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Original Investigation

Activation of Signal Transducer and Activator of Transcription 3 Signaling Attenuates Neurogenesis in a Rat Model of Intracerebral Hemorrhage

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ABSTRACT

AIM: To investigate the effect of signal transducer and activator of transcription 3 (STAT3) signaling on neurogenesis following intracerebral hemorrhage (ICH).

MATERIAL and METHODS: A rat model of ICH was induced via the stereotaxic injection of 100 μL autologous blood into the right globus pallidus. AG490 (0.25 mg/kg) was injected into the lateral ventricle after ICH to block STAT3 signaling. Brains were perfused to identify proliferating cell nuclear antigen (PCNA)+/ doublecortin (DCX) + nuclei by immunohistochemistry. In addition, the distribution and expression of phosphorylated STAT3 [p-STAT3 (Tyr705)] were evaluated by immunohistochemistry and western blot, respectively.

RESULTS: An enhanced level of p-STAT3 (Tyr705) was observed in neurons in the perihematomal region. An increase in DCX-positive cells and PCNA+/DCX+ nuclei was detected after ICH. Blocking STAT3 signaling with AG490 further increased the numbers of DCX-positive cells and PCNA+/DCX+ nuclei after ICH.

CONCLUSION: These results suggest that activation of STAT3 signaling attenuates ICH-induced neurogenesis and may delay neural recovery following hemorrhagic cerebral injury.

KEYWORDS: Intracerebral hemorrhage, Neurogenesis, Signal transducer and activator of transcription 3

ABBREVIATIONS: BSA: Bovine serum albumin, **DCX:** Doublecortin, **DMSO:** Dimethylsulfoxide, **ICH:** Intracerebral hemorrhage, **NSC:** Neural stem cells, **PCNA:** Proliferating cell nuclear antigen, **STAT:** Signal transducer and activator of transcription, **SVZ:** Subventricular zone

INTRODUCTION

Spontaneous intracerebral hemorrhage (ICH) is a common and fatal form of stroke with a high rate of mortality and morbidity. Survivors often suffer from prolonged neurological deficits (20,25); however, no definite treatment is available to promote recovery. Several lines of evidence suggest that neurogenesis, derived from neural stem cell (NSC) differentiation and proliferation, is associated with restored neurological function after hemorrhagic stroke (17,18). Accordingly, endogenous neurogenesis might offer a future cell-based therapy for ICH.

Signal transducer and activator of transcription 3 (STAT3), a member of the STAT family, is an important transcription factor involved in a wide variety of cellular functions (1).

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Accumulating evidence has shown that activation of STAT3 by phosphorylation induced the differentiation of NSCs into astrocytes rather than neurons (6,13,21), and eventually led to the formation of glial scar tissue (8,16,24,28). Furthermore, glial scar could restrain neurogenesis and hamper axonal regeneration (15,19). Several studies have demonstrated that STAT3 phosphorylation at tyrosine705 (Tyr705) negatively regulated neurogenesis (9,26). Recently, STAT3 activation was reported to contribute to ICH-induced astrogliosis (30). Accordingly, the purpose of this paper was to systematically evaluate the effect of STAT3 signaling on neurogenesis following ICH.

MATERIAL and METHODS

Animal Preparation

Seventy-eight male, 6-week-old, Sprague–Dawley rats were used in this study. The rats were individually housed in Perspex cages under a 12/12-hour light/dark cycle at a regulated temperature of 21 ± 0.5°C,with access to food and water ad libitum. All procedures were approved by the ethics committee of China Three Gorges University and performed in accordance with the National Institutes of Health guide for the care and use of laboratory animals.

Induction of ICH

Rats were subjected to ICH as described previously (27,30). In brief, rats were anesthetized with an intraperitoneal injection of chloral hydrate (400 mg/kg) and positioned in a stereotaxic frame. After exposing the skull with a midline skin incision, a burr hole was drilled 3.2 mm lateral and 1.4 mm posterior to bregma. A 26-gauge needle was inserted 5.6 mm below the surface of the skull, into the right globus pallidus. At this injection site, 100 μL non-heparinized autologous whole blood, which was withdrawn from the femoral artery, was infused over a period of 10 minutes. The needle was kept in place for 5 minutes after the infusion and then slowly withdrawn. The same surgical procedure (without blood injection) was performed for the sham group.

STAT3 Inhibitor Treatment

AG490 (Sigma-Aldrich, St.Louis, MO, USA) was used to inhibit STAT3 phosphorylation. It was dissolved in dimethylsulfoxide (DMSO) to a final concentration of 2g/mL. A dose of 0.25 mg/kg was stereotaxically injected into the right lateral ventricle (0.8 mm posterior and 1.5 mm lateral to bregma, at a dorsoventral depth of 4.8mm) of rats immediately after ICH. An equal volume of DMSO (vehicle) was given to the control group (5).

Sample Preparation

On day 14 after ICH, animals were anesthetized and transcardially perfused with saline and then with 4% paraformaldehyde. The brains were post-fixed in 4% paraformaldehyde for at least 2 hours, embedded in paraffin, and cut into 5-μm coronal sections for immunohistochemistry. For western blots, rats were transcardially perfused with saline, the brains were removed quickly, and the tissues around the hematoma were dissected and stored at −80°C.

Immunohistochemistry

After deparaffinization in xylene and rehydration in agraded alcohol series, the brain sections were immersed in 3% hydrogen peroxide to quench endogenous peroxidase. They were then incubated in 5% bovine serum albumin (BSA, Sigma, USA) to block non-specific binding, and exposed to a goat polyclonal antibody against phosphorylated STAT3 [p-STAT3 (Tyr705), 1:100 dilution] overnight at 4°C. The next day, the sections were incubated in a 1:100 dilution of anti-goat immunoglobulin G (Vector Laboratories) for 1 hour at 37°C. Diamino benzidine (DAB, Vector Laboratories) was used for staining.

Expression of p-STAT3

To identify whether p-STAT3 (Tyr705) was expressed in neurons, immune fluorescence double-labeling was used. The primary antibodies were anti-p-STAT3 (Tyr705) (goat polyclonal, 1:100 dilution, Santa Cruz Biotechnology, USA) and anti-NeuN (mouse monoclonal, 1:200 dilution, Abcam, USA). Alexa Fluor 488-conjugated donkey anti-goat antibody (1:100 dilution, Jackson Immuno Research Laboratories, USA) and Cy3-conjugated goat anti-mouse antibody (1:100 dilution, Jackson Immuno Research Laboratories, USA) were used as secondary antibodies. A negative control without primary antibody was included in the experiment to verify antibody specificity.

Evaluation of Neurogenesis

To assess neurogenesis, immunofluorescence for double cortin (DCX, an immature neuronal marker) was performed. The primary antibody was goat anti-DCX (1:200 dilution, Santa Cruz Biotechnology, USA),and the secondary antibody was a Cy3-conjugated donkey anti-goat antibody (1:100 dilution). To investigate the proliferation of newborn neurons, we co-stained DCX with proliferating cell nuclear antigen (PCNA, an endogenous cell proliferation marker) using a rabbit anti-PCNA primary antibody (1:250 dilution, Santa Cruz Biotech, USA) and an Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (1:100 dilution). DAPI was used to label nuclei Images were captured on a laser-scanning confocal microscope (LSM-510,Zeiss). In each experiment, negative-control sections were not incubated with the primary antibodies.

Cell Counting

To quantify p-STAT3, DCX-positive(DCX^+) cells and $PCNA^+$/DCX^+ nuclei were counted in the perihematomal region and expressed as cells per mm^2. Five sections per rat and four fields of view in each section were randomly selected for cell counts under magnification of a 40× objective. The positive cell or nuclei counts were performed by an investigator blinded to the experimental groups. All images were collected using Motic Images Advanced 3.2 image analysis software.

Western Blots

We performed the western blotting procedure as described previously (18,19). The primary antibodies used were goat anti-p-STAT3 (Tyr705) (1:250 dilution), goat anti-DCX (1:500

dilution), and goat anti-β-actin (1:200 dilution), all purchased from Santa Cruz Biotechnology, USA. Densitometry of the protein bands was performed with a digital image analysis system.

Statistical Analysis

All data were reported as the mean ± standard deviation (SD), and differences between groups were evaluated using one-way analysis of variance (ANOVA) followed by the LSD post-hoc test. *p*-values less than 0.05 were considered as statistically significant.

RESULTS

p-STAT3 Expression after ICH

The immunoreactivity of p-STAT3 (Tyr705) was very clear in the basal ganglia of sham-operated rats; however, the primarily nuclear-localized p-STAT3 (Tyr705) was significantly higher in ICH-injured rats (Figure 1A, C, $p<0.01$). Double-immunolabeling showed that p-STAT3 (Tyr705) was observed in NeuN-labeled neurons (Figure 1B). Similarly, western blots revealed that p-STAT3 (Tyr705) protein levels in the ipsilateral basal ganglia were significantly higher in the ICH group than in the sham group on day 14 post-ICH. After treatment with AG490 (STAT-3 inhibitor), both p-STAT3 (Tyr705)-immunopositive cells and protein levels were significantly lower in the ipsilateral basal ganglia of the ICH+AG490 group compared with the ipsilateral basal ganglia of the ICH group (Figure 1C, $p<0.01$).

Evaluation of Neurogenesis after ICH

We observed weak, scattered DCX labeling in sham animals. In contrast, the number of DCX-positive cells was notably increased in ICH animals (Figure 2A and C, $p<0.01$). Furthermore, we found $PCNA^{+}/DCX^{+}$ nuclei in the ipsilateral basal ganglia and subventricular zone (SVZ) (Figure 2B). Administration of AG490 augmented these ICH-induced increases further; the number of DCX-positive cells and $PCNA^{+}/DCX^{+}$ nuclei was markedly higher in the ICH+AG490 group than in the ICH group on day14 after injury (Figure 1C, $p<0.05$).

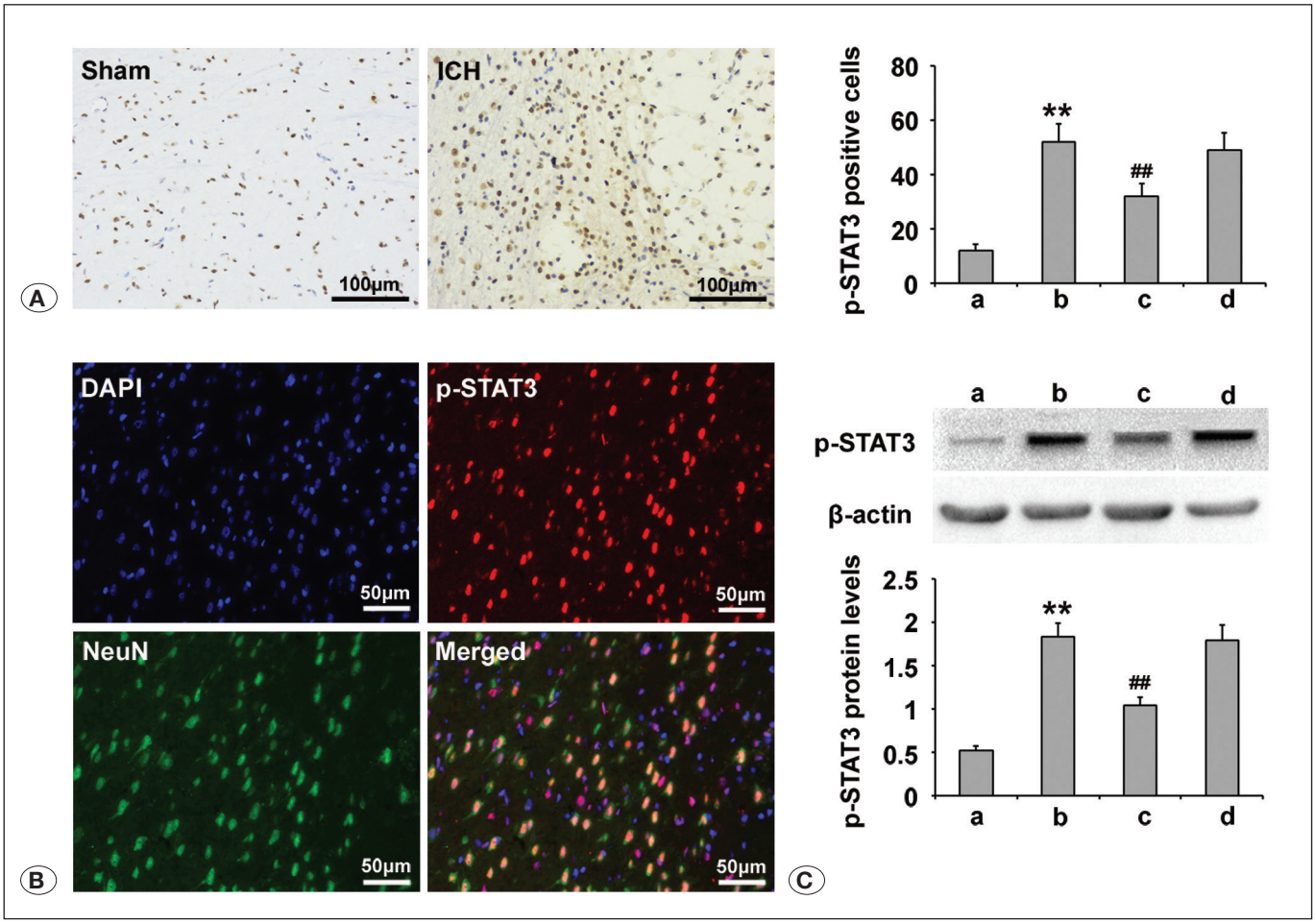

Figure 1: p-STAT3 expression after ICH. **A)** Immunohistochemistry of p-STAT3 (Tyr705) surrounding the hematoma on day 14 after the ICH or sham operation. **B)** Immunofluorescent double-staining was used to identify co-localization of p-STAT3 (Tyr705) and NeuN. **C)** The p-STAT3 antagonist AG490 significantly down regulated the expression of p-STAT3 after ICH. $^{*}p<0.05$, $^{**}p<0.01$ comparing the ICH and Sham group; $^{\#}p<0.05$, $^{\#\#}p<0.01$ comparing the ICH+AG490 group and ICH group (n=5, a: Sham group; b: ICH group; c: ICH+AG490 group; d: ICH+DMSO group).

■ DISCUSSION

This study found that ICH led to significant increases in the levels of p-STAT3 (Tyr705) in the ipsilateral brain, which was associated with increases in the numbers of DCX-positive and PCNA$^+$/DCX$^+$ cells. Intracerebroventricular injection of AG490, an inhibitor of p-STAT3, further augmented these increases in the ipsilateral brain, suggesting that p-STAT3 (Tyr705) may attenuate neurogenesis in the late phase of ICH.

Current treatment modalities in ICH, such as hematoma removal, edema attenuation, and intracranial pressure reduction, could decrease mortality. However, no effective therapy is available for promoting recovery from ICH-induced neurological deficits (2). Hence, various preclinical and translational studies have been conducted to understand the pathophysiology of ICH and explore treatment strategies, such as stem cell transplantation and iron chelation therapy. Nevertheless,

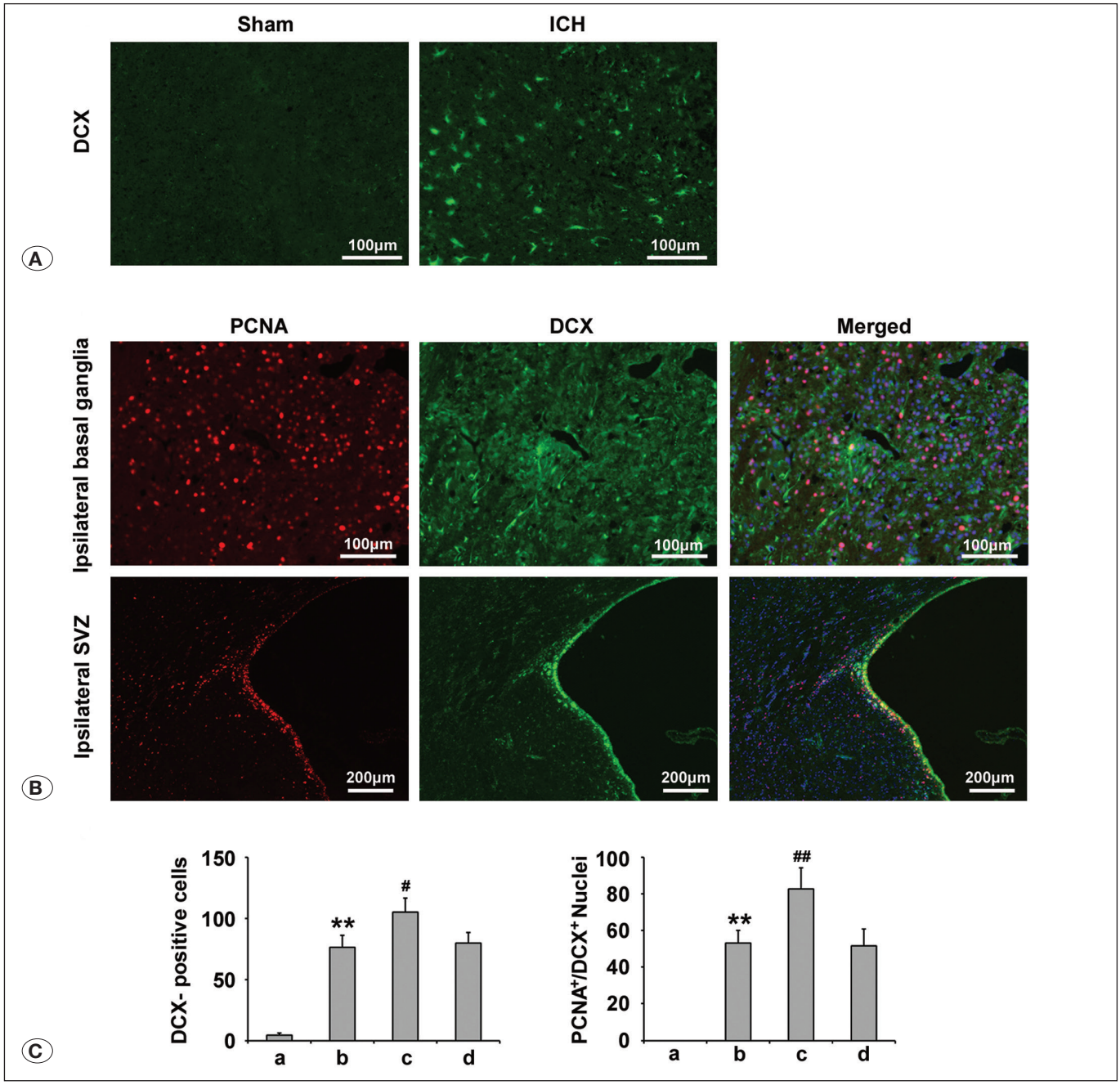

Figure 2: Neurogenesis after ICH. **A)** Immunofluorescence staining showed DCX immunoreactivity on day 14 after the ICH or sham operation. **B)** PCNA$^+$/DCX$^+$ nuclei were observed in the ipsilateral basal ganglia and SVZ after ICH. **C)** Blocking STAT3 signaling with AG490 significantly augmented the numbers of DCX-positive cells and PCNA$^+$/DCX$^+$ nuclei after ICH. $^*p<0.05$, $^{**}p<0.01$ comparing ICH group and Sham group; $^{\#}p<0.05$, $^{\#\#}p<0.01$ comparing the ICH+AG490 group and ICH group (n=5, a: Sham group; b: ICH group; c: ICH+AG490 group; d: ICH+DMSO group).

several challenges have yet to be overcome before these pre-clinical strategies can be applied for clinical use (22).

Neurogenesis has been shown to be necessary for tissue recovery after ICH (17,18). PCNA and DCX, two markers of neurogenesis, are often used to label newborn neurons (11). There is increasing evidence to show that many newly proliferated neurons appeared in the SVZ and the injured region after ischemic (22,29), and hemorrhagic (17,18) stroke. In line with these studies, we detected many $PCNA^+/DCX^+$ nuclei in the ipsilateral SVZ as well as the basal ganglia, which indicated that new neuronal cells migrated to the injured regions and contributed to brain recovery.

It has been well documented that p-STAT3 is expressed in astrocytes and neurons after cerebral ischemia (12). p-STAT3 dimerizes, translocates into the nuclei, binds to specific DNA elements, and finally controls the transcription of target genes involved in proliferation, apoptosis, and differentiation (7). p-STAT3 was found to be involved in the differentiation of NSCs into astrocytes, which led to glial scar formation (6,8,13,16,21). Furthermore, the p-STAT3 pathway has been reported to inhibit neurogenesis (9,20). Conditional deletion of STAT3 in NSCs enhanced neurogenesis and attenuated glial scar via a reduction in Notch1 signaling (3). Likewise, elimination of STAT3 expression by siRNA increased the expression of Mash1, a positive regulator of neurogenesis (4). In addition, the inhibition of STAT3 reduced ischemic (10), and hemorrhagic brain damage (14). Recent data from our own group showed that p-STAT3 was upregulated after ICH, expressed in astrocytes, and required for glial scar formation after ICH (30). In our current study, we further demonstrated that the upregulation of p-STAT3 was also observed in neurons and attenuated ICH-induced neurogenesis. Taken together, p-STAT3 signaling may play a dual role in determining the fate of the neural cell line age after ICH. However, whether p-STAT3 decreased neurogenes is in ICH via a Notch1-or Mash1-mediated mechanism is still unclear.

In summary, we showed that blocking p-STAT3 signaling with AG490 led to an increase in the number of DCX-positive cells and $PCNA^+/DCX^+$ nuclei after ICH. These findings suggest that p-STAT3 signaling is a critical negative regulator of neurogenesis during the recovery phase of ICH.

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