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ORIGINAL RESEARCH

Inhibition of Reactive Astrocytes with Fluorocitrate Ameliorates Learning and Memory Impairment Through Upregulating CRTC1 and Synaptophysin in Ischemic Stroke Rats

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Abstract

Ischemic stroke often causes motor and cognitive deficits. Deregulated glia gap junction communication, which is reflected by increased protein levels of glial fibrillary acidic protein (GFAP) and connexin 43 (Cx43), has been observed in ischemic hippocampus and has been associated with cognitive impairment in animal stroke models. Here, we tested the hypothesis that reactive astrocytes-mediated loss of synaptophysin (SYP) and CREB-regulated transcription coactivator 1 (CRTC1) contribute to dysfunction in glia gap junction communication and memory impairment after ischemic stroke. Male Sprague–Dawley rats were subjected to a 90-min middle cerebral artery occlusion (MCAO) with 7-day reperfusion. Fluorocitrate (1 nmol), the reversible inhibitor of the astrocytic tricarboxylic acid cycle, was injected into the right lateral ventricle of MCAO rats once every 2 days starting immediately before reperfusion. The Morris water maze was used to assess memory in conjunction with western blotting and immunostaining to detect protein expression and distribution in the hippocampus. Our results showed that ischemic stroke caused significant memory impairment accompanied by increased protein levels of GFAP and Cx43 in hippocampal tissue. In addition, the levels of several key memory-related important proteins including SYP, CRTC1, myelin basic protein and high-mobility group-box-1 were significantly reduced in the hippocampal tissue. Of note, inhibition of reactive astrocytes with fluorocitrate was shown to significantly reverse the above noted changes induced by ischemic stroke. Taken together, our findings demonstrate that inhibiting reactive astrocytes with fluorocitrate immediately before reperfusion may protect against ischemic stroke-induced memory impairment through the upregulation of CRTC1 and SYP.

Keywords Fluorocitrate · Reactive astrocytes · Cx43 · Memory · CRTC1 · Ischemic stroke

Abbreviations

CRTC1	CREB-regulated transcription coactivator 1
Cx43	Connexin 43
FC	Fluorocitrate
GFAP	Glial fibrillary acidic protein
GJC	Glia gap junction communication
HMGB1	High-mobility group-box-1
MBP	Myelin basic protein

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MCAO Middle cerebral artery occlusion
SYP Synaptophysin

Introduction

Stroke is one of the leading causes of morbidity and mortality worldwide (Baron 2018) and most stroke events were attributed to the occlusion of the middle cerebral artery (MCA). In addition to motor and sensory disturbances, cognitive deficit is the key problem for patients with compromised blood supply in the MCA (Bakker et al. 2000). However, the mechanism underlying ischemic stroke-induced memory impairment is not well understood and effective therapeutics to alleviate the deficits are not yet available in clinical practice.

Ischemic stroke-induced memory damage is accompanied by downregulation of synaptophysin (SYP) (Fonteles et al. 2016), which is a marker of synaptic plasticity (Smith et al. 2000). Although the hippocampus is supplied by the posterior cerebral arteries and not by the MCA (Xie et al. 2011), hippocampal neurons ipsilateral to the occluded MCA have been shown to undergo delayed death after ischemic stroke in both rats (Wang et al. 2004) and humans (Xie et al. 2011). CREB-regulated transcription coactivator 1 (CRTC1), a potent modulator of cAMP response element (CRE)-driven gene transcription (Xue et al. 2015), has been shown to play important roles in energy balance (Altarejos et al. 2008), life span extension (Mair et al. 2011), and neuronal survival after ischemia (Sasaki et al. 2011). In addition, CRTC1 has also been shown to play an important role in fear memory (Nonaka et al. 2014) and dysfunction of CRTC1 affects normal memory encoding and has been linked to neurodegeneration in Alzheimer's disease (Parra-Damas et al. 2016). More importantly, CRTC1 has been found to be upregulated after ischemic stroke and mediate the neuroprotective effects of Tanshinone IIA (Liu et al. 2010). Hence, CRTC1 may be a therapeutic target to improve ischemic stroke-induced memory impairment.

Astrocytes provide structural, trophic, and metabolic supports for neurons and regulate synaptic protein expression (Hayakawa et al. 2010a) and memory consolidation (Gibbs and Bowser 2009). Under ischemic stroke conditions, a large number of astrocytes are activated to produce and release a number of inflammatory mediators (IL-6, TNF-α and interferon) that leads to neuronal death and contribute to infarct progression (Hayakawa et al. 2010a; Liu and Chopp 2016). However, how and to what extent these activated astrocytes contribute to memory deficit after ischemic stroke remains unknown. As fluorocitrate (FC) is preferentially taken up by glial cells and can inhibit the Krebs cycle via targeting enzyme aconitase (Clarke et al. 1970; Hayakawa et al. 2010a), it has been widely used to inhibit astrocytes and study their roles under various pathological conditions (Gibbs and Bowser 2009; Paulsen et al. 1987; Wang et al. 2009, 2018). Hence, we hypothesize that inhibiting reactive astrocytes by FC could alleviate acute ischemic stroke-induced memory impairment.

To test this hypothesis, we employed the Morris water maze to assess hippocampus-dependent memory impairment in male Sprague–Dawley adult rats (Li et al. 2013; Ran et al. 2018; Xie et al. 2011) and examine the effect of FC treatment on stroke-induced memory impairment. Our results show that memory disruption due to middle cerebral artery occlusion (MCAO) was accompanied by an increase of reactive astrocytes and downregulation of CRTC1 and SYP. FC treatment could ameliorate this outcome through inhibiting reactive astrocytes and alleviating acute ischemic stroke-induced downregulation of CRTC1 and SYP.

Experimental Procedure

Focal Cerebral Ischemia and Reperfusion Model

Male Sprague–Dawley rats (SLAC Company, Shanghai, China) weighing 270–290 g (8–10 weeks) were kept under a 12-h light/dark cycle in an air-conditioned room (23 ± 1 °C) with ad libitum access to food and water. Rats were subjected to middle cerebral artery occlusion (MCAO) surgery using a suture model, as described previously (Sun et al. 2017). Reperfusion was produced by gently withdrawing the 4-0 silicone-coated monofilament nylon suture (Zhu et al. 2014). The University Committee on Animal Care of Soochow University approved all experimental protocols (IACUC-201611A355), and all animal procedures were performed according to the NIH Guide for the Care and Use of Laboratory Animals.

Treatment Protocol and Drugs

The detailed procedure was shown in Fig. 1. After a 90-min MCAO, the rats were randomly assigned into two treatment groups (the vehicle and FC groups) using a table of random numbers. Immediately before reperfusion, rats either received FC (Cat#f9634, Sigma-Aldrich) or vehicle infusion into the lateral ventricle of the brain. The same treatment was repeated on day 1, 3, 5, 7 at a dose of 1 nmol, which have showed irreversible ultrastructural alterations on glia, especially astrocytes, but not in neurons (Shang et al. 2015). The FC solution was prepared according to a previous study (Paulsen et al. 1987) and was microinjected into the right lateral ventricle (AP: −0.8 mm, ML: +1.3 mm, DV: −3.5 mm) (Shang et al. 2015). A total of 1 μL FC solution was injected continuously at a rate of 0.25 μL/min through a stainless steel cannula connected to a 5 μL syringe driven

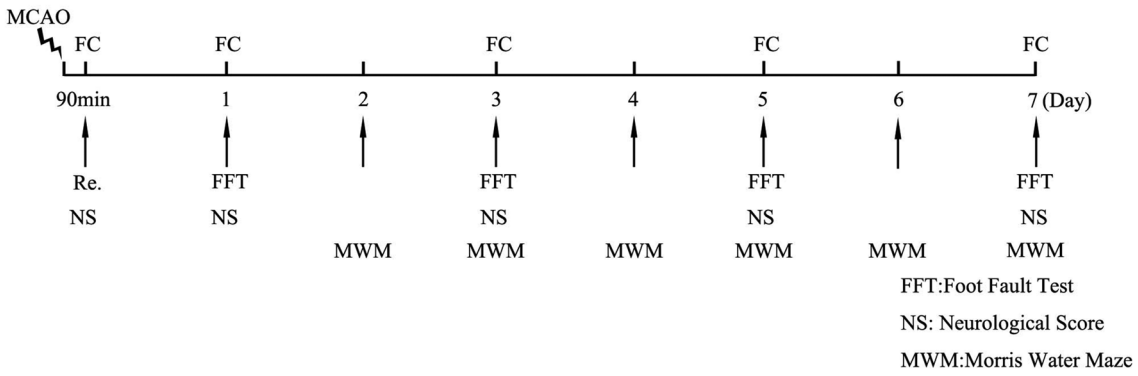

Fig. 1 Diagram shows the experimental procedure

by a slow-injection pump. Drug treatment experiments were performed in the Animal Facility between 9 and 11 a.m.

Behavioral Training and Testing

Morris Water Maze Task

Spatial learning and memory were evaluated by the Morris water maze task, as described previously with minor modifications (Ran et al. 2018). The observer was blinded to the experimental conditions and 48 h after the MCAO surgery and before formal training, each rat was allowed to swim freely in a pool (diameter, 160 cm; water depth, 70 cm) for 60 s without an escape platform. During formal training, each rat was placed in the pool with an invisible platform which had a 10 cm diameter and was submerged 2 cm below the water surface. The pool was divided into four quadrants and the platform was fixed within one quadrant. Each rat was placed in the water from four different starting points and the latency to escape onto the platform was recorded. Each daily training session included four trials and each rat was given a maximum time of 60 s to climb onto the platform during each trial. A trial would be terminated if a rat could not find the platform within 60 s, and a score of 60 s would be assigned. The rat was allowed to stay on the platform for 10 s after it was guided to the hidden platform by hand and the rat would be put back to the home cage. The training was performed for five consecutive days followed by a spatial probe trial on the sixth day, during which the escape platform was removed from the pool. The latency of first entry to the platform quadrant, which had contained the platform, was recorded and the ratio between the time taken to cross this quadrant and the total time taken to cross all quadrants was reflected as a percentage (Damodaran et al. 2014).

Behavioral Assessments Two types of behavioral functional tests were performed at 24 h and 3, 5, and 7 days after cerebral ischemia and reperfusion. Both the neurological deficits and the forelimb foot-fault-placing assessment were performed as previously described (Liu et al. 2017). Each animal was tested individually. Behavioral tests were performed during light cycle and the observer was blinded to the experimental conditions.

Neurological Deficit Scores Neurological scores evaluations were applied to verify that the MCAO was successful and assess neural function. An eight-point behavioral rating scale was used as described previously (Liu et al. 2006).

Foot-Fault-Placing Assessments The foot-fault-placing apparatus is consisted of an elevated (100 cm) grid surface (106 cm × 110 cm), with a square opening of 9 cm^2 and grid wire diameter of 1.0 mm) that is connected to platforms at each end. In each trial, the animal was encouraged, using either noise or prodding, to traverse the grid surface for 2 min. Occasionally, animals would place a forelimb inaccurately and the limb would fall through one of the openings in the grid. These mistakes were considered foot-faults. The rate of contralateral forelimb foot-faults made per meter over the 2 min period was calculated (Liu et al. 2017).

Western Blot

Immediately after the final behavioral test, rats were anesthetized with an overdose of chloral hydrate (60 mg/kg, i.p.) and perfused with ice-cold PBS. Ischemic (I) and nonischemic (NI) hippocampal tissues were collected for Western Blot as described previously (Yang et al. 2018). Protein concentrations were determined using a BCA protein assay kit (Beyotime, Haimen, Jiangsu, China). After homogenate aliquots (30 μg of total protein) were boiled, they were electrophoresed in 10% SDS-PAGE acrylamide gels, transferred onto 0.45 μm PVDF membranes (Millipore, Billenca, MA, USA) and incubated for 2 h in PBS-T (phosphate buffer saline and 0.1% Tween-20) containing 5% nonfat milk. Membranes were then incubated overnight at 4 °C

with primary antibodies against GFAP (RRID:AB_2109815, 1:2000, EMD Millipore Corporation, Temecula, CA, USA, Cat# MAB360), Cx43 (RRID: AB_2294590, 1:1000, Cell Signaling Technology, Boston, MA, USA, Cat#3512S), SYP (RRID: AB_10698743,1: 4000, Cell Signaling Technology, Boston, MA, USA, Cat#5461S), CRTC1 (RRID: AB_10563847,1: 1000, Abcam, Cat#ab92477), p-CRTC1 (1:500, Cell Signaling Technology, Boston, MA, USA, Cat#3359S), HMGB1(1:20,000, Abcam, Cat#ab79823), β-actin (1:5000, Boster, Wuhan, Hubei, China), and GAPDH (1:5000, Cat# BM1623, Boster, Wuhan, Hubei, China). After washing with PBS-T, the membranes were incubated for 2 h at room temperature with horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse secondary antibody (Boster, Wuhan, Hubei, China). The membranes were developed using a Super Signal West Pico HRP substrate kit (Thermo Fisher, Rockford, IL, USA) and photographed. Protein band intensities were quantitated after normalization to beta-actin. Each measurement was repeated in triplicate.

Immunofluorescence for GFAP, Cx43, MBP, HMGB1

Immediately after the final behavioral tests, rats were perfused with PBS followed by 4% PFA. The 20-μm-thick cryosection was fixed with 4% PFA for immunofluorescence analysis as described previously (Han et al. 2013; Wang et al. 2017a). Sections were incubated overnight with primary antibody against GFAP (RRID: AB_2109815, 1:1000, EMD Millipore Corporation, Temecula, CA, USA, Cat# MAB360), Cx43 (RRID: AB-2294590, 1:200, Cell Signaling Technology, Boston, MA, USA, Cat#3512S), MBP (RRID: AB_1141521,1: 400, Abcam, Cat# ab40390), and HMGB1 (1: 800, Abcam, Cat# ab79823) followed by incubation with CY3 (anti-mouse, RRID: AB_2338251, 1:800) and 488-conjugated (anti-rabbit, RRID: AB_143165, 1:1000) secondary antibody for 1.5 h at room temperature, respectively. Immunofluorescence was visualized under LSM 700 microscope (Zeiss), and images were taken from the hippocampus.

Statistical Analysis

The sample size was predetermined by analyzing our pre-experimental data with PASS (power analysis and sample size) software and based on prior experience (Shen et al. 2018; Shu et al. 2015). Two-way ANOVA with repeated measures was used for the escape latency, mean speed in the Morris water maze task, neurological scores, and the foot-fault-placing assessment. The factors were the treatment administered and testing time (days). A value of less than 0.05 was accepted as statistical significance. Allowing a type I error of 5%, $\alpha = 0.05$ with power of 90.9%, we calculated a size of 10 animals per group. One-way ANOVA was used to assess the Western blot results. All statistical analysis was conducted in SPSS 18.0 statistical programs (SPSS, Chicaco, IL, USA). Data in the text and figures are expressed as mean ± SEM.

Results

Treatment with FC Alleviates Ischemic Stroke-Induced GFAP Upregulation

The glial fibrillary acidic protein (GFAP) is a marker of reactive astrocytes (Hayakawa et al. 2010a), which play an important role in MCAO-induced memory impairment (Xie et al. 2011). As Ischemia–reperfusion has been shown to induce a significant increase in GFAP expression, we first examined the levels of this protein in the hippocampus of the MCAO rats. Western blot results showed that there was a significant increase of GFAP in the hippocampus of the MCAO rats ($F(1, 6) = 6.661$, $P = 0.042$) and treatment with FC minimized the GFAP upregulation ($F(1, 6) = 6.273$, $P = 0.046$, Fig. 2a). In addition, the immunofluorescence results showed that, while ischemic stroke had triggered a significant increase of reactive astrocytes in CA1 (Fig. 2b) and CA3 of hippocampus (Fig. 2c), FC treatment had significantly inhibited this increase.

Effect of FC on Stroke-Induced Memory and Motor Impairment

Ischemic stroke has been shown to impair hippocampus-dependent memory impairment assessed by the Morris water maze (Bingham et al. 2012; Bouet et al. 2007; Xie et al. 2011). Using the same approach, we investigated the effect of FC on stroke-induced memory impairment in the MCAO rats. Our results showed that FC treatment improved the spatial learning ability in the MCAO rats. As shown in Fig. 3b, the MCAO rats located the hidden platform over shorter durations during the acquisition trials when treated with FC. Two-way repeated ANNOVA revealed a significant difference in the escape latency between the vehicle and FC groups ($F(1, 19) = 7.807$, $P = 0.012$, Fig. 3b), and there was no significant difference in the average speed of the two groups ($F(1, 19) = 0.001$, $P = 0.986$, Fig. 3c).

Memory assessment was done using a probe trial test on the seventh day where the platform was removed and each rat was allowed to swim freely for 60 s. During this trial, the time spent by each rat within the target quadrant and latency to first entering the target quadrant were recorded. One-way ANOVA indicated that the time of first entry to the target quadrant during the probe trial was decreased significantly for the FC treatment group compared to the vehicle group ($F(1, 19) = 8.654$, $P = 0.008$, Fig. 3d). However, there was no

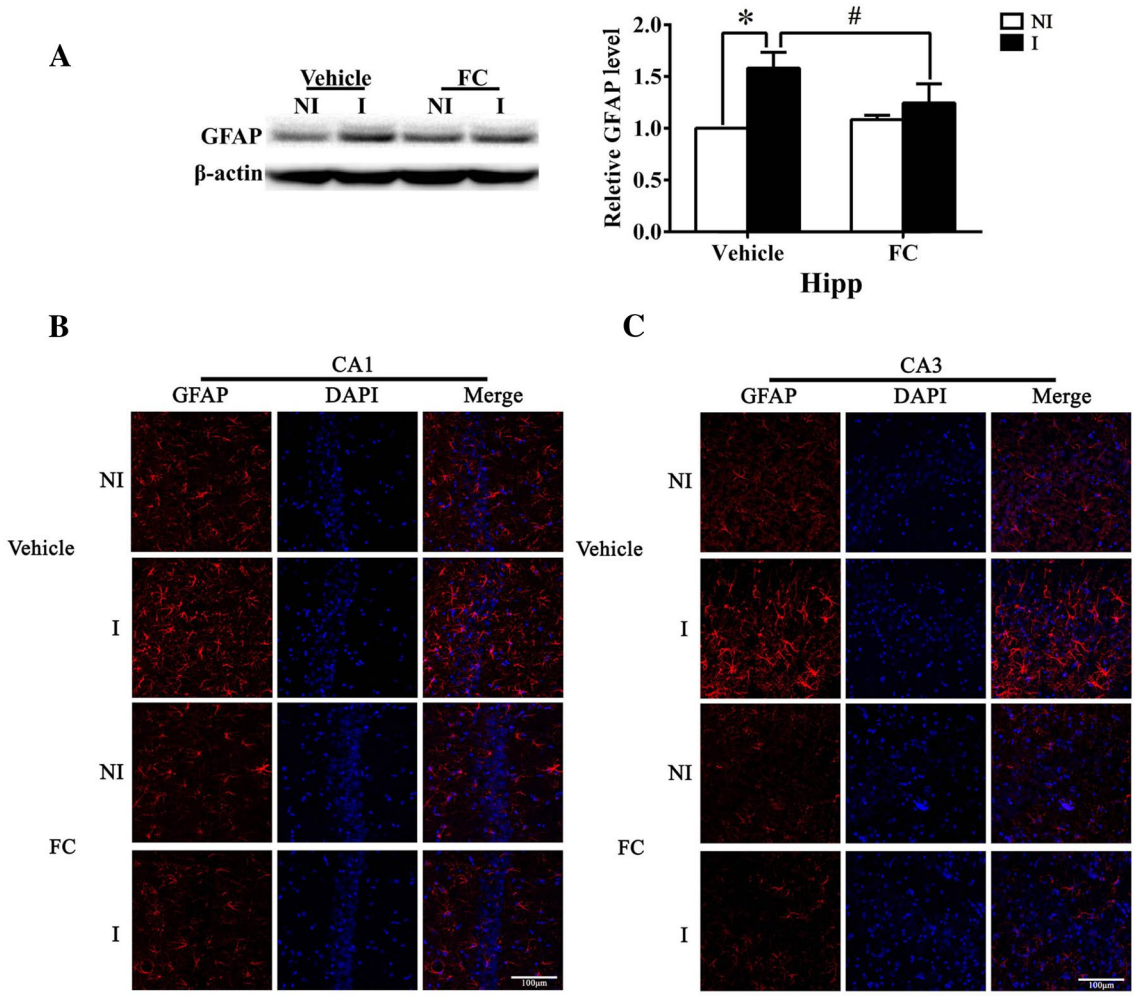

Fig. 2 Effect of FC treatment on stroke-induced GFAP expression in hippocampus after 90-min MCAO and 7-day reperfusion. Western blot and immunofluorescence were used to detect the GFAP expression and spatial distribution in the nonischemic (NI) and ischemic (I) hemispheric tissue. Representative western blot revealed that MCAO induced a significant increase in GFAP protein level in hippocampus (A, left panel) and the relative band intensities of GFAP were quantitated (**a**, right panel). FC treatment significantly prevented this increase ($^{\#}P<0.05$ versus vehicle group, n = 4/group). Representative photomicrographs of fluorescent staining of GFAP in CA1 (**b**) and CA3 (**c**) in hippocampus. MCAO induced a significant increase GFAP in the CA1 (**b**) and CA3 (**c**) and FC treatment significantly prevented this increase. N = 3/group, scale bar = 100 μm

significant effect on the time spent within the target quadrant ($F(1, 19) = 0.090$, $P = 0.768$, Fig. 3e).

Both the neurological scores and the foot-fault test have been used to evaluate motor function after ischemic stroke (Liu et al. 2017). Here, we did the same behavioral tests. Compared with the vehicle group, FC treatment significantly improved neurological scores ($F(1, 19) = 6.157$, $P = 0.023$, Fig. 3f). Assessment of motor function by using the foot-fault-placing test confirmed that ischemic stroke had induced severe functional deficits of the left forelimb. Our results showed that FC treatment markedly improved these behavioral deficits ($F(1, 19) = 14.241$, $P = 0.001$, Fig. 3g) and suggest that FC treatment also protects against ischemic stroke-induced motor dysfunction.

Treatment with FC Decreases Ischemic Stroke-Induced Cx43 Upregulation

Astroglial gap junction communication contributes to MCAO-induced remote hippocampal damage and cognitive impairment (Xie et al. 2011), in which Cx43 plays an important role (Xie et al. 2011). As shown in Fig. 4, ischemic stroke significantly upregulated Cx43 ($F(1, 8) = 17.425$, $P = 0.003$, $^{**}P < 0.01$, Fig. 4a) and FC treatment significantly prevented this increase ($F(1, 8) = 8.576$, $P = 0.019$. $^{\#}P < 0.05$, Fig. 4b).

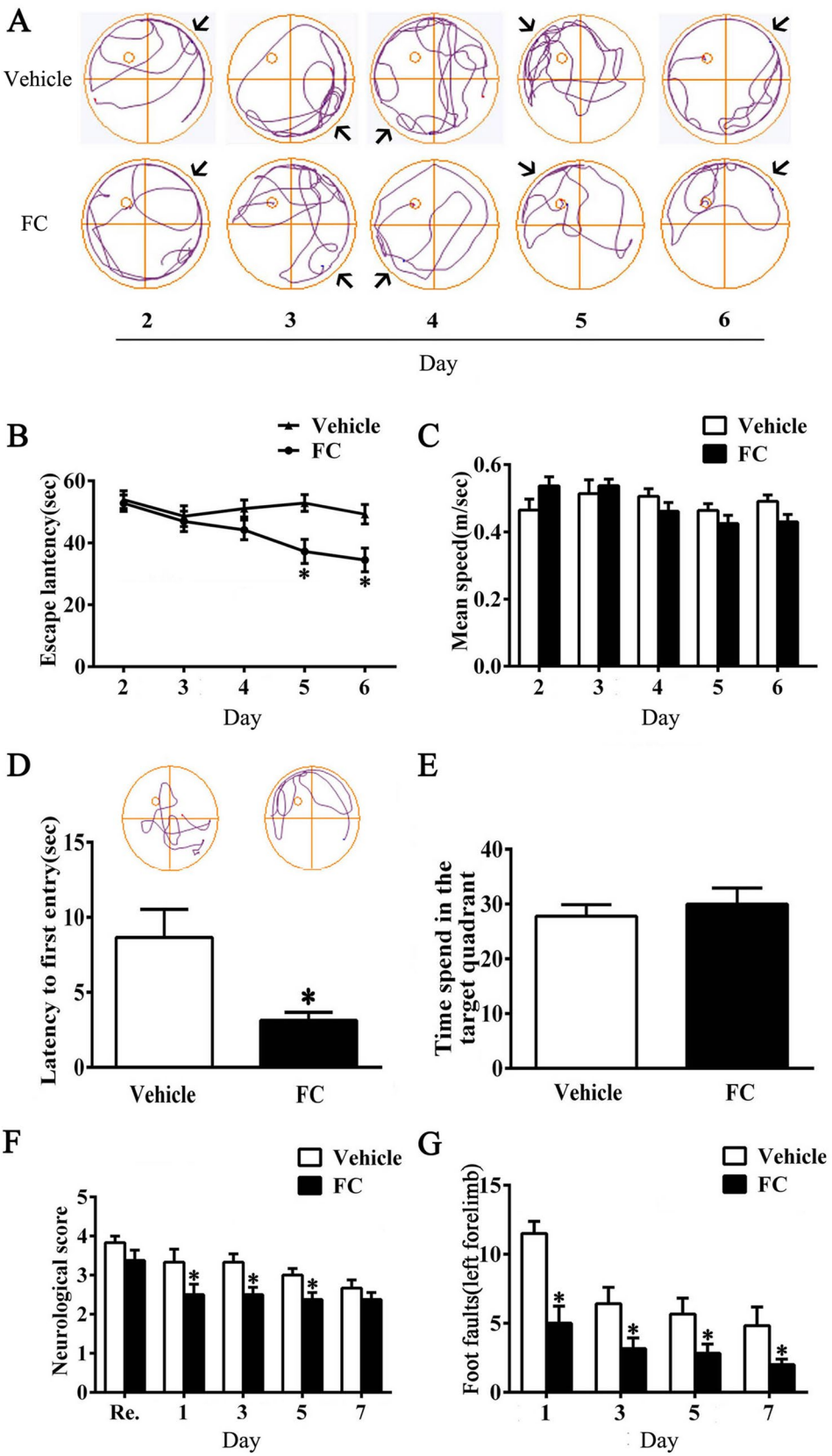

Fig. 3 Effect of FC treatment on stroke-induced learning, memory and motor impairment after 90-minute MCAO and 7-day reperfusion. Morris water maze was used to detect learning (**a**–**c**) and memory (**d**, **e**). Diagram shows the track of motion (**a**). Treatment with FC significantly decreased the escape latency ($P < 0.01$ versus vehicle group, **b**), but had no effect on mean speed ($P > 0.05$ versus vehicle group, **c**). Treatment with FC significantly decreased the latency to first entry ($P < 0.05$, **d**), but not the duration in the target quadrant ($P > 0.05$, **e**). Treatment with FC significantly decreased neurological scores ($P < 0.01$, **f**) and foot-faults ($P < 0.01$, **g**). N = 10 for vehicle group and n = 11 for FC group

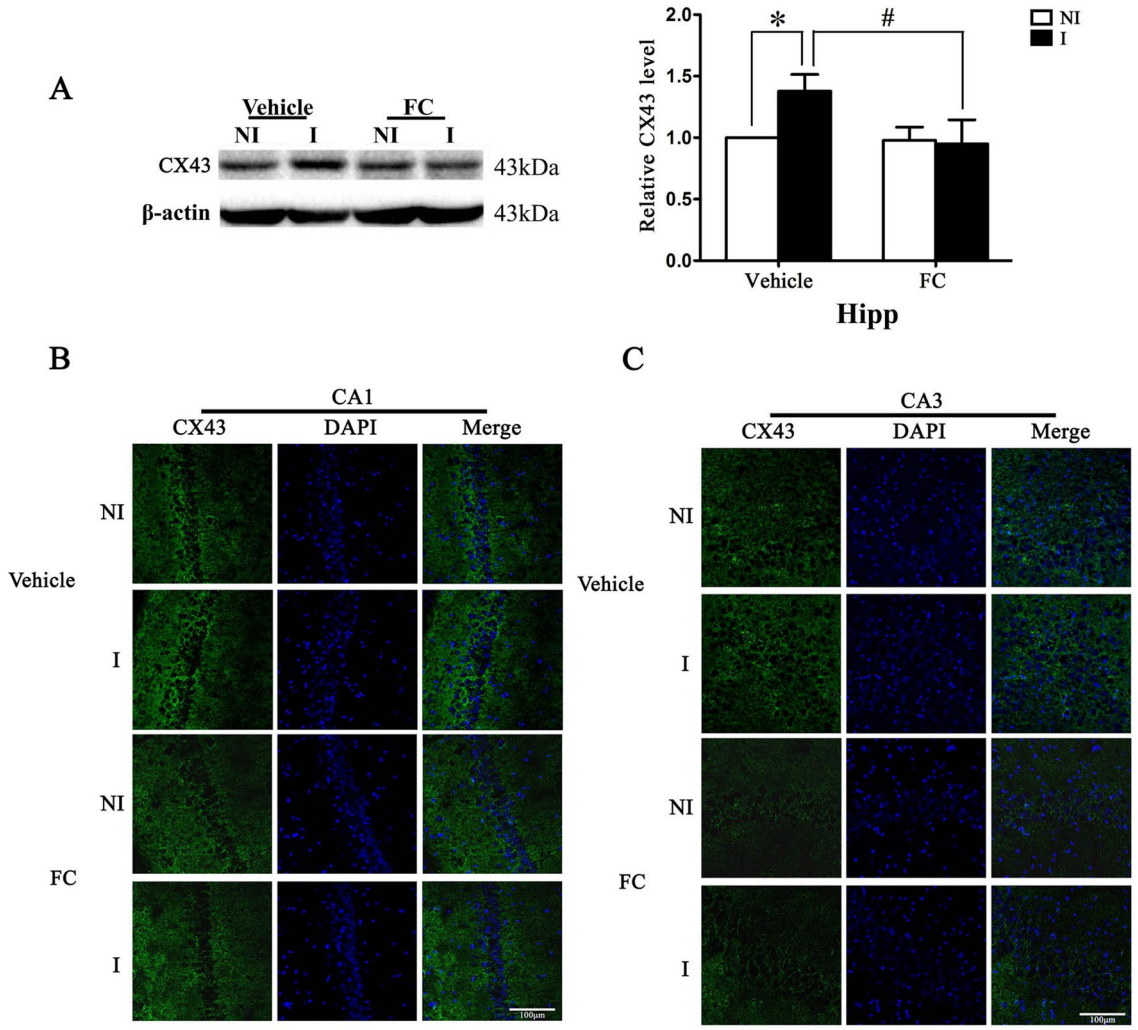

Fig. 4 Effect of FC treatment on stroke-induced Cx43 expression change in hippocampus after 90-minute MCAO and 7-day reperfusion. Representative western blot revealed that MCAO induced a significant increase in Cx43 protein level in hippocampus (**a**, left panel). The relative band intensities of Cx43 were quantitated (**a**, right panel). *$P<0.05$ versus NI hemisphere in vehicle group. Treatment with FC significantly prevented this increase.#$P<0.05$ versus vehicle group, n = 5/group. Representative photomicrographs of fluorescent staining of Cx43 in CA1 (**b**) and CA3 (**c**) in hippocampus. MCAO induced a significant decrease Cx43 in the CA1 (B) and CA3 (**c**). Treatment with FC significantly prevented this decrease. N = 3/group, scale bar = 100 μm

Treatment with FC Reduces Ischemic Stroke-Induced Decrease of SYP, CRTC1, and MBP Levels in Hippocampus

As SYP is a marker of synaptic plasticity (Shen et al. 2018; Smith et al. 2000) and stroke-induced memory damage is accompanied by the downregulation of SYP (Fonteles et al. 2016), we next explored SYP expression in the hippocampus of MCAO rats. Western blot analysis showed that ischemic stroke significantly downregulated SYP ($F(1, 6) = 6.280$, $P = 0.046$) and FC treatment significantly reduce this downregulation ($F(1, 6) = 6.152$, $P = 0.048$, Fig. 5a).

CRTC1 has been shown to play a role in neuronal survival after ischemic stroke (Sasaki et al. 2011) and memory protection (Nonaka et al. 2014; Parra-Damas et al. 2016). In addition, the phosphorylation status of CRTC1 determines the function of CRTC1 in ischemia (Sasaki et al. 2011) and associative memory (Espana et al. 2010; Parra-Damas et al. 2016; Xue et al. 2015). We assessed the effect of FC on CRTC1 expression in the MCAO rats. Our results showed that ischemic stroke leads to a significant decrease in protein level of CRTC1 ($F(1, 6) = 5.937$, $P = 0.031$) and FC treatment can significantly inhibit this decrease ($F(1, 6) = 5.574$, $P = 0.036$, Fig. 5b). Although there is a trend, ischemic stroke did not significantly increase the level of p-CRTC1, suggesting that phosphorylation of CRTC1 did not play a role in the motor and cognition impairment to observed 7 days after reperfusion.

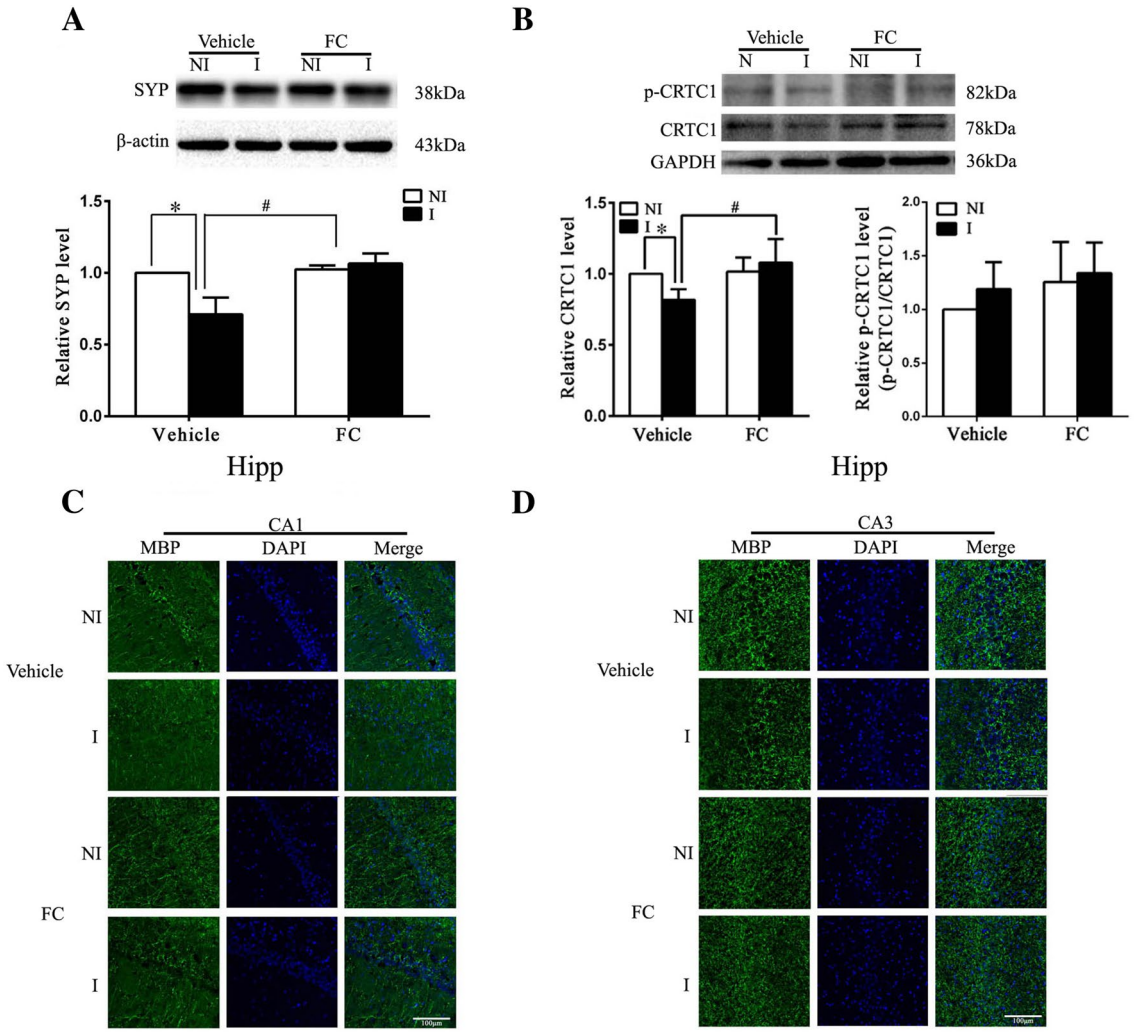

Fig. 5 Effect of FC treatment on stroke-induced SYP, CRTC1, p-CRTC1 and MBP expression in hippocampus after ischemic stroke. Representative western blot revealed protein level of SYP (**a**, upper panel) and p-CRTC1, CRTC1 (**b**, upper panel) in hippocampus. The relative band intensities of SYP (**a**, bottom panel) and CRTC1 (**b**, bottom panel) were quantitated. Ischemic stroke induced a significant decrease of SYP and CRTC1, but no significant change of p-CRTC1. Treatment with FC significantly prevented this decrease of SYP and CRTC1. $*P<0.05$ versus NI hemisphere in vehicle group.$\#P<0.05$ versus vehicle group, n = 4/group for SYP, n = 4/group for CRTC1. Representative photomicrographs of fluorescent staining of MBP from CA1 and CA3 in hippocampus. Ischemic stroke induced a significant decrease MBP in CA1 (**c**) and CA3 (**d**). FC treatment significantly prevented this decrease. N = 3/group, scale bar = 100 μm

In addition to gray matter injury, cerebral ischemia and reperfusion can also damage the white matter, which contributes to memory loss in ischemic stroke (Wang et al. 2017b). Our results showed that ischemic stroke resulted in a significant decrease of myelin basic protein (MBP) in CA1 and CA3 of hippocampus and FC treatment significantly inhibited this decrease (Fig. 5c).

Treatment with FC Reduces Ischemic Stroke-Induced Decrease of HMGB1 Levels in Hippocampus

High-mobility group-box-1 (HMGB1) is a novel cytokine-like mediator linking acute neuronal death and delayed neuroinflammation and memory deficit after ischemic stroke (Das et al. 2017; Kim et al. 2006; Lai et al. 2018). Here, we investigated the expression of HMGB1 in hippocampus and its colocalization with astrocytes in CA1 and CA3 of hippocampus after ischemic stroke. Our results showed that ischemic stroke induced a significant decrease of HMGB1 in the hippocampus and FC treatment inhibited this decrease (Fig. 6a). Immunofluorescence results showed that HMGB1 in CA1 (Fig. 6b) and CA3 (Fig. 6c) did not have a good colocalization with GFAP, suggesting that HMBG1 might have been released from affected astrocytes during cerebral ischemia and reperfusion.

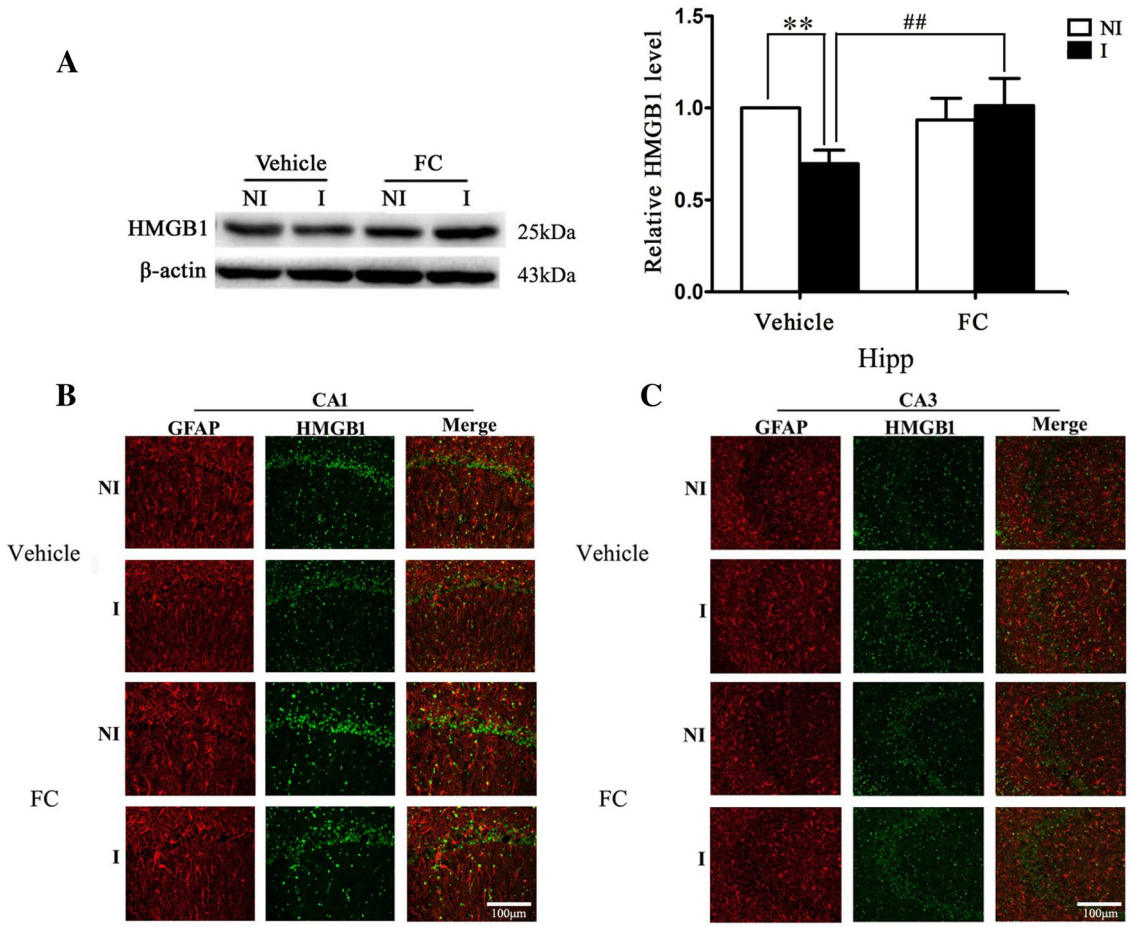

Fig. 6 Effect of FC treatment on stroke-induced HMGB1 expression in hippocampus after ischemic stroke. Representative western blot revealed protein level of HMGB1 (**a**, left panel) in hippocampus. The relative band intensities of HMGB1 (**a**, right panel) were quantitated. Ischemic stroke induced a significant decrease of HMGB1 in hippocampus and treatment with FC significantly prevented this decrease of HMGB1. $^{*}P<0.05$ versus NI hemisphere in vehicle group. $^{\#}P<0.05$ versus vehicle group, n = 4/group for HMGB1. Representative photomicrographs of fluorescent staining of HMGB1 and GFAP from CA1 and CA3 in hippocampus. Ischemic stroke induced a significant increase of GFAP and a significant decrease of HMGB1 in CA1 (**b**) and CA3 (**c**). FC treatment significantly prevented this change. HMGB1 did not have a good colocalization with GFAP. N = 3/group, scale bar = 100 μm

Discussion

Cognitive deficit is a key problem for patients with compromised blood supply to the MCA (Bakker et al. 2000). However, available therapeutics are limited and the mechanism underlying ischemic stroke-induced memory impairment is not well understood. In the present study, our results showed that: (1) memory disruption due to MCAO was accompanied by an increase of reactive astrocytes and downregulation of CRTC1 and SYP and that inhibiting reactive astrocytes may be a strategy to alleviate stroke-induced memory impairment, (2) since HMGB1 can mediate both detrimental and beneficial effects on plasticity and recovery in the neurovascular unit, application of FC to reduce ischemic stroke-induced memory impairment should be very careful in order to optimize the inhibition of HMGB1 during acute stage of injury without interfering with beneficial endogenous mechanisms of neurovascular remodeling, and (3) CRTC1 is a potential therapeutic target for improving ischemic stroke-induced memory impairment.

Consistent with a previous study showing that transient focal cerebral ischemia induced a deficit in long-term cognitive function in an experimental ischemic stroke model (Li et al. 2013), our results showed that MCAO induced a significant decrease of the expression of memory-related proteins SYP and CRTC1 in the hippocampus. Therefore, in addition to frontal and/or temporal cortex damage, hippocampus damage also played an important role in cognition impairment in stroke patients, despite that the region is not supplied by the MCA (Xie et al. 2011).

Astrocytes have the capability to modulate synaptic transmission (Pena-Ortega et al. 2016) and memory (Adamsky

and Goshen 2018) via secreting gliotransmitters and neurotrophic and neuroinflammatory factors. For example, inhibition of glial cells by FC treatment has been shown to decrease the expression of several memory-related proteins (Shang et al. 2015) and alleviate working memory impairment in rats (Wang et al. 2009), as well as ameliorating memory deficits in a color discrimination task performed on chicks (Gibbs and Bowser 2009). Our results showed that inhibition of reactive astrocytes by FC alleviated the ischemic stroke-induced memory impairment, indicating that astrocytes played dual roles in regulating memory under different conditions.

Reactive astrocytes have biphasic effects on brain injury after ischemic stroke. Early detrimental effects at the acute stage of stroke result from excessive release of inflammatory mediators, which aggravate ischemic neuronal death (Liu and Chopp 2016). Later effects are beneficial and promote recovery (Ding 2014; Hosoi et al. 2006). Here, we administered FC immediately before reperfusion to inhibit the detrimental effect of reactive astrocytes during the acute stage and showed that FC could improve ischemia–reperfusion-induced memory impairment. Due to the biphasic effects of active astrocytes in ischemic stroke, achieving a beneficial effect hinges on the timing of FC administration. A previous study using similar MCAO model reported that when FC was administered 5 days after cerebral ischemia, functional recovery was impaired (Hayakawa et al. 2010a). It is worth pointing out that the selected dose of FC (1 nmol) in this study has been previously shown to inhibit reactive astrocytes after stroke without affecting GFAP expression in normal mice (Hayakawa et al. 2010a; Wang et al. 2018). In addition, 6 h or 30 h after the lateral ventricle injection of 1 nmol FC, there was no alteration in the baseline behavior, as reflected by the tail suspension test, forced swimming test, and the number of crossings and rearings (Wang et al. 2018). Administration of 1 nmol FC also showed no brain cell damage in normal rats (Hosoi et al. 2004). Therefore, 1 nmol FC is not expected to have a detrimental effect on normal astrocytes or memory.

We have shown that FC treatment can alleviate stroke-induced memory impairment at a dose of 1 nmol which has been used to investigate the role of astrocytes in the central nervous system (Paulsen et al. 1987). This dose of FC has been shown to be preferentially taken up by astrocytes, reversibly inhibit the tricarboxylic acid cycle in astrocytes (Shang et al. 2015), and selectively induced reversible ultrastructural alterations in glial cells (especially astrocytes) without affecting the neurons in adult male rats. Therefore, at this dose, the change in level of memory-related proteins should be affected by inactivating astrocytes, but not neurons.

Another consideration in using FC is the possibility of glutamate-induced brain damage. A dose of 1 mM FC can induce a fast and marked increase of extracellular nonsynaptic glutamate (Rodriguez Diaz et al. 2005), with 0.5 µM FC causing less than 30% reduction of glutamate ($P > 0.05$) (Swanson and Graham 1994). However, an earlier study demonstrated that, while doses above 2 nmol FC can induce irreversible damage to glial cell ultrastructure and neurons, an intracerebral injection of 1 nmol results in a large loss of glutamine and glutathione, but only smaller changes in glutamate, aspartate and GABA (Paulsen et al. 1987). Therefore, a concentration of 1 nmol FC should avoid glutamate-induced toxicity and brain damage.

Cx43 hemichannels are necessary for fear memory consolidation (Stehberg et al. 2012) and have been shown to play a role in MCAO-induced remote hippocampal damage and cognitive impairment (Xie et al. 2011). Our results showed that stroke-induced memory impairment was accompanied by a significant increase in Cx43. Therefore, manipulating interastrocytic communication via the gap junction channels or inhibiting reactive astrocytes could be a strategy to reduce ischemia-induced memory impairment.

Our results showed that the level of HMGB1 was significantly decreased after ischemia and 7-day reperfusion. This is not consistent with a previous study showing that HMGB1 expression was increased in reactive astrocytes after white matter injury (Hayakawa et al. 2013) and focal cerebral ischemia (Hayakawa et al. 2012). In addition, there is also evidence demonstrating that the level of HMGB1 in the brain was significantly decreased 3 days post-reperfusion, but then upregulated 14 days post-reperfusion (Hayakawa et al. 2012), suggesting a biphasic actions of HMGB1 signaling in inflammation and recovery after stroke (Hayakawa et al. 2010b). Accumulating evidence supports the hypothesis that inflammation after stroke can be both detrimental and beneficial, depending on the cellular situations involved (Stoll et al. 2002). As a nuclear weapon in the immune arsenal, HMGB1 is normally presented in the nucleus (Lotze and Tracey 2005) and released extracellularly from many types of cells upon different stresses, including oxygen glucose deprivation (Li et al. 2014), MCAO (Halder and Ueda 2018) and lipopolysaccharide (Xie et al. 2016). During the acute phase of post-stroke, HMGB1 promotes necrosis and influx of damaging inflammatory cells (Qiu et al. 2010) and worsens ischemic neurodegeneration, as observed after release in vitro and in vivo (Faraco et al. 2007). However, during the delayed phase of post-stroke, HMGB1 can mediate beneficial plasticity and recovery in many cells of the neurovascular unit (Hayakawa et al. 2012). Our results showed that FC treatment inhibited HMGB1 decrease following ischemic stroke. Since HMGB1 can mediate both detrimental and beneficial responses, depending on the stages after cerebral ischemia–reperfusion (Hayakawa et al. 2010b), careful application of FC is needed to reduce ischemic stroke-induced memory impairment and

optimize the inhibition during the acute stage of stroke without interfering with beneficial endogenous mechanisms of neurovascular remodeling. FC treatment should be avoided when HMGB1 is upregulated during the recovery stage after ischemic stroke.

The phosphorylation status of CRTC1 has been shown to play an important role in neuronal survival after ischemia (Sasaki et al. 2011) and associative memory (Nonaka et al. 2014; Parra-Damas et al. 2016). In the current study, our results showed that there was a significant decrease of the level of CRTC1 but not p-CRTC1 following ischemia and reperfusion. It is known that in unstimulated cells, CRTC1 is phosphorylated and binds to 14-3-3 proteins in the cytoplasm (Sasaki et al. 2011). During oxygen glucose deprivation, p-CRTC1 remains in the cytoplasm, but is quickly dephosphorylated and translocated into the nucleus after reoxygenation (Xue et al. 2015). In our study, there was a 7-day reperfusion with results suggesting that CRTC1 may either be translocated to the cytoplasm or be degraded during prolonged reperfusion.

Several studies have described the occurrence of cognitive and motor impairment following MCAO (Bingham et al. 2012; Bouet et al. 2007). However, the deficits observed for the Morris water maze task have often been confounded by disturbed sensorimotor functions (Cain and Boon 2003). MCAO has been shown to induce substantial damage to the striatum, which is known to affect performance in the Morris water maze by impairing motor function, response flexibility and procedural aspects of the task (D'Hooge and De Deyn 2001). The MCAO-induced motor impairments may account for the decreased performance in the Morris water maze. However, in our study, the swimming speed in the Morris water maze was similar, indicating that the deficit of the rat's performance in water maze is attributable to an impaired memory function and is not a result of confounding motor disturbances.

Conclusions

Our results demonstrate that inhibition of reactive astrocytes after ischemia and reperfusion significantly improved stroke-induced memory impairment through upregulating CRTC1 and SYP in the hippocampus. Hence, CRTC1 may be a potential therapeutic target for the development of therapeutics to improve ischemic stroke-induced memory impairment.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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