Baicalin Regulates Neuronal Fate Decision in Neural Stem/Progenitor Cells and Stimulates Hippocampal Neurogenesis in Adult Rats

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Keywords

Baicalin; Cognitive functions.; Ischemic stroke; Neural stem/progenitor cells; Neurogenesis; Neuronal fate.

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Received 3 September 2012; revision 19

November 2012; accepted 20 November

2012.

SUMMARY

Background: Recent studies revealed that baicalin, a flavonoid compound derived from the root of *Scutellaria baicalensis Georgi*, could promote neuron differentiation of NSPCs after commencing the differentiation process *in vitro*. However, this may not be the most efficacious strategy to determinate cell fate. Here, we have investigated whether baicalin can influence early events of neuron generation and stimulate adult neurogenesis. **Results:** Transient exposure of NSPCs to baicalin during proliferation could activate Mash1 to alter the differential fate and increase the proportion of cells expressing neuronal markers. Seven days after, rats were exposed to transient cerebral ischemia, they were treated for 3 weeks with baicalin, BrdU labeling study showed that exposure to baicalin increased the number of newly generated cells in hippocampus, BrdU/NeuN double staining analysis indicated that baicalin could promote new neuron production after cerebral ischemia. Additionally, Morris water maze test showed that delayed posts ischemic treatment with baicalin improved cognitive impairment. **Conclusions:** These results identify the existence of a single molecule, baicalin, which can specify the neuronal fate of multipotent NSPCs and stimulate neurogenesis, making it a promising candidate for developing clinically relevant strategies to manipulate neuronal fate of NSPCs for brain repair.

doi: 10.1111/cns.12050

The first two authors contributed equally to this work.

Introduction

Neural stem/progenitor cells (NSPCs) are excellent candidates for developing therapeutic strategies to repair the injured central nervous system (CNS) [1]. The neurons in the hippocampus would be injured following with the proliferation of NSPCs after cerebral ischemia. However, a current limitation in this strategy is that the majority of NSPCs tend to differentiate into astrocytes, which are thought to be undesirable as they can inhibit neurite outgrowth and contribute to regenerative failure when a glial scar is formed [2]. The ability to predetermine the neuronal fate of NSPCs would be beneficial to NSPCs therapy. Inducing a stable and predictable program of neural cell fate in NSPCs is an important goal for utiliz-

ing these cells for the treatment of neurological disorder. Recent studies have demonstrated that local signaling factors specify cell fate [3–5]. And Åkerblom et al. [6] reported that miR-124 is a neuronal fate determinant in the subventricular zone. These findings indicated that the ability to direct NSPCs cell fate may have therapeutic potential.

NSPCs fate can be determined by intrinsic signals and affected by small molecules [7–10]. The main mechanisms of the neuronal fate specification would be regulating the proneural genes. Genetic studies have provided evidence that proneural genes are necessary to restrict the neuronal cell fate of NSPCs [11]. Neurogenin and Mash are bHLH transcription factors, which initiate neurogenesis [12–14]. These findings indicate that bHLH transcription

factors are the key points in the fate specification of NSPCs. Small molecules have been shown to be useful chemical materials in promoting neurogenesis and manipulating cell fate [9,15]. A variety of priming agents used to direct neuronal fate in NSPCs, such as lithium chloride [16], fibroblast growth factor-2 [17], and insulin-like growth factor 1 [18] had been identified.

In the search for new therapeutics of brain injury or neurodegenerative disease, the herbs being used in traditional medicines for neurogenesis are promising candidates. Baicalin (Figure 1A) is one of the predominant flavonoid derivatives isolated from the dry roots of *Scutellaria baicalensis* Georgi, which has protective effects against cerebral ischemia, brain inflammation, and other brain injuries [19–21]. Our previous studies have showed that baicalin treatment could induce neuron differentiation of NSPCs after its commencing the differentiation process [22–24], which has been confirmed by a latest study [25]. However, this may not be the most efficacious strategy to determinate cell fate. As emphasized by Vazey and Connor [16], alternative avenue for directing fate may be to pretreat NSPCs while they maintain their undifferentiated phenotype. Although a variety of active ingredients of herbs, such as ginsenosides [26–28], curcumin [29], ferulic acid [30], salvianolic acids [31,32], could affect the biological properties of NSPCs. However, due to the presence of blood–brain barrier, some of the effective drugs *in vitro* cannot produce efficacy *in vivo*. Interestingly, some researchers had demonstrated that baicalin could pass through the blood–brain barrier [33], making it more likely to promote neurogenesis in the central nervous system.

In the present study, we demonstrated that after transient exposure to baicalin during proliferation could increase the new neurons production and improve the cognitive functions in a rat model of stroke. The neuronal fate determination and neurogenesis stimulating effects of baicalin makes it a promising candidate

for developing clinically relevant strategies to stimulate NSPCs for brain repair.

Materials and Methods

Animals

Pregnant rats and Adult male Wistar rats (Certificate No SCXX 2006-0009; Vital River Lab Animal Technology Co., Ltd., Beijing, China) were used in this study. The rats were housed in temperature-controlled conditions with a 12-h light/dark cycle following surgery. They had access to food and water *ad libitum*. All experimental procedures were evaluated and approved by the animal ethics committee of Tianjin University of Traditional Chinese Medicine (TCM-2009-034-E02), and the animal care was according to the NIH Guide for the Care and Use of Laboratory Animals.

Neurosphere Culture and Cell Viability Assay

NSPCs were isolated from the cerebral cortex of 13.5-day-embryonic Wistar rats as previously described [34]. NSPCs were cultured in neurosphere proliferation media consisting of DMEM/F12 supplemented with 2% (v/v) B27 Supplement (Invitrogen, Carlsbad, CA, USA), 20 ng/mL EGF (Millipore, Temecula, CA, USA), 20 ng/mL FGF2 (Millipore), and 100 µg/mL heparin (Sigma, St. Louis, MO, USA). For the *in vitro* cell viability assay, Cell Titer 96® Aqueous One Solution Cell Proliferation Assay kit (Promega, Charbonnières-les-Bains, France) was used in this study. NSPCs were plated at 30,000 cells/well in a 96-well plate in the presence and absence of baicalin (1, 1.875, 3.75, 7.5, 15, 30 µM). Cell proliferation was assessed at day 2 of cell culture. According to the manufacturer's recommendations, 40-µL MTS solution was then added into each of the wells, and data were obtained at a wavelength of 490 nm using a microplate reader (FlexStation 3; Molecular Devices, Sunnyvale, CA, USA). The same volume of medium without cells was used as blank.

RT-PCR Analysis

NSPCs were cultured with or without baicalin (7.5, 15, 30 µM) in proliferation medium for 3 days. Cells were harvested and total RNA was isolated from treated and untreated NSPCs with TRIzol (Roche, Mannheim, Germany). The first strand of cDNA was synthesized from 1 µg of total RNA using reverse transcriptase (TIAN-GEN) and random primer as described in the manufacture's instructions. After synthesis, 1 µL of cDNA were used in PCR reaction with gene-specific primers. The sequences of the PCR primer pairs (5' to 3') that were used for each gene are as follows: β -tubulin III, 5'-GGACCTCAACCACCTTGTGT-3' (forward), and 5'-AAC-ATGCCCGTAAACTGCTC-3' (reverse); GFAP, 5'-ATTCCGCGCCT-CTCCCTGTCTC-3' (forward), 5'-GCTTCATCCGCCTCCTGTCTGT-3' (reverse); GAPDH, 5'-CCAAGGTCATCCATGACAA-3' (forward), and 5'-TGTCATACCAGGAAATGAGC-3' (reverse); RT-ve control were seted for RT-PCRs analysis (RT-ve control = reaction with H₂O instead of RNA as control). Products were analyzed on 1.5% agarose gel and visualized by ethidium bromide staining. The relative quantitative expressions of tissue-specific markers were calculated after normalization with GAPDH as a housekeeping gene.

Figure 1 Effect of baicalin on the cell viability of NSPCs. Dissociated NSC/NPCs were cultured on a 96-well plate with the indicated baicalin (A) concentration (1, 1.875, 3.75, 7.5, 15, 30 µM) for 2 days. (B) Cell proliferation was measured using a MTS assay kit. Values were expressed as means $\pm$ SD of three individual experiments. $n = 6$, * $P < 0.05$, ** $P < 0.01$ compared with control.

Real-time RT-PCR Analysis

Real-time RT-PCR was performed in a reaction mixture containing Power SYBR green PCR master mix (Applied Biosystems, Carlsbad, CA, USA), gene-specific primers (Mash1, 5'-CTCG-TCTCTCCGGAAGTATG-3' (forward), and 5'-ATGCTCCGGA-GGGTGGCAAAA-3' (reverse); Ngn1, 5'-ATGCCTGCCCT-TTGGAGACCT-3' (forward), and 5'-TGTCAGCTGGCACAGTC-CTCCT-3' (reverse); Ngn2, 5'-CGGGTCAGACGTGGACTACT-3' (forward), and 5'-GGCGGGAGAAGGATGGGAAGA-3' (reverse). Quantitative real-time PCR reactions were run in a 7500 Fast real-time PCR system (Applied Biosystems) with 7500 Fast System Sequencing detection software version 1.3.1 and the thermal profile of 95°C for 10 min followed by 40 cycles of 95°C for 15 second and 60°C for 1 min. The expression level of each gene was enumerated in three independent treatments. The relative gene expression between baicalin treatment and control was calculated by the $\Delta\Delta C_t$ method with GAPDH as the internal control.

In Vitro Differentiation Assay

For the *in vitro* differentiation assay, NSPCs were cultured with or without baicalin (7.5, 15, 30 μ M; Baicalin > 98.5% was purchased from Tianjin Zhongxin Pharmaceutical Group Co., Ltd., Tianjin, China) in proliferation medium for 3 days. Then, neurospheres were collected and plated into poly-L-lysine-coated 12-well plates in differentiation culture medium without growth factors or baicalin. Neurospheres were allowed to differentiate for 3 days before being fixed for immunocytochemistry. For fluorescence immunostaining, cells were fixed with 4% paraformaldehyde for 30 min. After fixing, cells were incubated overnight at 4°C with the following primary antibody: Rabbit anti-MAP-2 (1:500; Millipore), Mouse anti-GFAP (1:500; Millipore), followed by FITC-conjugated goat anti-mouse IgG (1:100; Boster, Wuhan, China), and Cy3-conjugated goat anti-rabbit IgG (1:100; Boster). 4',6-Diamino-2-phenylindole (DAPI; Sigma) was used as a fluorescent nuclear counterstain. Immunostained cells were visualized by indirect fluorescence under the fluorescent microscope (Leica, Wetzlar, Germany). MAP-2 or GFAP-positive cells were counted in 10 randomly selected fields from three different chambers.

Western Blotting Assay

Protein extracts were prepared and subjected to Western blot analysis. The protein concentrations of the lysates were determined with a Bradford protein assay kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. An equal amount of protein was fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene difluoride membranes. After blocking with 5% skim milk in TBS-T, the membrane was probed with goat anti-actin (1:2000; Santa Cruz, CA, USA); mouse anti-mash1 (1:1000; Millipore) primary antibody in a blocking solution of nonfat milk (5%). Secondary horseradish peroxidase-conjugated goat anti-mouse, goat anti-rabbit (Boster) antibody in nonfat milk blocking solution (5%) was then applied. The immunoreactivity was visualized with

ECL Western blotting detection reagents (Millipore). The total protein content was normalized using mouse anti-actin antibodies.

Induction of the Transient Global Ischemia

Adult male Wistar rats weighing 200–250 g were subjected to transient forebrain ischemia by a method combining those described previously [35–37]. In brief, 12 h before the induction of ischemia, rats were anesthetized with sodium pentobarbital, the bilateral carotid arteries were exposed to facilitate the occluding on the following day, and then the vertebral arteries were irreversibly occluded by electrocoagulation. The next day, ischemia was induced by bilaterally occluding carotid arteries with aneurysm clips, and carotid arteries were clamped for 6 min exactly. Rats lost their righting reflex during ischemic, the clips were removed to restore cerebral blood flow. The rats that remain their righting reflex in 1 min after occluding of the both carotid arteries were considered to be the failure of ischemia and eliminated. Sham-operated rats were anesthetized, the carotid arteries were isolated, but they were not clamped.

Drug Administration

The study was carried out on rats divided into three groups. (1) Sham group + vehicle (sham), (2) Ischemic group + vehicle (vehicle), and (3) Ischemic group + baicalin (Baicalin). Baicalin was diluted in saline at the concentration of 100 mg/mL (Stock solution) and incubated under agitation for 1 h at 37°C. The solution was then filtered with 0.22-mm filter. Baicalin were administered intraperitoneally (i.p.) at a dose of 50 mg/kg body weight, once daily for 3 weeks. All controls received an amount of vehicle equivalent to drug treatment conditions.

BrdU Labeling and Analysis of In Vivo Neurogenesis

BrdU (50 mg/kg; Sigma) was injected i.p. to the rats once every 2 h over a period of 8 h at the 20th day after baicalin treatment to label the proliferating cells after ischemia. The next day (21th), rats were anesthetized with ether and perfused with PBS, followed by a cold 4% paraformaldehyde solution. Brains were collected and postfixed overnight in a 4% paraformaldehyde solution at 4°C. Coronal sections (20 μ m thickness) were obtained throughout the hippocampus. For analysis concerning BrdU or BrdU/NeuN double stain immunohistochemistry, sections were incubated in 50% formamide/2 $\times$ saline sodium citrate for 2 h at 65°C, followed by a rinse with PBS. Sections were then incubated in 2 N HCl for 30 min at 37°C to denature double-stranded DNA, and rinsed in 0.1 M borate buffer (pH 8.5). After blocking for 2 h with 1% BSA in PBS, sections were incubated overnight at 4°C with mouse anti-BrdU monoclonal antibody (1:100; senta), or rabbit anti-NeuN polyclonal antibody (1:100; Millipore). Followed by rinsing in PBS, sections were incubated for 2 h at RT with FITC-conjugated goat anti-mouse IgG (1:100; Boster) and Cy3-conjugated goat anti-rabbit IgG (1:100; Boster). BrdU or BrdU/NeuN double-positive cells were visualized under the fluorescent microscope (Lica, Germany).

Morris Water Maze Task

The cognitive functions were examined using the Morris water maze. A black circular pool 1.5 m in diameter was filled with water ($22 \pm 1^\circ\text{C}$), and a hidden platform 10 cm in diameter was placed at a constant position in the center of one of the four quadrants within the tank. Rats in each groups ($n = 10$) were allowed to swim freely for 2 min to become habituated to the apparatus. From the next day, in the hidden platform trials, acquisition trials were carried out two times per day for 5 days. In each trial, rats were placed into the water at a fixed starting position, and the time taken to escape onto the hidden platform was measured. Rats were given 90 second to find the hidden platform during each acquisition trial. If it failed to locate the platform within 90 second, it was guided there, and the rat was allowed to stay on the platform for 20 second. The latency to finding the escape platform was analyzed as an index of spatial learning. Performance was tested 24 h after the final training day in a probe trial during which the platform was removed, the rat was placed in the start and its behavior was monitored for 90 second.

Data Analysis

Data were expressed as mean $\pm$ SD. Statistical comparison between different treatments was carried out by one-way analysis

of variance, and then *post hoc* least significant difference (LSD) test was employed for comparisons between control and other groups. To analyze water maze place-navigation performance, the average escape latency was calculated and evaluated by repeated-measures ANCOVA. Differences were considered statistically significant for $P < 0.05$.

Results

Baicalin Upregulated Neuronal Genes Expression

To investigate the effect of baicalin on the viability of NSPCs, NSPCs were exposed to baicalin for 2 days. The effect of baicalin treatments on the growth rates of the NSPCs was compared using a cell viability measurement. As demonstrated in Figure 1B, baicalin had a promoting activity for NSPCs proliferation and exhibited a dose-dependent effect. Based on the cell viability study, the doses of 7.5, 15, 30 μM were chosen in the following study. Mash1 and Ngn1 are thought to positively regulate neuronal development [38,39]. The expression of proneural transcription factors (Mash1, Ngn1 and Ngn2) in the proliferating NSPCs in the presence and absence of baicalin was measured. Changes in gene expression in the NSPCs treated and untreated with baicalin were evaluate by real-time RT-PCR technique. In our study, the results showed that Mash1 mRNA increased after baicalin treatment (Control $0.50 \pm 0.10\%$ vs. 15 μM baicalin $1.22 \pm 0.38\%$, 30 μM

Figure 2 Effect of baicalin on the bHLH transcription factors expression and the neural fate of NSPCs. The mRNA or protein levels of Mash1 treated with designated concentrations (7.5, 15, 30 μM) of baicalin analyzed by real-time RT-PCR or Western blotting. **(A)** The mRNA levels quantified were calculated by the $\Delta\Delta\text{Ct}$ method, normalized with GAPDH internal control. Mash1 expression were significantly upregulated in baicalin-treated NSPCs compared with control ($n = 6$); **(B)** Semiquantified scores of Mash1 expression were significantly upregulated in baicalin-treated NSPCs compared with control, normalized with Actin internal control ($n = 6$); **(C)** The mRNA levels of β -tubulin III, GFAP treated with designated concentrations (7.5, 15, 30 μM) of baicalin for 3 days were analyzed by RT-PCR. The mRNA levels were semiquantified by densitometric measurements, normalized with GAPDH internal control ($n = 6$), and expressed as means $\pm$ SD of three individual experiments. Values were expressed as means $\pm$ SD of three individual experiments. * $P < 0.05$, ** $P < 0.01$ compared with control. [Correction made after online publication on 24 Jan 2013: 'percent of total gene' was removed from the y-axis label in part A and 'percent of total protein' was removed from the y-axis label in part B.]

baicalin 1.54 ± 0.09 , $n = 6$, $P < 0.05$, $P < 0.01$, respectively; Figure 2A). To provide further evidence that baicalin upregulated *mash1* expression, *Mash1* protein was detected by Western blotting assay with anti-*mash1* antibody. As shown in Figure 2B, baicalin significantly increased *mash1* expression compared with untreated control (Control 0.34 ± 0.08 vs. $7.5 \mu\text{M}$ baicalin 0.83 ± 0.11 , $15 \mu\text{M}$ baicalin 0.82 ± 0.13 , $30 \mu\text{M}$ baicalin 0.87 ± 0.10 , $n = 6$, $P < 0.01$). These results indicated that baicalin stimulates neuronal differentiation by activating bHLH transcription factors.

Baicalin Specified the Neural Fate of NSPCs

Given the induction of bHLH factors by baicalin, in the following research we investigated whether baicalin treatment during proliferation would increase neuronal fate determination. Cells were subjected to baicalin treatment for 3 days under proliferative con-

ditions and the ability of their neuronal fate commitment was compared by RT-PCR technique, using specific neuronal markers. The expressions of β -tubulin III (neuron marker) and GFAP (astroglia marker) were measured by RT-PCR technique. Results showed that no bands were detected in RT-ve control samples, and after 3 days of baicalin treatment, NSPCs significantly decreased the mRNA expressions of GFAP (Control 0.98 ± 0.21 vs. $15 \mu\text{M}$ baicalin 0.57 ± 0.12 , $30 \mu\text{M}$ baicalin 0.48 ± 0.09 , $n = 6$, $P < 0.01$), and baicalin increased the expression of β -tubulin III (Control 1.02 ± 0.16 vs. $15 \mu\text{M}$ baicalin 1.89 ± 0.37 , $30 \mu\text{M}$ baicalin 2.80 ± 0.64 , $n = 6$, $P < 0.01$; Figure 2C).

Immunostaining was also employed to evaluate the ability of neuronal fate determination of baicalin. NSPCs were subjected to baicalin treatment under proliferative conditions for 3 days, after that we withdrew the baicalin, and made the NSPCs adhere to differentiate without baicalin for 3 days. Based on the differentiation assay, we also found that, along with elevated proneural gene

Figure 3 Baicalin primed toward a neuronal fate of NSPCs during their subsequent differentiation. (A) Fluorescence microscopic photos showing the neural differentiation of NSPCs treated with designated concentrations (7.5, 15, 30 μM) of baicalin for 3 days in proliferation medium. Red, MAP-2; Green, GFAP; blue, DAPI. Scale bar, 100 μm . (B) Baicalin significantly increased the fraction of MAP-2-positive cells. (C) There was no significant difference in the fraction of GFAP-positive cells. The data expressed as means $\pm$ SD of three individual experiments. $n = 8$, $*P < 0.05$, $**P < 0.01$ compared with control.

expression, the fraction of MAP-2-(neuron marker) positive cells was also highly upregulated compared with control (Figure 3A). Our immunocytochemistry approach identified the percentage of MAP-2-positive cells with more than twofold changes between baicalin (30 μ M) exposure and control (Control $11.8 \pm 3.4\%$ vs. baicalin $24.9 \pm 4.3\%$, $n = 8$, $P < 0.01$; Figure 3B). There was no significant difference in the fraction of GFAP-positive cells (Control $71.8 \pm 10.5\%$ vs. 7.5 μ M baicalin $75.6 \pm 8.5\%$, 15 μ M baicalin $73.3 \pm 14.6\%$, 30 μ M baicalin $79.0 \pm 10.6\%$, $n = 8$, $P > 0.05$; Figure 3C). These results suggested that exposed to baicalin during proliferation was sufficient to specify the neuronal fate of NSPCs.

Baicalin Increased the Number of Newly Generated Cells in the Dentate Gyrus

To elucidate the possible effects of baicalin on adult NSPCs of the hippocampus, in the following study, we employed experimental cerebral ischemic rats. Newly generated cells in hippocampus dentate gyrus after transient cerebral ischemia were evaluated in the absence or presence of baicalin. The dividing cells labeled with BrdU were visualized by BrdU immunohistochemistry. As showed in Figure 4A–B, a few BrdU-positive cells were observed in the SGZ of hippocampal dentate gyrus in the sham, and BrdU immunoreactivity did not significantly differ between vehicle and

sham-operated animals (Sham 10.4 ± 3.0 vs. Vehicle 12.9 ± 6.3 , $n = 3$, $P > 0.05$). The numbers of BrdU-positive cells in the dentate gyrus were significantly greater in baicalin-treated rats compared with the rats in vehicle (Vehicle 12.9 ± 6.3 vs. Baicalin 23.8 ± 4.3 , $n = 3$, $P < 0.05$). The results suggested that baicalin increased the number of newly generated cells in the hippocampus dentate gyrus in adult rats after cerebral ischemic injury.

Baicalin Enhanced Adult Hippocampal Neurogenesis

To determine neurons from the newly generated cells, we performed double-label fluorescence immunohistochemistry on the hippocampus using antibodies against the neuron-specific protein (NeuN) in combination with the BrdU antibody. BrdU/NeuN double staining-positive cells were seemed to be new neurons. As shown in Figure 4C–D, BrdU/NeuN double staining-positive cells were seldom found in sham group in the dentate gyrus of the hippocampus. A few BrdU/NeuN double staining-positive cells were observed in the SGZ of hippocampal dentate gyrus in the vehicle-treated rats. We observed a significant increase in the numbers of BrdU/NeuN double staining-positive cells in the SGZ of hippocampal dentate gyrus in the baicalin-treated group (Vehicle 4.8 ± 2.6 vs. Baicalin 11.5 ± 3.8 , $n = 3$, $P < 0.05$), which was consistent with our *in vitro* findings that baicalin could prime proliferating

Figure 4 Baicalin increased the number of BrdU and BrdU/NeuN double staining-positive cells *in vivo*. (A) Representative photomicrographs showing BrdU-positive cells in the hippocampus of each group. BrdU-labeled cells (Green) were indicated by arrows. (B) Quantification of BrdU-positive cells in the hippocampus. (C) Representative photomicrographs showing BrdU/NeuN double staining-positive cells (newly generated neurons) in the hippocampus of each group. New generated neurons were indicated by arrows. (D) Quantification of new generated neurons in the hippocampus. Scale bar: 200 μ m. Each column represents the represent the mean $\pm$ SD ($n = 3$). * $P < 0.05$, ** $P < 0.01$. Significant difference from the vehicle. [Correction made after online publication on 24 Jan 2013: a bar indicating the scale of 200 μ m was added to the Baicalin image in part A.]

Figure 5 Effects of baicalin on spatial learning in Morris water maze. **(A)** The ordinate indicated escape latency to the hidden platform, and the abscissa represented the training time. There was a decrease in escape latencies with training in all three groups. Baicalin-injected rats showed improved performance in the Morris water maze task compared with vehicle on day 5. Data are expressed as means $\pm$ SD of 10 rats in each group. *Significant difference from the Sham at $P < 0.05$, #Significant difference from the vehicle at $P < 0.05$. **(B)** Representative examples of search patterns in the pool of each group rats on day 6 after the platform removed. [Correction made after online publication on 24 Jan 2013: the images for Vehicle and Baicalin were replaced in part B.]

NSPCs to be a neuronal fate during their subsequent differentiation. The results suggested that baicalin could promote new neurons production after cerebral ischemic injury.

Baicalin Improved the Learning and Memory Functions

Next, we used the Morris water maze task to examine the effect of baicalin on the cognitive functions after cerebral ischemic injury. Figure 5A showed the escape latencies of the three groups of animals during acquisition training (5 days). As expected, in all the groups, escape latencies became shorter with the training time prolonged. The decreased escape latency during the training process in the three groups clearly reflected memory for the escape platform place. However, differences were observed between the three groups, sham-operated animals memorized the position of the hidden platform very quickly, thereby reached it in a shorter latency with the training time prolonged. In contrast, the vehicle-treated ischemic animals showed a deficiency compared with the sham group. Although they were gradually trained and showed better performance, their escape latencies were still much longer than those of the sham group animals, and there were a significantly difference in the day 4 (Sham 18.37 ± 11.60 vs. Vehicle 44.38 ± 30.82 , $n = 10$, $P < 0.05$) and day 5 (Sham 14.63 ± 5.03 vs. Vehicle 38.23 ± 24.29 , $n = 10$, $P < 0.01$). Administration of baicalin significantly ameliorated such deficiencies. *Post hoc* analysis revealed that baicalin-injected rats showed significantly faster

learning rates compared with vehicle-treated animals in the day 5 (Vehicle 38.23 ± 24.29 vs. Baicalin 20.05 ± 10.35 , $n = 10$, $P < 0.05$). Figure 5B presents representative performances by the rat when the platform was removed on day 6. Clearly, the swim pattern of sham-operated rats revealed a direct route to the platform, and continued to search in the original platform location. While the vehicle-treated animals showed a complex search pattern in the pool. The search pattern in baicalin treatment group was more similar to the sham. The results indicated that baicalin treatment facilitated performance in the water maze task.

Discussion

Previously studies had demonstrated that baicalin treatment could promote neuronal differentiation of C17.2 neural stem cells or primary cultured NSPCs after its commencing the differentiation process *in vitro* [22–25]. However, an alternative avenue for directing fate may be to pretreat NSPCs while they maintain their undifferentiated phenotype. In the present study, we demonstrated that a short-time exposure of baicalin during proliferation was sufficient to induce neuronal fate commitment of NSPCs and stimulate neurogenesis *in vivo*.

While baicalin-induced neuronal differentiation is prominent in our and others *in vitro* system [22–25]; in the present study, we found that baicalin could not only promote the neuronal differentiation, but also specify a neuronal fate of NSPCs during the proliferation process and then facilitate the neuron differentiation. From our results on the fate commitment of NSPCs, it is clear that baicalin activates the expression of proneural genes such as Mash1. Reports in various researches suggest that bHLH transcription factors direct neural differentiation [12,13,40]. In line with this notion, our results indicate that increased expression levels of Mash1 point to a shift toward neuronal fate determination targeted by baicalin. Cell fate determination of NSPCs and subsequent successive steps of differentiation should be under strict control at the molecular and cellular levels. To provide more clear mechanisms that baicalin could prime the neuronal fate of the NSPCs, further study will be carried out.

There was evidence to suggest that proneural genes are involved in lineage restriction of cortical progenitors [41]. The neuronal gene β -tubulin III is a terminal differentiation gene [42]. Considering the hierarchic order of these genes and proneural genes, it was not surprising that upregulation of mash1 leads to a similar effect on β -tubulin III expression. To allow the dissection of the events that occur during neural cell-fate determination, NSPCs were first subjected to baicalin treatment under proliferative conditions for 3 days, after that we withdrew the baicalin. In our differentiation system, after 3 days, the fraction of MAP-2-positive cells was also highly increased, as indicated by upregulation of the expression of the proneural genes. However, the fraction of GFAP-positive cells showed no difference between baicalin treatment and control. Although this was in contradiction to the RT-PCR data, this might be due to the proliferation of astroglia. Taken together, these data suggested that baicalin could efficiently induce neural specification of NSPCs during proliferation.

Baicalin could induce the NSPCs differentiate into neurons, and specify a neuronal fate of NSPCs *in vitro*; however, its role on promoting neurogenesis *in vivo* was still unclear. Because cerebral

ischemia can cause prolonged spatial memory disturbance in rats [43]. In order to examine the *in vivo* effects of baicalin on NSPCs, experimental cerebral ischemic rats were employed in the present study. Forebrain ischemia was performed by four-vessel occlusion in this work. We demonstrated that baicalin significantly increased the numbers of newly generated cells in the dentate gyrus of the hippocampus after transient forebrain ischemia. To determine the neurons from the newly generated cells in the baicalin-treated animals, we assessed the differentiated cells by colocalization of NeuN, a neuron phenotypic marker, in BrdU-labeled cells. Animals treated with baicalin displayed an increase in the fraction of BrdU/NeuN double-positive cells compared with vehicle-treated animals (Figure 5). The *in vivo* BrdU/NeuN double staining analyses support our conclusion that chronic baicalin treatment enhanced the neurogenesis. In parallel, our *in vitro* data showed that baicalin could prime the NSPCs toward a neuronal fate during their proliferation and then facilitate the subsequent differentiation to the neurons.

To test baicalin that enhanced hippocampal memory function, Morris water maze had to be employed. The major finding of the present study was that baicalin facilitated spatial learning in cerebral ischemic rats. Interestingly, baicalin increased the newly generated neurons in the hippocampal compared with the vehicle-treated animals. Considering that the hippocampus is an essential structure for spatial learning, it seems reasonable to assume that increased hippocampal neurogenesis induced by baicalin injection

contributed at least partly to improving spatial learning. Thus, our data indicated that baicalin enhanced the neurogenesis and improved the cognitive functions after cerebral ischemic injury.

In conclusion, the present study demonstrated that baicalin could prime the NSPCs toward a neuronal fate and then facilitate the subsequent differentiation to the neurons and stimulate adult hippocampal neurogenesis after cerebral ischemic injury. Baicalin might be developed into a promising candidate for developing clinically relevant strategies to stimulate NSPCs for brain repair.

Acknowledgments

This study was supported by National Natural Science Foundation of China (30873395 and 30472177), Key Technologies R&D Program of China (NO. 2011BAI07B08) and Program for Changjiang Scholars and Innovative Research Team in University (PCSIRT, No. IRT0973). We would thank Dr. Hui Wang and Prof. Yan Zhu for their helpful discussion and critical reading of our manuscript. We would thank Mr. Huaian Bu for technical assistant of the Statistical analysis.

Conflict of Interest

The authors declare no conflict of interest.

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