

Electroacupuncture promotes neural stem cell proliferation and neurogenesis in the dentate gyrus of rats following stroke via upregulation of Notch1 expression

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Abstract. Neural stem cells (NSCs) are important in rehabilitation following stroke. Electroacupuncture (EA) treatment has been observed to promote the recovery of neurological functions subsequent to stroke, however, the effects of EA on the proliferation and differentiation of NSCs and its potential mechanisms remain to be elucidated. In the present study, rats, in which a stroke was induced through middle cerebral artery occlusion (MCAO), were treated with EA or control manipulation for 21 days. The modified Neurological Severity score and Morris water maze tests were used to assess the neurological functions of the rats. Bromodeoxyuridine (BrdU)/glial fibrillary acidic protein (GFAP) or BrdU/neuronal marker (NeuN) double immunofluorescence staining were used to examine the proliferation and differentiation of the NSCs. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) and western blot analyses were performed to detect the expression levels of Notch1 and Hes1 in the dentate gyrus (DG) of the hippocampus of rats following MCAO. The results demonstrated that EA treatment significantly improved the neurological functional recovery of rats following stroke. A significant increase was observed in the number of BrdU⁺/GFAP⁺ and BrdU⁺/NeuN⁺ cells in the DG area in the EA-treated rats compared with that of the control group. RT-qPCR analysis revealed that EA treatment significantly increased the expression levels of Notch1 and Hes1, which may account for the enhanced proliferation and differentiation of NSCs. In conclusion, to the best of our knowledge, the present study was the first to demonstrate that EA treatment promoted

NSC proliferation and neurogenesis in the DG area through the upregulation of Notch signaling following a stroke; therefore, EA may be a useful novel therapeutic strategy in future stroke treatment.

Introduction

Cerebrovascular diseases are one of the prominent causes of mortality and permanent disability worldwide, the occurrence of which is predicted to further increase due to increasing life expectancies (1). The pharmaceutical industry has developed numerous potential neuroprotective drugs for the treatment of stroke, which have been observed to decrease cerebral damage and enhance the recovery of motor and cognitive abilities following ischemic insult in preclinical studies using animal models. However, these results have not been reproduced in phase III clinical trials and none of the assessed compounds led to the successful post-ischemic treatment of patients (2,3). Therefore, further investigation in this field is required in order to examine novel therapeutic strategies, which may ameliorate the recovery of post-ischemic patients.

Stem cells, with self-renewal and pluripotent differentiation potentials, are a promising source for tissue regeneration, including neural regeneration (4). Neural stem cells (NSCs) have been identified in the subventricular zone (SVZ) and dentate gyrus (DG) of adult brain and these NSCs proliferate and generate neurons throughout the adult life (5-9). It is now generally considered that cerebral ischemia results in the expansion of endogenous NSCs, as has been previously demonstrated using various ischemia models, including transient global ischemia (10), transient focal ischemia (11,12) and permanent focal ischemia (13). Stroke, induced by middle cerebral artery occlusion (MCAO), leads to the increased proliferation of NSCs in SVZ and the formation of neuroblasts, which migrate towards the damaged striatum, where they differentiate into mature striatal neurons (12). In addition to replacing lost neurons, NSCs appear to contribute to regeneration following stroke by providing neuroprotection and trophic support, which reduces neuroinflammation and induces remodeling (14,15). However, the spontaneous proliferation and differentiation of endogenous NSCs stimulated by stroke is often insufficient to

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overcome the neural damage (14). Therefore, it is important to develop novel strategies to enhance neurogenesis in order to improve rehabilitation following stroke.

Acupuncture has been used in traditional Chinese medicine for >3,000 years as a treatment for numerous diseases, including stroke. The use of acupuncture has become more prevalent in Western countries, and has been suggested to have potential therapeutic benefits for the treatment of cerebral ischemia-associated disorders (16,17). A previous meta-analysis confirmed that acupuncture may be effective in the treatment of post stroke rehabilitation (18). Electroacupuncture (EA) is a form of acupuncture which involves a small electric current between pairs of acupuncture needles. EA is considered to augment the use of regular acupuncture (19) and several clinical trials have demonstrated that EA treatment improves limb function following stroke (20,21). Studies involving murine models have demonstrated that EA treatment promotes neurological functional recovery through a series of mechanisms (22). Ke *et al.* (23) reported that EA treatment upregulates the expression of brain-derived neurotrophic factor (BDNF), an important peptide, which supports the growth and maintenance of brain neurogenesis and facilitates motor recovery following stroke (23). However, whether EA treatment promotes neurogenesis through stimulating the proliferation of NSCs remains to be elucidated.

The present study aimed to examine the effects of EA treatment on the proliferation and differentiation of NSCs in the DG area of the adult rat brain following stroke, and to assess the impact of EA treatment on the expression of Notch1, an important molecule maintaining the proliferation of stem cells.

Materials and methods

Animal care. The experimental procedure used in the present study was approved by the Ethics Committee for Animal Experimentation of Sun Yat-sen University (Guangzhou, China) and was performed according to the Guidelines for Animal Experimentation of Sun Yat-sen University. All efforts were made to minimize animal suffering and the number of animals used. Male specific pathogen-free (SPF) Sprague-Dawley rats (n=160), weighing 310±30 g, were provided by the Experimental Animal Center of Sun Yat-sen University and housed under controlled conditions with a 12 hour light/dark cycle, temperature of 24±1°C and humidity of 50±5% for at least 1 week prior to drug treatment or surgery. The rats were allowed free access to a standard rodent diet and tap water.

Experimental stroke in rats. The rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (3%; Sigma-Aldrich, St. Louis, MO, USA) at a dose of 30 mg/kg. Core body temperature was monitored using a rectal probe (Zhengzhou Haorunqi Electronic Sci-Tech Co., Ltd., Henan, China) and were maintained at 37±0.5°C using a heating lamp and a heating pad (Zhengzhou Haorunqi Electronic Sci-Tech Co., Ltd.). The arterial blood gases, pH, PaO₂, PaCO₂ and blood pressure were closely monitored via catheterizing the right femoral artery using a RM-6240BD physiological monitoring system (Chengdu Instrument Factory, Sichuan, China). MCAO

was achieved using the Intraluminal Filament method, as previously described (24). Following exposure of the external carotid artery, the internal carotid artery (ICA) and the pterygopalatine artery of the ICA, a piece of monofilament nylon suture (diameter, 280 μm; Shandong Biotek, Beijing, China), with its tip rounded by gentle heating (diameter, 380±20 μm), was introduced via the lumen of the left external carotid artery stump and left ICA to embed into the left anterior cerebral artery, resulting in the occlusion of the right middle cerebral artery at its origin. Following surgery, the rats were transferred to their cage, in which the temperature was maintained at 37°C until the animals were completely conscious. Sham-operated rats (S group) were manipulated in the same way, however the ICA was not occluded.

Measurement of neurological deficits. Subsequent to the regaining consciousness, neurological deficits were preliminarily determined using a modified Bederson's scoring system (25,26) as follows: 0, no observable deficit; 1, forelimb flexion; 2, forelimb flexion with decreased resistance to lateral push; 3, unidirectional circling; 4, unidirectional circling with decreased level of consciousness. Rats with a score of 2-3 were selected for use in the subsequent experiments.

Electroacupuncture treatment. A total of 128 eligible rats with successful MCAO were randomly assigned into either the electroacupuncture treatment group (EA group) or control group (M group). In the EA group rats, stainless acupuncture needles (diameter of 0.3 mm) were inserted into the acupuncture points Baihui (DU20) and Shuigou (DU26) at a depth of 2-3 mm. Stimulation was then generated by the EA apparatus (Model G6805; SMIF, Shanghai, China), and the stimulation parameters were set as follows: Disperse wave, 4 and 20 Hz; electric current, 1-2 mA; voltage, 2-4 mV; 15 min of each treatment; once a day. EA treatment was performed at 72 h following surgery and continued until the animals were sacrificed for tissue preparation. The rats in the M group were subjected to the same manipulation procedure without any electric stimulation.

Behavioral assessment. Behavioral assessments of the rats in the two groups were performed by an investigator in a blinded-manner. The assessments consisted of the modified Neurological Severity Score (mNSS) and Morris water maze. All measurements were completed in a dedicated behavioral investigation facility during this interval in order to minimize the environmental impact associated with transfer between home cage and the test arenas.

The mNSS test was performed at 3, 7, 14 and 21 days of EA or control treatment. Table I shows the mNSS scores (27). Neurological function was graded on a scale of 0 to 18, with 0 indicating a normal score and 18 indicating the maximal deficit score. The mNSS is a composite of motor, sensory, reflex and balance tests. In the severity scores of injury, 1 score point is awarded for the inability to perform the test or for the lack of a reflex; thus, the higher the score, the more severe the injury (27).

The Morris water maze test was used to measure spatial learning and memory at days 7, 14 and 21 EA or control treatment, as previously described (28). Each rat was placed

individually in a black water tank (136 cm in diameter and 60 cm in height) in a well-lit room and filled with water (50 cm height at 27°C). The tank was visually separated into four quadrants and, in the center of one quadrant, a plexiglass platform (diameter, 10 cm; Zhengzhou Haorunqi Electronic Sci-Tech Co., Ltd.) was hidden 2 cm below the waterline. Numerous extra-maze cues surrounding the maze were fixed at specific locations and were visible to the rats. Following a single habituation trial, the rats were trained over 4 days and received four trials each day. On each trial, the rat had 120 sec to escape to the submerged platform; rats that failed to escape were led to the platform and remained there for 30 sec prior to being removed from the maze and dried off. On the fifth day, a probe trial was performed following removal of the platform. The latency of quadrant search, path length and swim speed were measured by a TSE video tracking system (TSE Systems, Bad Homburg, Germany) interfaced to a computer on days 3, 7, 14 and 21 of treatment.

Immunofluorescence confocal microscopy. In order to track the fate of proliferating cells, the rats were administered with intraperitoneal (i.p.) injections of 75 mg/kg body weight bromodeoxyuridine (BrdU; Sigma-Aldrich) dissolved in phosphate-buffered saline (PBS; Whiga Technology Co., Ltd., Guangzhou, Guangdong, China) twice a day for 5 days prior to sacrifice. At days 3, 7, 14 and 21 of EA or control treatment, rats (n=5 per experiment subgroup per time-point) were sacrificed through deep anesthetization with 45 mg/kg sodium pentobarbital and perfused intracardially with cold PBS and 4% buffered paraformaldehyde. Brains were paraffin-embedded (Whiga Technology Co., Ltd.) and sliced into 5- μ m thick coronal sections, starting at 3 mm posterior to the anterior pole. The sections were then incubated with the mixture of monoclonal mouse antibodies (1:400) for BrdU (cat. no. MCA2060T; Oxford Biotech, Oxford, UK) with polyclonal mouse antibodies (1:400) for neuronal nuclei (NeuN; cat. no. SAB4300883; Sigma-Aldrich) or polyclonal mouse antibodies (1:10,000) for glial fibrillary acidic protein (GFAP; cat. no. SAB1405864; Sigma-Aldrich) overnight at 4°C, followed by a mixture of fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin (Ig)G and cyanine dye (Cy)3-conjugated goat anti-mouse IgG (1:100; Jackson Laboratory, Bar Harbor, ME, USA). The sections were washed in Tris-buffered saline (TBS; 0.2% Triton X-100 in PBS; Whiga Technology Co., Ltd.) and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Confocal images were captured using an LSM710 confocal spectral microscope (magnification, x400; ZEISS, Oberkochen, Germany). Image analysis was performed using Leica QWin software (Leica Microsystems, Wetzlar, Germany). The number of cells showing double immunostaining was estimated by counting cells in five random fields of the peri-necrotic cortex in five non-continuous sections.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR). Following evaluation of neurological deficits, rats (n=6 per experiment group per time-point) were sacrificed with an overdose of sodium pentobarbital at days 3, 7, 14 and 21 of EA or control treatment, as described above. Total RNA was extracted from the perinecrotic cortex using TRIzol

reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA (2 μ g) was then reverse transcribed into first-strand cDNA using M-MLV Reverse Transcriptase (Promega Corp., Madison, WI, USA) according to the manufacturer's instructions. Notch1, Hes1 and GAPDH were amplified by qPCR using the following primers: Notch1, forward 5'-TCGTGCTCCTGTTCTTTGTG-3' and reverse 5'-GGGTTCTCTCCGCTTCTTCT-3'; Hes1, forward 5'-GCTGGAGAAGGCAGACATTC-3' and reverse 5'-GTCACCTCGTTCATGCACTC-3'; and GAPDH, forward 5'-TGCCACTCAGAAGACTGTGG-3' and reverse, 5'-TTCAGCTCTGGGATGACCTT-3' (BGI Tech, Guangdong, China). Gene-specific amplification was performed in an ABI 7500 real-time PCR system (Life Technologies, Carlsbad, CA, USA) using 15 μ l PCR mix containing 0.5 μ l cDNA, 7.5 μ l 2x SYBR Green master mix (Invitrogen Life Technologies) and 200 nM of the appropriate primers. The mix was preheated at 95°C for 30 sec and amplified in 45 cycles of 95°C for 5 sec and 60°C for 34 sec. The resolution curve was measured at 95°C for 15 sec, 60°C for 15 sec and 95°C for 15 sec. The threshold cycle (Ct) value of each sample was calculated and the relative expression of Notch1 and Hes1 mRNA was normalized to the GAPDH value ($2^{-\Delta C_t}$ method) (29).

Statistical analysis. All values are expressed as mean \pm standard deviation. A repeated measurement analysis of variance was performed to analyze differences in the behavioral assessment data and relative mRNA expression levels. Analysis of variance and the Least Significant Difference test were used to further compare the differences at each time-point. SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA) was used for all statistical analyses. P<0.05 was considered to indicate a statistically significant difference between values.

Results

EA treatment improves the functional recovery of rats following stroke. In order to examine the effects of EA treatment on the functional recovery of MCAO rats, the mNSS was used to assess neurological deficits. The mNSS of the rats in the S group were 0 at all time-points. As shown in Fig. 1A, the rats in the EA group had a significantly lower mNSS compared with that in the M group at 14 (5.33 \pm 1.22 and 7.00 \pm 0.87, respectively; P<0.05) and 21 (2.78 \pm 0.67 and 4.78 \pm 0.67, respectively; P<0.05) days of treatment. Compared with those in the M group, rats in EA group only had significant improvement in the motor test score (P<0.05; Fig. 1B) but not in scores of other tests at 14 and 21 days of treatment. The cognitive recovery of MCAO rats was further assessed using the Morris water maze test. As shown in Fig. 1C, rats in the EA group had significantly decreased escape latency compared with those in the M group at 14 (90.63 \pm 2.62 and 98.13 \pm 2.90, respectively; P<0.05) and 21 (84.63 \pm 1.92 and 94.13 \pm 1.46, respectively; P<0.05) days of treatment. These results suggested that EA treatment effectively improved the neurological dysfunction of cerebral ischemia in rats, promoting nerve functional recovery.

EA treatment promotes the proliferation and differentiation of NSCs following stroke. BrdU/GFAP and BrdU/NeuN

Table I. Modified Neurological Severity Score tests and points.

Test	Points
Motor	
Raising rat by tail	3
Flexion of forelimb	1
Flexion of hindlimb	1
Head moved >10° to vertical axis within 30 sec	1
Placing rat on floor (normal=0; maximum=3)	3
Normal walk	0
Inability to walk straight	1
Circling toward paretic side	2
Falls down to paretic side	3
Sensory	2
Placing test (visual and tactile test)	1
Proprioceptive test (deep sensation, pushing paw against table edge to stimulate limb muscles)	1
Beam balance (normal=0; maximum=6)	6
Balances with steady posture	0
Grasps side of beam	1
Hugs beam and 1 limb falls down from beam	2
Hugs beam and 2 limbs fall down from beam, or spins on beam (>60 sec)	3
Attempts to balance on beam but falls off (>40 sec)	4
Attempts to balance on beam but falls off (>20 sec)	5
Falls off; no attempt to balance or hang on to beam (<20 sec)	6
Reflex absence and abnormal movements	4
Pinna reflex (head shake when auditory meatus is touched)	1
Corneal reflex (eye blink when cornea is lightly touched with cotton)	1
Startle reflex (motor response to a brief noise from snapping a clipboard paper)	1
Seizures, myoclonus, myodystony	1
Maximum points	18

One point was awarded for inability to perform the task or for lack of reflex: 13-18, severe injury; 7-12, moderate injury; 1-6, mild injury.

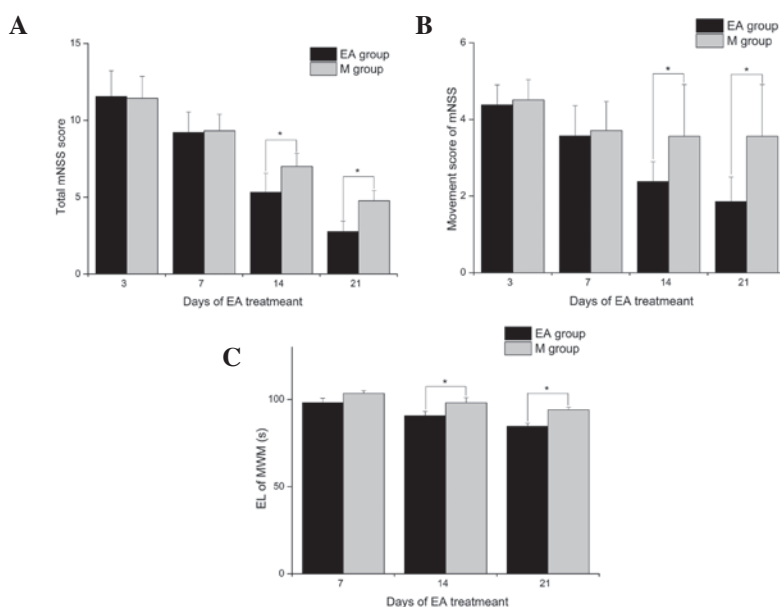


Figure 1. EA treatment significantly improves the recovery of neurological function in rats following stroke. (A) Neurological deficits and (B) motor deficits assessed by mNSS. (C) Recognitive deficits assessed by MWM. Values are presented as the mean \pm standard deviation. * $P < 0.05$ vs. M group. EA, electroacupuncture; M, middle carotid artery occlusion control; mNSS, modified Neurological Severity Score; MWM, Morris water maze; EL, escape latency.

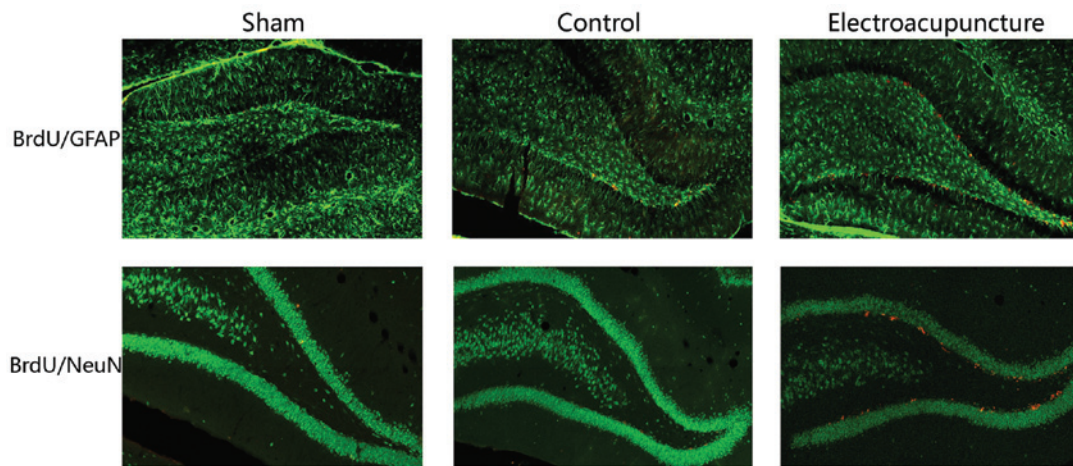


Figure 2. Representative images of BrdU⁺/GFAP⁺ and BrdU⁺/NeuN⁺ cells in the dentate gyrus area of rats on day 14 of electroacupuncture treatment, following immunofluorescence labeling. Green, BrdU; red, GFAP or NeuN. BrdU, bromodeoxyuridine; GFAP, glial fibrillary acidic protein; NeuN, neuronal nuclei.

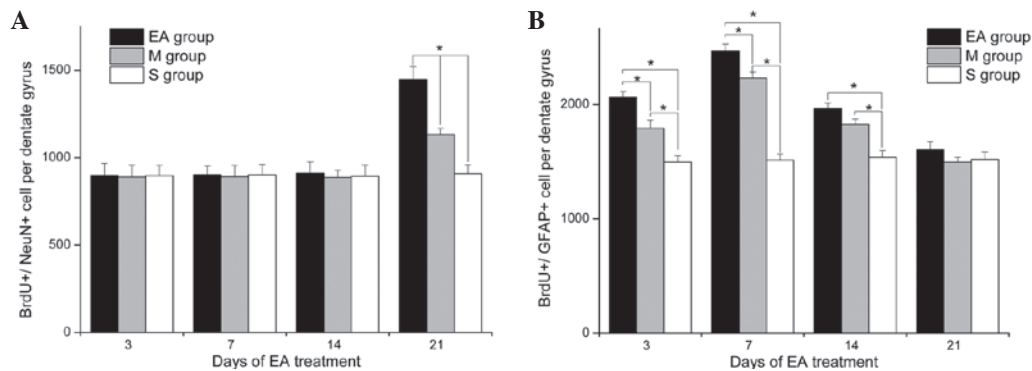


Figure 3. EA treatment significantly increases the number of BrdU⁺/NeuN⁺ cells in the dentate gyrus of rats following stroke. Quantitative analyses of (A) BrdU⁺/NeuN⁺ and (B) BrdU⁺/GFAP⁺ cells on days 3, 7, 14 and 21 of EA treatment. Values are presented as the mean \pm standard deviation. * $P < 0.05$ between the indicated groups. BrdU, bromodeoxyuridine; GFAP, glial fibrillary acidic protein; NeuN, neuronal nuclei; EA, electroacupuncture; M, middle carotid artery occlusion control; S, sham-operated.

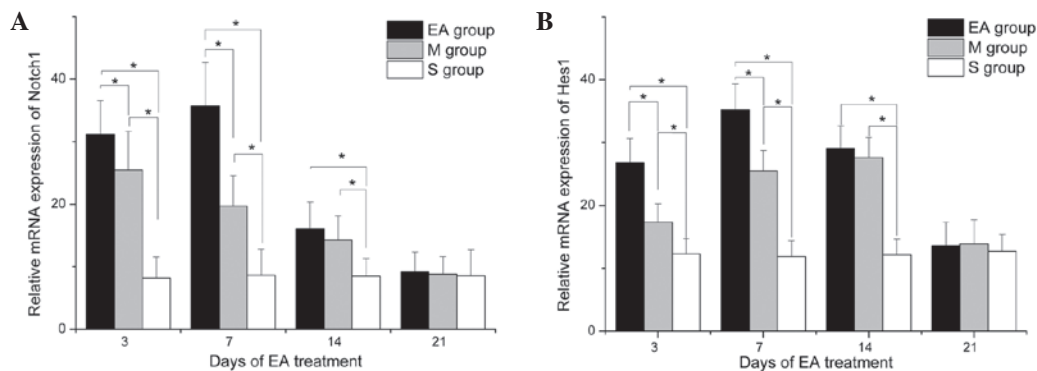


Figure 4. EA treatment significantly enhances the expression levels of Notch1 and Hes1 in the dentate gyrus area of rats following stroke. The mRNA expression levels of (A) Notch1 and (B) Hes were assessed by reverse transcription quantitative polymerase chain reaction at days 3, 7, 14 and 21 of EA treatment. Values are presented as the mean \pm standard deviation. * $P < 0.05$ between the indicated groups. EA, electroacupuncture; M, middle carotid artery occlusion control; S, sham-operated.

double-labeling immunofluorescence was performed to evaluate the proliferation and differentiation of NSCs following MCAO in each group. As shown in Figs. 2 and 3, MCAO rats had significantly higher frequencies of BrdU⁺/GFAP⁺ and BrdU⁺/NeuN⁺ cells in the DG area compared with those in the S group ($P < 0.05$), indicating that the proliferation and

differentiation of NSCs was stimulated by ischemic stress. In addition, EA treatment significantly elevated the frequencies of BrdU⁺/GFAP⁺ cells at 3, 7 and 14 days of treatment compared with the control treatment ($P < 0.05$), indicating the enhanced effects of EA treatment on the differentiation of NSCs into glia cells. Furthermore, EA treatment enhanced the neuronal

differentiation of NSCs, as indicated by increased frequencies of BrdU⁺/NeuN⁺ cells in the EA group, compared with that of the M group at 21 days of treatment ($P < 0.05$).

EA treatment enhances the expression of Notch1 and Hes1 following stroke. In order to examine the underlying molecular mechanisms by which EA treatment promoted the proliferation and differentiation of NSCs, the expression of Notch1 and its target molecule, Hes1, were analyzed by RT-qPCR. The results revealed that MCAO stress significantly stimulated the expression of Notch1 and Hes1 in the DG area compared with that of the S group at 3, 7 and 14 days of treatment ($P < 0.05$; Fig. 4).

Discussion

In the present study, the effects of EA treatment on the recovery of neurological functions and on the proliferation and differentiation of NSCs following stroke were investigated. The results revealed that EA treatment significantly improved the recovery of neural function in rats with MCAO. In addition, to the best of our knowledge, the present study was the first to demonstrate that EA treatment significantly promoted the proliferation and differentiation of NSCs into glial cells and neurons, possibly through the activation of Notch1 signaling pathways.

Previous studies have demonstrated that acupuncture or EA treatment promoted the recovery of neurological functions following stroke (22). In accordance with these findings, the present study confirmed that EA treatment significantly improved the mNSS and the Morris water maze performance of rats at 14 and 21 days of treatment following MCAO. It has been reported that EA treatment exerted its neuroprotective effects through a series of mechanisms, including reducing the apoptosis of neural cells and inhibiting the inflammatory responses (22). In addition, EA treatment has been found to enhance neurogenesis via the upregulation of neurotrophic factors, including BDNF (30). However, the impact of EA treatment on the proliferation and differentiation of NSCs remains to be elucidated.

NSCs in the DG area of hippocampus are important in neurogenesis (31). In normal physiological conditions, these cells gradually generate neurons to renew the granular layer of the cortex throughout adult life. Pathological stress, including ischemic insult, markedly stimulates the neurogenesis of NSCs in the DG area. These characteristics suggest NSCs may offer a promising therapeutic strategy in stroke treatment. However, difficulties in the availability of abundant sources of NSCs and issues in legislation and ethics impede the clinical application of NSC transplantation treatment for stroke (32). Therefore, enhancing the expansion and differentiation of endogenous NSCs offers a promising alternative strategy for NSC treatment. In the present study, EA treatment was found to significantly promote the proliferation and differentiation of NSCs into glial cells and neurons, as indicated by increased BrdU⁺/GFAP⁺ and BrdU⁺/NeuN⁺ cell frequencies, respectively. However, in line with previous studies, the proliferation and differentiation of NSCs in the DG area in the M or EA groups only lasted for 3 weeks. These data highlight the requirement for the development of novel strategies to extend the duration and intensity of neurogenesis following stroke.

Notch1 is an essential regulator of NSC maintenance and self-renewal during development. In addition, components of the Notch signaling pathway are expressed in neuroproliferative regions of the postnatal brain (33). Previous studies have reported that Notch signaling may augment the expansion and differentiation of adult NSCs following stroke. Activated Notch1 (NICD) and its downstream transcriptional targets, including Hes1, have been identified in SVZ cells following ischemic injury (34-36). The expression levels of NICD and Hes1 are significantly upregulated in the SVZ at 4 h following focal cerebral ischemia. In addition, Notch1 signaling activation enhances SVZ cell proliferation, whereas inhibition of Notch1 signaling results in the reduced proliferation of cells in the SVZ. It has also been reported that, during differentiation, the expression levels of Notch and Hes1 are downregulated in neural progenitor cells following ischemia, which is associated with a significant increase in the size of the neuronal population. This suggests that Notch signaling is involved in mediating adult SVZ neural progenitor cell proliferation and differentiation following stroke (37). In line with these findings, the present study confirmed that stroke significantly increased the expression levels of Notch1 and Hes1. In addition, EA treatment significantly enhanced the expression levels of Notch1 and Hes1 stimulated by ischemia insult, which may, at least in part, account for the higher frequencies of BrdU⁺/GFAP⁺ and BrdU⁺/NeuN⁺ cells in the DG area of rats in the EA group.

In conclusion, to the best of our knowledge, the present study was the first to demonstrate that EA treatment promoted the neurogenesis of NSCs through upregulation of the expression of Notch1 following stroke. These findings contribute to the current understanding of the mechanisms by which acupuncture promotes the recovery of neurological functions following stroke and may provide support for novel integrative therapeutic strategies for stroke treatment.

Acknowledgements

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