were recorded. ICH mice were impaired compared to Sham mice, while Notch1 activator treatment rescued this deficit significantly. Data are presented as mean ± SEM (n = 5). *P < 0.05, **P < 0.01. BrdU, 5-bromo-2-deoxyuridine; ICH, intracerebral hemorrhage; SGZ, subgranular zone.
number of NSCs, asymmetric cell division could produce a single daughter neuron (or astrocyte) and a mother cell that remains a progenitor cell [24,25]. Interestingly, we found that ICH decreased the ratio of symmetric/asymmetric cell division in SGZ significantly on 3 days after ICH (Fig. 1f). This result may indicate that ICH alters the NSCs division mode, thereby influencing the hippocampal NSCs pool.

Activating Notch1 signaling modulated ICH-induced imbalances between symmetric and asymmetric division patterns of NSCs in the SGZ and enhanced hippocampal neurogenesis

To confirm the effect of Notch1 signaling on the division patterns of NSCs and neurogenesis, we designed ICH model and then the Notch1 activator was administered. As shown in Fig. 2a, b, compared to Vehicle group, the Notch1 and NICD protein expression level in hippocampus were increased significantly in the Notch1 activator-treated group at 3 days post-ICH (Notch1: $F_{2,13} = 20.271$, $P < 0.001$; NICD: $F_{2,13} = 31.556$, $P < 0.001$). Importantly, the number of BrdU$^+$ and the ratio of symmetric/asymmetric cell division in SGZ also were increased significantly in the Notch1 activator group at 3 days post-ICH (Fig. 3a–c). Moreover, after Notch1 activator administration, we found that the number of Sox2$^+$GFAP$^+$ cells/GFAP$^+$ cells (Fig. 3d, e) and the percent of BrdU$^+$NeuN$^+$ cells (Fig. 3f, g) increased significantly in SGZ at 3 days post-ICH. These findings indicated that Notch1 activator enhanced the proliferation of NSCs significantly in hippocampus of post-ICH mice.

DCX immunoreactivity is a method to identify immature neurons; therefore, we assessed DCX positive immature neurons in the SGZ. To evaluate the effects of Notch1 signaling on new integrated neurons post-ICH, we analyzed the number of DCX$^+$ cells and performed co-staining with NeuN and BrdU at 28 days post-ICH. Compared to ICH + Vehicle group, we found that both DCX$^+$ cells and BrdU$^+$NeuN$^+$ cells were increased significantly at 28 days post-ICH in ICH + Notch1 group (DCX$^+$: $F_{2,13} = 31.722$, $P < 0.001$; BrdU$^+$NeuN$^+$: $F_{2,13} = 32.467$, $P < 0.001$) (Fig. 4a–d).

**Activating Notch1 signaling alleviated the motor function, learning and memory ability deficits of post-intracerebral hemorrhage mice**

To assess the effects of Notch1 activator on the mice motor function after 28 days of ICH, we performed the rotarod fatigue test and beam walking test. As shown in Fig. 4e, compared to Sham group, the motor coordination was reduced following ICH, but Notch1 activator could rescue this deficit, which was caused by ICH ($F_{2,13} = 57.707$, $P < 0.001$). Among the beam walking test, compared to Sham group, the number of foot slips increased significantly in ICH group. Treatment with Notch1 activator could rescue the number of foot slips among ICH mice ($F_{2,13} = 83.521$, $P < 0.001$) (Fig. 4f).

Besides, we used a Morris water maze to assess the effect of Notch1 activator on the mice learning and memory ability after ICH. ICH group mice had significant learning impairments on 25–27 days post-ICH compared to Sham group mice, while Notch1 activator administration alleviated the learning impairments significantly on 26–27 days post-ICH (Fig. 4g). Indeed, spatial memory was assessed in a probe trial on 28 days post-ICH. The time spent searching correct quadrant was recorded and the numbers of entries into correct quadrant were measured. As shown in Fig. 4h, i, ICH group spent more time in searching correct quadrant and shown less numbers of entries into correct quadrant compared to Sham group. Interestingly, Notch1 activator treatment rescued parts of the spatial memory deficit of mice post-ICH ($F_{2,13} = 43.326$, $P < 0.001$; $F_{2,13} = 38.432$, $P < 0.001$).

**Discussion**

In the present study, we found activating Notch1 signaling could increase the rate of symmetric division pattern of NSCs in hippocampus of ICH mice. Importantly, we found that activating Notch1 signaling could influence the recovery of long-term neurological function and spatial memory. Our data suggested that activating Notch1 signaling can regulate division pattern of NSCs, hippocampal neurogenesis and improve functional outcome after ICH.

Notch1 signaling pathway is one of the major regulators of neural development, which is mainly expressed in neural germinal zone of adult brains, including SGZ and SVZ [26]. Numerous studies found that Notch1 signaling could regulate SGZ and SVZ neurogenesis both in physiological and pathological conditions. For example, Sun et al. found that Notch1 signaling decreased with aging, but ischemia-induced cell proliferation in the SVZ of aged brain was enhanced by activating the Notch1 pathway [13]. Moreover, electroacupuncture treatment also could enhance hippocampal NSCs proliferation and neurogenesis after ischemic stroke by upregulating Notch1 signaling [27]. However, Zhong et al. showed that the application of a Notch1 inhibitor, DAPT, improved the neurological outcomes of ICH rats [28]. Probably, the different animal species and the volume of autologous blood used in ICH model could produce different effects on intact animals.

Among the capacity of adult hippocampal neurogenesis, which was determined by the available NSCs pool [29], the NSCs pool was related to the two types of division patterns of NSCs in SGZ: asymmetrical and symmetrical patterns. The asymmetrical division pattern of NSCs could produce differentiation of NSCs into astrocytes or neurons, thus exhausting the NSCs pool in hippocampus, while symmetrical division increases the number of NSCs and enhances neurogenesis [24,25]. As previous studies found, the Notch1 signaling is essential for cells fate control, the maintenance and proliferation of NSCs [12,30]. Interestingly, coupled with the rate of symmetric division pattern of NSCs decreased, the Notch1 signaling
also reduced significantly in hippocampus of post-ICH mice, while Notch1 activator treatment increased the rate of symmetric division pattern of NSCs significantly in ICH mice. These findings may indicate that ICH modulates the division pattern of NSCs via Notch1 signaling. Indeed, consisted with previous reports [31], we also found ICH enhances the number of BrdU-positive cells significantly in the acute phase following ICH, even though the Notch1 signaling decreased. This may be explained by many kinds of compensatory mechanism existed in the acute phase after ICH, including high-mobility group box1 pathway, Wnt/β-catenin pathway, etc. [32,33]. Moreover, Notch1 signaling could integrate with numerous other pathways thereby to regulate the maintenance of NSCs, including vascular niche factor PEDF and protein S [34]. Indeed, Ehm et al. found that the PI3 kinase pathway and the Wnt/GSK-3β/β-catenin pathway could cooperate with Notch1 signaling, thereby enhancing Notch-induced promoting effect on adult NSCs [35]. Thus, Notch1 may regulate the NSCs fate by complex cross-talking pathways but not work alone.

In a human study, it has been identified that ICH-induced endogenous neurogenesis and migration of neuroblasts into perihematoma regions were enhanced following ICH [11]. Moreover, the transplantation of human amniotic mesenchymal stem cells could promote neurological recovery following ICH in a rat model [36]. Cognitive impairments after stroke are very common that lead to a decline in everyday functioning and in social functioning, life satisfaction and quality of life of patients and caregivers [37]. Thus, promoting neurogenesis may alleviate cognitive impairments post-ICH. Importantly, Notch1 activator enlarged the NSCs pool, and enhanced the continuous neurogenesis via symmetrical division. These may indicate that modulating the division pattern of NSCs by Notch1 signaling could promote neurological functions recovery after ICH in a long term. However, further research is needed, in order to clarify the mechanisms of Notch1 modulation of division patterns and mediated neurological functions recovery following ICH.

**Conclusion**

In conclusion, our data provide a novel method to enhance the endogenous neurogenesis and rescued parts of neurological functions deficits via activating Notch1 signaling after ICH. Thus, Notch1 may be a potential target to promote neurological functions regenerative for ICH patients.

**Acknowledgements**

Authors’ contributions: X.Y.Y. conceived the study and drafted the article. J.C. conducted the animal studies and collected the data. X.Z. revised the article and language.

Availability of data and materials: All data generated or analysed during this study are included in this published article.

**Conflicts of interest**

There are no conflicts of interest.

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