Functional electrical stimulation-facilitated proliferation and regeneration of neural precursor cells in the brains of rats with cerebral infarction

Yun Xiang1,2, Huihua Liu, Tiebin Yan3, Zhiqiang Zhuang3, Dongmei Jin3, Yuan Peng1

1 Department of Rehabilitation Medicine, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou, Guangdong Province, China
2 Department of Rehabilitation Medicine, Shenzhen Sixth People’s Hospital, Shenzhen, Guangdong Province, China
3 Department of Rehabilitation Medicine, Guangzhou First People’s Hospital, Guangzhou, Guangdong Province, China

Abstract
Previous studies have shown that proliferation of endogenous neural precursor cells cannot alone compensate for the damage to neurons and axons. From the perspective of neural plasticity, we observed the effects of functional electrical stimulation treatment on endogenous neural precursor cell proliferation and expression of basic fibroblast growth factor and epidermal growth factor in the rat brain on the infarct side. Functional electrical stimulation was performed in rat models of acute middle cerebral artery occlusion. Simultaneously, we set up a placebo stimulation group and a sham-operated group. Immunohistochemical staining showed that, at 7 and 14 days, compared with the placebo group, the numbers of nestin (a neural precursor cell marker)-positive cells in the subgranular zone and subventricular zone were increased in the functional electrical stimulation treatment group. Western blot assays and reverse-transcription PCR showed that total protein levels and gene expression of epidermal growth factor and basic fibroblast growth factor were also upregulated on the infarct side. Prehensile traction test results showed that, at 14 days, prehension function of rats in the functional electrical stimulation group was significantly better than in the placebo group. These results suggest that functional electrical stimulation can promote endogenous neural precursor cell proliferation in the brains of acute cerebral infarction rats, enhance expression of basic fibroblast growth factor and epidermal growth factor, and improve the motor function of rats.

Key Words: nerve regeneration; brain injury; functional electrical stimulation; neural precursor cells; neurogenesis; basic fibroblast growth factor; epidermal growth factor; nestin; stroke; rats; NSFC grant; neural regeneration

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Introduction
The brain has great potential to reorganize its functions and modulate its behavior after insults, including stroke[1-3]. In recent decades, therapeutic interventions to improve the functional recovery of subjects with stroke have been developed by scientists and clinicians such as neurodevelopment facilitation representative of the Bobath, Brunstrom and Rood approaches[4], but none of these interventions has demonstrated global superiority over the others. More recently, developing new therapeutic strategies has become a focus in stroke rehabilitation, and approaches such as motor relearning programs[5-6], repetitive task training[7-8], constraint-induced movement therapy[9-10] and neuromuscular electrical stimulation[11-12] have all received attention. However, it remains unknown which of the available treatment options for stroke rehabilitation is most effective.

Functional electrical stimulation is one neuromuscular electrical stimulation technique that supplements or replaces function that was lost in neurologically impaired individuals. Functional electrical stimulation applies an electric current to activate muscles that have been paralyzed as a result of central nervous system injury such as stroke[13]. In recent decades, functional electrical stimulation has been used in the improvement of motor recovery in subjects with stroke and has now advanced from being an electrophysiological brace to a treatment modality for improving muscle control, for the neuroaugmentation of residual movement, and to support “spontaneous” recovery of motor control[14], especially in stroke survivors. Several randomized, controlled trials have demonstrated that functional electrical stimulation can improve limb function in terms of decreasing spasticity, increasing muscle strength, and enhancing walking ability[14-15].
Why functional electrical stimulation has effects in stroke patients and the exact mechanism underlying the effects of such treatment remain poorly understood. One explanation for the positive effects is that functional electrical stimulation-evoked afferent activity may play an important role in facilitating the reorganization of sensory and motor systems early after stroke. To date, however, few investigations have focused on the mechanisms underlying the effectiveness of functional electrical stimulation. Although some investigators have concluded that functional electrical stimulation treatment may facilitate neural plasticity, few experiments have explored the role that neural precursor cells in the brain may have in functional electrical stimulation-induced neural plasticity.

Neural precursor cells proliferate and differentiate into neurons and glia, and are important for proper generation, repair and maintenance of the nervous system. Moreover, neural precursor cells seem to be an effective tool for brain repair after injury. They are mainly located in the subgranular zone of the hippocampal dentate gyrus and in the subventricular zone in the brains of adult mammals. They can be mobilized in response to certain pathological conditions such as stroke, especially with the help of some crucial neural factors such as two specific mitogens of the neural precursor cells: basic fibroblast growth factor and epidermal growth factor. Epidermal growth factor primarily stimulates mitotic division of all neural stem/progenitor cells and epidermal growth factor-related peptide neuregulins also stimulate neural stem cell proliferation in the subventricular zone via ErbB4 receptors. Basic fibroblast growth factor also stimulates proliferation of neural stem/progenitor cells, but in a less rigid fashion than epidermal growth factor. In vitro analysis of epidermal growth factor-basic fibroblast growth factor cross-talk in primary subventricular zone neurosphere cultures has shown that basic fibroblast growth factor acts subsequent to epidermal growth factor and that basic fibroblast growth factor receptors are induced secondarily by epidermal growth factor signaling. Owing partly to these effects of the two growth factors, neural precursor cells are thought to be involved in the repair of impaired neurons. However, the spontaneous proliferation of endogenous neural precursor cells stimulated by stroke is in both of the known neurogenic sites (the subgranular zone and subventricular zone) is not normally sufficient to completely restore the function of affected neurons. Subsequently, how to facilitate neurogenesis from these precursor cells is a challenge currently facing scientists and clinicians, and functional electrical stimulation may enhance that neurogenesis.

Recent studies have demonstrated that functional electrical stimulation activates both motor and sensory axons under the stimulating electrodes and that muscle contractions are either generated by activation of motor axons through peripheral pathways, or initiated by sensory axons through central pathways. Generating contractions through central pathways recruits motor units through the synaptic activation of motor neurons. Central pathway stimulation might induce afferent-efferent changes to help to resume normal movement patterns by altered activation in central nervous system circuits, including the ischemic penumbra area and motor cortex. This suggests that functional electrical stimulation-evoked sensory volleys would increase activities in central nervous system circuits that control movement, and this could help produce contractions that are more fatigue resistant in the recovery of neuromuscular function after central nervous system damage. Published studies raise the question of whether recovery from stroke might be related to neuroplasticity, or more specifically to neurogenic neural precursor cells. The present study was designed to observe the impact of functional electrical stimulation on endogenous neural precursor cells after focal cerebral infarction and to explore the mechanism underlying any observed functional recovery.

Results
Quantitative analysis of experimental animals
One-hundred and eight rats were equally and randomly divided into functional electrical stimulation (middle cerebral artery occlusion + functional electrical stimulation), placebo (middle cerebral artery occlusion + attached to the same functional electrical stimulation device but without electrical stimulation) and sham-operated (no artery occlusion) groups. Neural stem cell numbers are known to increase slightly early after an insult, to peak at 7 days, and to persist for at least 4 weeks. Thus, each group was further subdivided into four subgroups (baseline, and 3, 7 and 14 days of functional electrical stimulation treatment; n = 9 in each group). At each time point, three rats were selected for immunohistochemistry, three for immunoblotting, and three for PCR. All 108 rats were included in the final analysis, with no drop outs.

Functional electrical stimulation improved the behavioral function of stroke rats
In a beam-walking test and the rotating pole test, stroke rats showed impaired neurological function compared with the sham-operated group (P < 0.05). Significant differences were found between the scores of rats in the sham-operated group and those of rats in both the functional electrical stimulation and placebo groups, but not between the scores of rats in the functional electrical stimulation and placebo groups at each assessment point (data not shown).

In the prehensile traction test, the stroke rats clearly showed impaired prehensile traction compared with the rats in the sham-operated group. Significant improvement was observed in the functional electrical stimulation group compared with the placebo group at 14 days after treatment, but not at baseline or at 3 or 7 days (Figure 1). In addition, no seizures were observed in any of the rats during the whole experiment.

Functional electrical stimulation enhanced the numbers of nestin-positive cells in the brains of stroke rats
By immunohistochemical detection, the number of nestin-positive cells in the placebo group was greater than that in the functional electrical stimulation group at 7 and 14 days (P < 0.05; Figure 2).
Functional electrical stimulation upregulated basic fibroblast growth factor and epidermal growth factor protein levels in the brains of stroke rats
Western blotting demonstrated that the levels of basic fibroblast growth factor and epidermal growth factor proteins were significantly greater in the functional electrical stimulation group than in the placebo group at 7 and 14 days ($P < 0.05$; Figure 3).

Functional electrical stimulation upregulated basic fibroblast growth factor and epidermal growth factor mRNA expression in the brains of stroke rats
Reverse-transcription PCR demonstrated that the expression levels of basic fibroblast growth factor and epidermal growth factor mRNA expression were increased by more in the functional electrical stimulation group than in the placebo group at 7 and 14 days compared with the levels in the sham-operated group ($P < 0.05$; Figure 4).

Discussion
Studies have demonstrated that ischemia in the brain causes irreversible limb paralysis. Various rehabilitation approaches have been applied to improve limb paralysis in clinical practice, including functional electrical stimulation. On the other hand, the discovery of neural precursor cells and neurogenesis in adult brains has shown that the post-ischemic brain can be repaired, at least to a certain extent. Nestin was selected as a marker of neural precursor cells, and no seizures were observed during the procedures in the present study. This is important because seizures can change the levels of growth factors and endogenous precursor cell proliferation.

Functional electrical stimulation treatment facilitated behavioral recovery
No significant differences were found in the scores on the beam-walking test and rotating pole test between the functional electrical stimulation group and the placebo group at each assessment point. The reason for this is because the scores on the prehensile traction test were significantly higher in the functional electrical stimulation group than in the placebo group after 14 days of functional electrical stimulation treatment, because the prehensile traction test reflects forelimb function and functional electrical stimulation device electrodes were set on the paralyzed forelimbs of rats. Therefore, any improvement in forelimb function presented in the prehensile traction test should be contributed by functional electrical stimulation.

Although functional electrical stimulation resulted in significantly better outcomes compared with placebo at 14 days, the functional electrical stimulation-treated rats still had significantly worse outcomes than did the sham controls. The functional electrical stimulation treatment lasted only 14 days in our experiment, and 2 weeks may not be long enough for functional electrical stimulation treatment to demonstrate its full effects on behavioral recovery. If so, the scores of functional electrical stimulation-treated rats might eventually have almost equaled those of the sham controls if the treatment had lasted for 21 or 28 days.

Functional electrical stimulation treatment promoted the proliferation of neural precursor cells
There were no significant differences in nestin-positive cell numbers between the different assessment time points among rats in the sham-operated group. This suggests that neurogenesis in normal rats continued at a steady rate. The number of nestin-positive cells in rats in the placebo group increased after middle cerebral artery occlusion, which suggests the occurrence of stroke-induced neural precursor cell proliferation, at least to some extent, in rats, consistent with the results of many other studies.

More importantly, the number of nestin-positive cells in rats in the functional electrical stimulation group was upregulated significantly compared with the numbers in the placebo and sham-operated groups after 7 and 14 days (though not after 3 days) of functional electrical stimulation treatment. This strongly indicates that functional electrical stimulation can promote the proliferation of neural precursor cells, and moreover, it hints that functional electrical stimulation might show its effects only if the treatment is maintained for at least 7 days with at least a 10-minute treatment in each session. This significant difference between the functional electrical stimulation group rats and the others indicates that functional electrical stimulation activated central pathways and had a persistent effect on brain pathophysiology after stroke. Central pathway stimulation might induce afferent-efferent changes to help to resume normal movement patterns. This treatment effect of functional electrical stimulation was obvious after being applied for some time, which must be considered when planning functional electrical stimulation treatment after stroke, which is consistent with evidence in clinical practice.

Neural precursor cell proliferation slowed between 7 and 14 days in the treatment group, although the levels remained higher than in the placebo and sham-operated groups. This indicates that the effect of functional electrical stimulation...
treatment on neural precursor cell numbers may be only temporary, or that longer functional electrical stimulation treatment in each session might be helpful (such as three 10-minute sessions a day) and might amplify its effects. This could be a fruitful topic for future study. The effects of functional electrical stimulation on neurogenesis will perhaps be more obvious with a better optimized treatment protocol. We will investigate such an optimized protocol in future studies.

**Functional electrical stimulation treatment increased basic fibroblast growth factor and epidermal growth factor protein and mRNA expression levels**

The protein levels and gene transcripts for basic fibroblast growth factor and epidermal growth factor were also in-
Increased significantly after 7 and 14 days of functional electrical stimulation treatment. These findings suggest that the functional electrical stimulation treatment-induced promotion of neural precursor cell proliferation might be at least partially explained by the enhancement of basic fibroblast growth factor and epidermal growth factor expression.

Interestingly, basic fibroblast growth factor expression, epidermal growth factor expression and neural precursor cell proliferation were all increased significantly in the functional electrical stimulation group at 7 days, even though no significant improvement was yet evident in the prehensile traction test results. This time gap indicates that functional electrical stimulation treatment first enhances the production of growth factors, then the growth factors show their effects on neural precursor cell proliferation, and only then does macroscopic function improve (at 14 days).

Furthermore, epidermal growth factor and basic fibroblast growth factor expression levels and neural precursor cell proliferation in the functional electrical stimulation group peaked at 7 days and then declined at 14 days, although they were still higher than those in the control group at 14 days. This trend means that functional electrical stimulation aids neural precursor cell proliferation, since neural precursor cells are known to peak at 7 days and then decrease after cerebral ischemia in the absence of functional electrical stimulation treatment[31,32]. This encourages us to consider more suitable treatment parameters or longer treatment durations to amplify the treatment effects on neural precursor cell proliferation.

Overall, this study has provided evidence that functional electrical stimulation can augment stroke-induced neurogenesis in rat brain, suggesting that stimulation of the brain’s self-repair mechanisms through functional electrical stimulation treatment has an effect on endogenous neural precursor cell proliferation. It should be noted, however, that only 2 weeks of treatment was performed. Additional studies are called for to specify the ideal timing and further define the functional electrical stimulation parameters to maximize the protective effect documented here. It also remains to be determined whether the neuroprotective effect observed after 14 days of functional electrical stimulation treatment endures for any longer than observed.

Materials and Methods

Design
A randomized, controlled laboratory study.

Time and setting
Experiments were performed at the Research Laboratory of Sun Yat-sen Memorial Hospital, Sun Yat-sen University, China, from March 2009 to May 2010.

Materials
A total of 108 male Sprague-Dawley rats (weighing 250–300 g, about 2 months of age) from the Experimental Animal Center of Zhongshan University, with experimental animal production license No. SCXK (Yue) 2004-0011 and experimental animal use permit license No. SYXK (Yue) 2007-0081, were used. Rats were allowed free access to food and water in an air-conditioned room in a 12-hour light/dark
cycle at 23 ± 2°C and a relative humidity of 60 ± 5%. The experimental procedures were conducted in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, formulated by the Ministry of Science and Technology of China[39].

Methods
Establishment of middle cerebral artery occlusion model
After being acclimatized for 5 days, rats in the functional electrical stimulation and placebo groups were intraperitoneally anesthetized with chloral hydrate (0.4 mg/kg) and a stroke was simulated through permanent middle cerebral artery occlusion as described by Hasegawa et al.[33]. The rats were anesthetized with 10% chloral hydrate (0.4 mg/kg, i.p., Milipore, MA, USA). Under a dissecting microscope (San Jose CA SZX12, Olympus, Tokyo, Japan), the left common carotid artery was exposed through a ventral midline neck incision and the occipital and thyroid artery branches of the external carotid artery were ligated and dissected. The pterygopalatine artery was isolated and ligated close to its origin and the external carotid artery was ligated and dissected. The pterygopalatine artery was isolated and ligated close to its origin and the external carotid artery was ligated and dissected. The pterygopalatine artery was ligated and ligated close to its origin and the external carotid artery was ligated approximately 3–5 mm distal to its origin. The common carotid artery and internal carotid arteries were then temporarily clamped using microvascular clips to allow insertion of a 4-0 nylon filament (30 mm in length) with a blunt tip into the internal carotid artery by way of the external carotid artery. The external carotid artery was dissected and the nylon filament was loosely secured inside the external carotid artery using 5-0 sutures. This allowed a gentle movement of the nylon filament into the internal carotid arteries and eventual occlusion of the middle cerebral artery. The average depth to which the nylon filament reached was 18.5 ± 0.5 mm. The sham-operated rats were subjected to the same surgery but without filament occlusion.

Evaluation of neurological function in rats
After recovery from anesthesia, neurological function was scored using the scale described by Longa[34]. The neurologic findings were scored on a 5-point scale: a score of 0 indicated no neurologic deficit; a score of 1 (failure to extend the left forepaw fully) indicated a mild focal neurologic deficit; a score of 2 (circling to the left) indicated a moderate focal neurologic deficit; and a score of 3 (falling to the left) signaled a severe focal deficit. Rats with a score of 4 did not walk spontaneously and had a depressed level of consciousness.

Implantation of treatment electrodes
Three days after the middle cerebral artery occlusion procedure, a second operation was carried out on the paralyzed right forelimbs of the rats in the functional electrical stimulation and placebo groups. After anesthesia, two Teflon-coated stainless steel wires (AW633; Cooner Wire, Chatsworth, CA, USA) were passed subcutaneously from an incision on the limb to the scalp. Electrodes were formed by stripping insulation off the ends of the wires (about 3 mm) and looping them around the dorsal deep fascia (and thus, the dorsi-extension muscles underneath). The wires were embedded subcutaneously and emerged from the scalp between the ears. The other ends of the wires were sutured between the ears so that they could not be scratched off, but could still easily be connected with the electrodes of an external functional electrical stimulation stimulator NeuroTrac Continence (Verity Medical Corporation, Braishfield, UK). When the rats received functional electrical stimulation treatment, the wires were entwined with the electrodes inducing stim-
ulation in the paralyzed forelimb. The stimuliators around the dorsal fascia were fastened beneath the skin to resist scratching, as the rats were conscious while receiving treatment. The fingers and wrists of the rats’ paralyzed forelimbs dorsi-extended slowly and steadily in response to functional electrical stimulation, mimicking to some extent normal functional movement. Since stimulation was not applied to the motor limb, seizures were not often induced by the functional electrical stimulation treatment.

Evaluation of behavior changes in rats

Beam-walking, rotating pole and prehensile traction tests were carried out at each assessment session as described by Ohlsson et al. [36] and Johansson [37]. Behavior evaluations were carried out at each time point.

Beam-walking test: The beam used was 1,700 mm long and 19 mm wide and was placed 700 mm above the floor. A wall was placed 13 mm to the left or the right of the beam (rats are more willing to walk when a wall is placed next to the beam). Scoring was as follows: 0, the rat falls down; 1, the rat is unable to traverse the beam but remains sitting across the beam; 2, the rat falls down while walking; 3, the rat can traverse the beam, but the affected hind limb does not aid in forward locomotion; 4, the rat traverses the beam with more than 50% foot slips; 5, the rat crosses the beam with a few foot slips; and 6, the rat crosses the beam with no foot slips.

Rotating pole test: The pole, 45 mm in diameter and 1,500 mm in length, was rotated alternately to the left or the right at three turns per minute. The same scoring system was used as described in beam-walking test except that the score 3 was given if the rat jumped with both hind limbs together, apparently supporting the weak hind limb with the opposite strong limb, and a score of 4 was given if the affected hind limb was used for fewer than 50% of the steps.

Prehensile traction test: A 600 mm long, stainless steel wire (with a diameter of 1 mm) was fixed horizontally and 400 mm above a foam pad. After placing the forepaws of a rat on the rope, the rat was released and the time until falling was measured. Rats were recorded as: 6 points for 91–120 seconds on the wire; 5 points for 61–90 seconds; 4 points for 31–60 seconds; 3 points for 21–30 seconds; 2 points for 11–20 seconds; 1 point for 1–10 seconds; and 0 points if they were unable to hang on the rope.

Detection of nestin-positive cells by immunohistochemical methods

At each time point, three rats from each group were randomly selected for sacrifice by a computer program. Rats were anesthetized with 10% chloral hydrate (350–400 mg/kg, i.p., Milipore). The chest of a rat was cut open to expose the heart. A perfusion needle was inserted into the heart from the apex, and fixed. The right atrial appendage was cut open to obtain vein blood. Saline (100–200 mL) was given drop by drop, followed by reperfusion with 4% paraformaldehyde until rat limbs were stiff. Rat brains were removed quickly and fixed in 4% paraformaldehyde. The ischemic brains were collected from 1 mm anterior and 4.5 mm posterior to the bregma (approximately 10 µm in thickness) containing the cortex, hippocampus and subventricular zone in ice-cold methanol. The brain samples were sectioned in the coronal plane at approximately 8 µm intervals and the tissue sections were fixed in chilled fresh aceton for 10 minutes. They were then incubated with 3% hydrogen peroxide for 10 minutes and blocking reagents (Solution A, SP kit, Maixin, Fuzhou, Fujian Province, China) for 15 minutes, and then incubated with rabbit-anti nestin polyclonal antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. The sections were then incubated with goat anti-rabbit IgG (1:200; Invitrogen, Carlsbad, CA, USA), at room temperature for 2 hours. No-primary-antibody controls were also prepared to verify the experimental procedures. The antibodies were diluted with phosphate-buffered saline. The numbers of nestin-positive cells were counted under an optical microscope (Axioplan 2; Zeiss, Tokyo, Japan) in five sections (1 mm anterior and 4.5 mm posterior to the bregma). In the 10 × 40 magnification field and with the same brightness level, the numbers of nestin-positive cells were counted in five non-overlapping views of subgranular zone and subventricular zone. The stained cell counts of these sections were quantified using Image-Pro Plus 5.0 software (Media Cybernetics, Bethesda, MD, USA).

Measurement of basic fibroblast growth factor and epidermal growth factor protein level by western blot assay

At each time point, an additional three rats were randomly selected for sacrifice by a computer program in each group. Ischemic brain samples were again collected from the same locations, snap-frozen in liquid nitrogen and stored at −80°C. Analysis was performed according to Ueda’s method [37].

Frozen tissue was transferred to ice-cold buffer containing 0.1 mol/L NaCl, 0.05 mol/L Tris-HCl (pH 7.6), 0.001 mol/L ethylenediamine tetraacetatic acid (pH 8.0), 0.1% Tween-20, aprotinin (1 µg/mL) and phenylmethyl sulfonifluoride (100 µg/mL) and homogenized on ice. The homogenate was centrifuged at 12,000 × g for 10 minutes, and the supernatant was stored at 4°C. Total protein concentrations were determined using an ultraviolet spectrophotometer (Beckman Coulter, Fullerton, CA, USA) with a modified Bradford assay. Equal amounts of protein from each sample (20 µg) were determined using an ultraviolet spectrophotometer (Beckman Coulter, Fullerton, CA, USA) with a modified Bradford assay. Equal amounts of protein from each sample (20 µg) were determined using an ultraviolet spectrophotometer (Beckman Coulter, Fullerton, CA, USA) with a modified Bradford assay. Equal amounts of protein from each sample (20 µg) were determined using an ultraviolet spectrophotometer (Beckman Coulter, Fullerton, CA, USA) with a modified Bradford assay.
mixed with 5 µL of sample buffer and boiled for 5 minutes at 95°C. Samples were separated by electrophoresis on 7.5–12.0% polyacrylamide gels. Separated proteins were transferred onto polyvinylidene difluoride membrane at 80 V for 1.5 hours. The membrane was blocked with 5% dried, defatted milk in Tris-buffered saline buffer (50 mmol/L Tris-HCl, pH 7.5, containing 150 mmol/L NaCl) with 0.1% Tween-20 for 2 hours at room temperature. After washing with Tris-buffered saline with Tween-20, the membranes were cultured overnight at –4°C with primary antibody. The primary antibodies were mouse anti-basic fibroblast growth factor monoclonal antibody (1:500; Abcam Plc, Cambridge, UK), mouse anti-epidermal growth factor monoclonal antibody (1:1,000, Abcam) and mouse anti-GAPDH monoclonal antibody (1:500, Santa Cruz Biotechnology). Negative controls were prepared without primary antibody. The membranes were incubated with second antibody (rabbit anti-mouse, 1:150, Millipore) at room temperature for 1 hour. The optical densities of the specific bands were scanned and measured by Image Pro Plus 5.0 software (Media Cybernetics), and were represented as absorbance values according to the basic fibroblast growth factor/GAPDH ratio. The absorbances of specific bands were scanned and quantified using Image Pro Plus 5.0 software. The basic fibroblast growth factor/GAPDH and epidermal growth factor/GAPDH absorbance values were evaluated in duplicate by two observers working independently.

**Statistical analysis**

Data were analyzed using SPSS 13.0 software (SPSS, Chicago, IL, USA) and the results were expressed as mean ± SD. Comparisons between groups were conducted by one-way analysis of variance followed by a Dunnett-t post hoc test. A value of P ≤ 0.05 was considered statistically significant.

**Author contributions:** Yan TB, Zhuang QZ and Xiang Y designed the experiment. Xiang Y and Liu HH implemented the experiment. Jin DM evaluated experiment. Peng Y collected data. Xiang Y drafted and Yan TB revised the manuscript. Yan TB and Xiang Y were responsible for the article. All authors approved the final version of the paper.

**Conflicts of interest:** None declared.

**Peer review:** Functional electrical stimulation as a mature rehabilitative therapy has been applied in clinic for a long time, but few studies concerned its theoretic mechanisms, especially on the basis of animal models. This study deeply explored the mechanisms of electrical stimulation-induced neurogenesis and had innovative and a high value for clinical application.

**References**


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**Primers for genes are shown as below:**

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<tr>
<td>Epidermal growth factor</td>
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<td>GAPDH</td>
<td>Upstream: ACA GTC TTC TGA GTG GCA GTG AT Downstream: ACA GTC TTC TGA GTG GCA GTG AT</td>
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**Examination of basic fibroblast growth factor and epidermal growth factor mRNA expression levels by RT-PCR analysis**

At each time point, the remaining three rats in each group were sacrificed and fresh brain tissue was sampled from the same areas. Total RNA was isolated using Trizol (Promega, Madison, WI, USA) according to the manufacturer’s protocol. Total RNA (1 µg) was reverse-transcribed into first-strand cDNA using oligo-dT primer (Takara, Tokyo, Japan). Reverse transcription was performed using avian myeloblastosis virus reverse transcriptase (Takara) for 1 hour at 42°C, then the reaction mixture was inactivated for 10 minutes at 95°C and cooled to 4°C. The cDNA was diluted to a final volume of 25 µL, and a 2 µL aliquot was used in a PCR reaction containing 1 × DNA polymerase buffer, 1.5 mmol/L MgCl₂, 0.2 mmol/L dNTPs, 10 pmol primers and 2.5 units of Taq polymerase (Takara). The cDNA was amplified using 33 PCR cycles and RT-PCR products were separated electrophoretically on 1.2% agarose gel containing ethidium bromide and visualized under ultraviolet light.

The absorbance values for specific bands were scanned and quantified using Image Pro Plus 5.0 software. The basic fibroblast growth factor/GAPDH and epidermal growth factor/GAPDH absorbance values were evaluated in duplicate by two observers working independently.


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