# Electroacupuncture Intervention Combined with Rat Nerve Growth Factor on Expression of Nestin and NeuN in MCAO Rats

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## Abstract

Objective: To observe the effect of electroacupuncture (EA) on the expression of Nestin and Neuron specific nuclear protein antigen (neuron- specific protein, NeuN) at different time points after cerebral ischemia reperfusion injury. Methods: One hundred and fifty SD male rats were used to generate a one- side middle cerebral artery occlusion (MCAO) model using thread embolism method, then randomly divided them into the sham operation group, MCAO group, EA group, Nerve Growth Factor (NGF) group and EA+NGF group. The three benzene chloride tetrazolium (TTC) staining was used to detect the volume of cerebral infarction in the rats. Immunohistochemical staining was used to observe the expression of immune positive cell markers such as endogenous neural stem cell (NSC) marker protein Nestin, bromodeoxyuridine (Brdu) and NeuN on 1, 7 and 14 days after cerebral ischemia in rats, and compared with the sham operation group. Results: TTC detection of the cerebral infarction volume showed that the nerve function injury score of the EA+ NGF group was significantly lower than MCAO group ($P < 0.01$). Ischemia led to severe loss of brain function, and EA+ NGF helped in the recovery of cerebral ischemia. Immunohistochemical results showed almost no expression of Nestin in the sham operation group; positive cells was expressed significantly higher Nestin on the 7th day ($P < 0.05$); then was peaked on the 14th day. Nestin/BrdU labeled cells in the sham operation group showed minor expression, which was increased on the 7th day ($P < 0.05$) and was peaked on the 14th day after ischemia. The differences were statistically significant on the 7th and 14th day among the EA group, NGF group, EA+NGF group and MCAO group ($P < 0.05$). NeuN expression was higher in the sham operation group, and was increased on the 7th day after ischemia. NeuN/BrdU labeled cells showed higher expression in the sham operation group, the most obvious improvement was in the EA group, and the NGF and EA+NGF groups also showed significantly increased expression, while MCAO group showed the least. Conclusion: Protective effect of EA combined with NGF on cerebral ischemia reperfusion injury may induce nerve cell regeneration, accelerate proliferation of newborn cells, and promote differentiation of newborn cells, which is important for the recovery of nerve function.

Keywords: Cerebral ischemia reperfusion; Rat nerve growth factor; Electroacupuncture

## Introduction

The study found that cerebral ischemia stimulated endogenous Neural stem cell (NSC) proliferation, migration and integration, which are correlated and conductive to the recovery of neural function. The effect of electroacupuncture (EA) combined with rat nerve growth factor (NGF) on the recovery of neurological function, whether this treatment can activate endogenous NSC, or the effect of EA combined with NGF in Nestin and Neuron specific nuclear protein antigen (neuron- specific protein, NeuN) expression in middle cerebral artery occlusion (MCAO) rats, and the effect of EA combined with NGF on endogenous NSCs proliferation, were investigated on rats after cerebral ischemia reperfusion injury.

## Materials and Methods

### Modeling and Grouping

A total of 150 male SPF SD rats weighing 220- 250 g

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were provided by the experimental animal center of Southern Medical University. The MCAO model was established using the Longa EZ [1] method. With 3.5% pentobarbital sodium (0.18mL/100g) intraperitoneal injection, incision after anesthesia, the right common carotid artery (CCA) was separated from the tissue layer by layer, and gradually exposed. The external carotid artery (ECA) and internal carotid artery(ICA), the ECA and its branches were ligated and released, along the ICA separation of the pterygopalatine artery(PPA). The prepared suture was gently inserted, fast arrival PPA, the origin of PPA was gently gripped with a pair of tweezers, the suture was placed smoothly into the intracranial ICA, and the inserting depth to the ECA bifurcation was calculated to be about(18 + 0.5) cm. The skin was sutured. The reperfusion by the external pull bolt ball end back to the ECA can be restored within ICA and MCA. Insulation for screening in ischemia time of left limbs intact or dead were eliminated. After the completion of the operation, the line bolt was pulled out, ICA and the Willis ring without thrombosis and hemorrhage was determined for successful reperfusion [2]. To maintain the validity of the order, in the event of death of an animal, the number of animals were replenished to before, to ensure that the accurate number of animals in each group.

The MCAO model was successfully established according to the Longa EZ[1] standard evaluated the degree of neurological impairment. Grade 0, no defects, and normal; 1, the contralateral forelimb can not freely extend; 2, normal ipsilateral forelimb, contralateral forelimb flexion; 3, contralateral circling, but not serious; 4, serious side around; 5, cannot walk spontaneously, and loss of consciousness.

The experimental animals were divided into five groups, 30 rats in each group. Each group was subdivided into three subgroups according to the time points of MCAO at 1d, 7d and 14d (10 rats in each group). Sham operation group: blank control group, and the operated group with the neck incision along the inner edge of the separation of the sternocleidomastoid muscle and fascia, followed by the right CCA with no treatment. Model group: the MCAO model with no treatment. EA group: the MCAO model treated with EA once a day for 15min, for a total of 14 days. NGF group: the MCAO model treated with femoral vein injection of NGF once a day, for a total of 14 days. EA +NGF group: the EA and NGF treatments, the method and the parameters were the same as the EA +NGF groups.

Each group was subdivided into three subgroups according to of the cerebral infarction model rats.

### EA Therapy

According to the standard of acupuncture and moxibustion for positioning in rats on *TESTS CHINESE ACUPUNCTURE AND MOXIBUSTION*, we selected the Du Meridian "Baihui" and "Dazhui" and acupuncture treatment based on the needs of the experiment. Acupuncture: No. 31 with 1 inch needle was used, the location identified, along the skin acupuncture "Baihui" and "Dazhui" two points, the depth of the needle to moderate was about 2.5-3.5 mm. The needle handle was connected to the G68051 type electro acupuncture instrument electrode, and the intensity 3.5 V, frequency 4 Hz, and then under close observation, the appropriate standard showed a minor muscle spasm with no struggle called degree, with duration of 10 min. The treatment was started once a day when the animal was awake, for a total of 14 days.

### Drug Administration

Rat NGF administration: Once daily femoral vein injection of NGF for a total of 14 days.

Bromodeoxyuridine (Brdu) mark: All the rats were sacrificed 3 days before injection of Brdu of proliferative cell markers SVZ and SGZ in ischemic area, thrice, once every four hours, each time 50mg/kg, the last injection was sacrificed.

### Index Detection

Neurological score: Evaluation of the degree of neurological impairment after Longa EZ[1] evaluation in rats for success of model production.

Exclusion criteria: Any animal that met one of the following criteria was excluded: (1) scoring 1 point for assessment of neurological symptoms in rats; (2) subarachnoid hemorrhage in the brain; (3) did not meet the processing requirements of dead rats.

Volume of cerebral infarction rats (TTC staining): The MCAO rats after 1 d, 7 d and 14 d were adminitested with 10% chloral hydrate (2.5-3.0 mL/kg) after intraperitoneal anesthesia, saline perfusion was given through ascending

aorta to the right atrium, outflow clear blood. The brains were removed, cleaned with PBS, placed in 1.5% TTC solution, and then incubated for 25 min in a 37℃ $CO_2$ incubation box in complete darkness. After incubation, the brains were placed in 4.5% paraformaldehyde solution. The focal ischemia area was observed with TTC staining. TTC is colorless, but is reduced by the catalase present in the mitochondria, leading to the formation of four triazole nitrogen lipophilic red, which helps to identify viable mitochondria in the non-infarcted area, while the ischemic area is clearly distinguished as white by the naked eye. TTC staining can prove the reliability of the suture method in this experiment. TTC staining showed that the ischemic area was mainly in the right and back sides of the brain. The results of the experiment were recorded, and then calculated with Image Pro, infarction area image processing software Plus.

We observe the ischemic markers at 1.5 h and 1 d, 7 d, 14 d by immunohistochemical method after SVZ and SGZ cells were reperfused. Endogenous NSC related markers Brdu, Nestin, the number of NeuN positive cells, and the expression of cells were observed under the microscope.

Statistical analysis: Experimental data shown as the average and standard deviation and behavioral data were analyzed by SPSS22 statistical software. Repeated measurements were analyzed by variance analysis of Mauchly's W-Sphere Differences Test and repeated measurements of the group of main effect, and the Bonferroni test was used to compare among the two groups. $P < 0.05$ was considered to be statistically significant.

## Results

### TTC Staining of Cerebral Infarction Volume

Data showed that the model can cause cerebral ischemic injury, the damage at different time points was different, EA and rat NGF stimulation can improve the ischemic injury area.

### Expression of Brdu Positive Cells

After cerebral ischemia reperfusion, expression of BrdU labeled newborn cells in ischemic brain tissue was peaked at 7d of reperfusion and the expression at 14 d was increased. As compared to the model group, EA+NGF group was significantly increased($P < 0.05$). As compared to the sham operation group, the expression of Brdu of SGZ in EA group was significantly increased.

Analysis of variance of the group, indicated that there were statistical differences ($P < 0.05$), and the results of multiple comparisons showed that there was significant difference between each group($P < 0.05$).

Table 1 Comparison of the Infarct Volumes at Different Time Points($\bar{x} \pm s$)

| Group | Time | | |
|---|---|---|---|
| | 1 d | 7 d | 14 d |
| Sham Operation group | 0 | 0 | 0 |
| Model group | 73.40± 1.27 | 107.20± 1.23 | 69.30± 1.64 |
| EA group | 70.80± 1.03 | 101.60± 1.265 | 68.10± 1.66 |
| NGF group | 69.30± 1.34 | 85.50± 2.17 | 60.00± 2.59 |
| EA+NGF group | 53.50± 0.85 | 59.80± 1.93 | 53.90± 1.10 |

SVZ and SGZ cells emploied self-renewal, nerve regeneration ability exist, but gradually weaked with age. The experiment using BrdU labeled split neurons showed that Brdu neurons was widely expressed in SVZ and SGZ cells. The number of BrdU positive cells in EA + NGF group was much higher than that in the model group ($P < 0.05$). The levels of Brdu positive cells in the EA group at the same period were more than those in the EA+NGF group ($P < 0.05$). These results indicated that EA and rat NGF can promote SVZ cell proliferation and SGZ area.

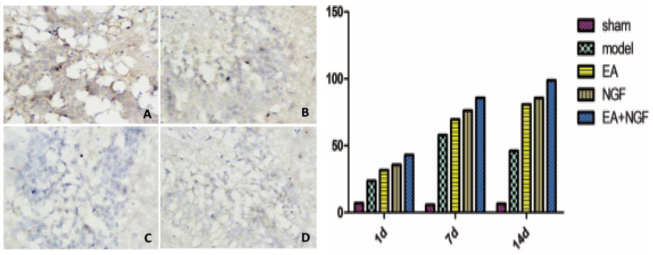
Compared with the model group, Brdu expression in EA + NGF group was increased significantly ($P < 0.05$). Compared with sham operation group, Brdu expression in SGZ of EA group was significantly higher($P < 0.05$).

Table 2 Comparison of Brdu Dentate Gyrus (DG) Positive Cell Numbers at Different Time Points($\bar{x} \pm s$)

| Group | Time | | |
|---|---|---|---|
| | 1 d | 7 d | 14 d |
| Sham Operation group | 7.00± 0.67 | 5.80± 0.79 | 6.50± 0.85 |
| Model group | 23.70± 1.25 | 57.80± 1.48 | 46.00± 1.764 |
| EA group | 31.70± 3.89 | 69.60± 2.17 | 80.80± 2.10 |
| NGF group | 35.70± 1.42 | 76.20± 1.48 | 85.50± 4.20 |
| EA+NGF group | 43.10± 1.45 | 85.70± 1.25 | 98.80± 1.30 |

### Number of Nestin Positive Cells of MCAO

The results showed that the expression levels of Nestin in the brain of sham operation group was lower than those of the model group, and there was no significant change at all time points. The expression levels of Nestin in rat brain of




A. Model group; B. EA group; C. NGF group; D. EA+NGF group

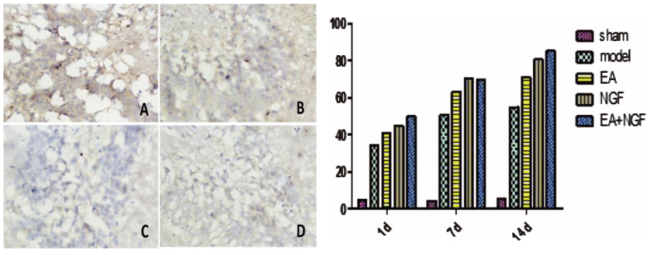
Figure 1 Number of Brdu Positive Cells at Different Time Points in Each Group

EA group, NGF group and EA+NGF group were significantly higher than those in sham operation group at the same time point ($P < 0.01$), indicating that the neurons in brain only stimulated the proliferation of ischemic factors.There was no significant difference between EA group and NGF group at all time points ($P > 0.05$), but there was significant difference between EA group and EA+NGF group ($P < 0.05$), which indicated that EA combined with NGF could promote the proliferation of neurons, and keep the neurons proliferation for a long time in the model group at the same time.

Table 3 Comparison of the Nestin DG Positive Cell Numbers at Different Time Points ($\bar{x} \pm s$)

| Group | Time | | |
|---|---|---|---|
| | 1 d | 7 d | 14 d |
| Sham Operation group | 4.80± 0.92 | 4.30± 0.95 | 5.50± 1.18 |
| Model group | 34.10± 1.10 | 50.80± 1.135 | 54.90± 1.52 |
| EA group | 40.80± 1.14 | 63.20± 0.92 | 71.10± 2.02 |
| NGF group | 44.80± 1.48 | 70.40± 1.43 | 80.70± 1.64 |
| EA+NGF group | 49.70± 1.42 | 69.70± 2.11 | 85.10± 1.10 |

As compared to the sham operation group, $P < 0.01$.




A. Model group; B. EA group; C. NGF group; D. EA+NGF group

Figure 2 Number of Nestin Positive Cells at Different Time Points in Each Group

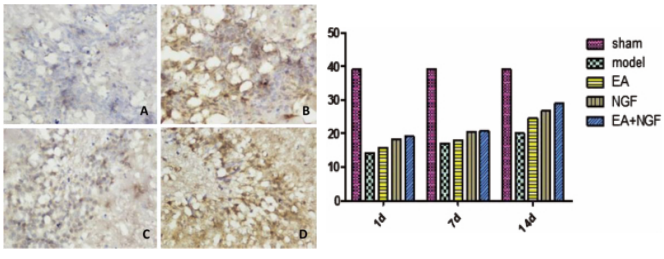
### Number of NeuN Positive Cells

The number of NeuN present cerebral SVZ and SGZ area in model group was less than that in sham operation group, the difference being statistically significant ($P < 0.05$). The expression of NeuN in EA + NGF group was relatively increased as compared to the model group ($P > 0.05$). The number of NeuN in EA + NGF group and NGF group at 14 d after reperfusion was significantly higher than that in EA group ($P < 0.05$).

Table 4 Comparison of NeuN DG Positive Cell Numbers at Different Time Points ($\bar{x} \pm s$)

| Group | Time | | |
|---|---|---|---|
| | 1 d | 7 d | 14 d |
| Sham Operation group | 39.10± 0.99 | 39.10± 0.88 | 39.00± 0.82 |
| Model group | 14.20± 0.92 | 17.10± 0.74 | 20.20± 0.63 |
| EA group | 15.70± 0.67 | 17.90± 0.74 | 24.60± 0.70 |
| NGF group | 18.30± 0.82 | 20.50± 0.53 | 26.70± 0.68 |
| EA+NGF group | 19.10± 1.10 | 20.90± 0.99 | 28.90± 0.99 |

As compared to the sham operation group, $P < 0.05$. Data were represented as Mean ± SEM.




A. Model group; B. EA group; C. NGF group; D. EA+NGF group

Figure 3 Number of NeuN Positive Cells at Different Time Points in Each Group

## Discussion

A recent study found that SVZ and SGZ still have nerve regeneration phenomenon in the adult animal brain. SVZ continuous proliferation of NSCs can produce new neurons, intermediate, and SGZ in neural stem progenitor cells will not become interneurons, but continue to produce new granule cells [3]. NSC self-renewal and multipotential differentiation ability can promote the recovery of neural function in ischemic cerebral infarction. Cerebral ischemic injury may cause SVZ and SGZ NSC proliferation of immature neurons at the same time, cerebral ischemia can activate the hippocampus adjacent periventricular posterior neural progenitor cells, and lead to the proliferation of SGZ cells. After ischemia, the newborn orthotopic and heterotopic nerve cells lose proliferation, migration and differentiation to replace damaged neurons in the brain. Therefore, it is important to promote the recovery of

neurological function, in order to help the brain to maintain its basic functions.

Several recent reports have confirmed that EA has a good regulatory effect on ischemic brain injury neurotrophic factor, thereby supporting its use in cerebral infarction [4]. Stimulation of endogenous NSCs after nerve regeneration by EA actively contributed to neural function recovery and ischemic neuronal proliferation. Clinical rehabilitation experience has shown significant curative effect of EA on nerve dysfunction after cerebral infarction. The molecular mechanism underlying EA treatment remains unknown. Nerve regeneration after cerebral infarction has become a research hotspot in recent years.

NGF is the most important biological substance in the central nervous system. Ischemic brain damage reduces the expression of endogenous NGF [5]. NGF can increase neuronal protection in hypoxia, hypoglycemia, and other abnormal conditions such as nitric oxide damage. Hence, injection of exogenous NGF in the abdominal cavity can effectively protect rats, and reduce the probability of hypoxic-ischemic brain injury. This study confirmed that cerebral ischemia reperfusion damaged the rat neural function. However, NGF could improve the neurological function after cerebral ischemia reperfusion damage in rats and reduce the extent of the damage.

The number of NSCs, differentiation and functional changes are important for selecting the appropriate NSC marker method. In this study, we tracked the markers of Brdu, observed brain tissue in MCAO rats by changes of Brdu labeled cells, to reflect the effect of EA and NGF after cerebral infarction. The results showed that EA and NGF in cerebral ischemia stimulation could cause the proliferation of neurons. Nestin is mainly expressed in the embryonic stage during the development of Central Nervous System. This research showed that nestin positive cells were neural precursor cells in the adult mammalian brain area SGZ, with strong regenerative ability. Nestin plays an important role in neural precursor cell differentiation. From pluripotent cells to NSCs, NSCs to neural precursor cells, differentiation in this continuous differentiation of nestin, skeleton is formed at this stage, and relevant information in cells under the command of a transformation leads to emergence of precursor cells. The high expression of nestin appeared to be transient, the neural precursor cells through mitosis differentiate into mature neurons [2]. EA and NGF group showed significantly increase in the loss of Nestin expression, which peaked at 10 d, then decreased rapidly, and the sham operation group at each time point showed significant difference ($P < 0.05$), that can promote brain neuron proliferation in the normal state, and only when the brain is damaged, it can induce prolonged proliferation and differentiation of neurons. After the statistical analysis, the expression in EA+NGF group 1d was higher than that in the model group, but showed no significant difference ($P > 0.05$), and the expression in EA+NGF group 7d was significantly higher than that in the model group ($P < 0.05$), which showed favorable proliferation due to EA and NGF. The promoting effect was not obvious in the early stage, but was very significant at 7 d. NeuN is a specific protein expressed in most neurons. NeuN is considered to be a marker of a stable and sensitive mature neuron specific antigen, and widely used in the detection of mature neurons [6]. In the Central Nervous System during the developing process of the immune response, increase in the number of mature neurons and the expression of NeuN could reflect the neurons at different developmental stages [7]. NeuN nuclear expression in this study showed comparable expression between the model group and the sham operation group, and ischemic NeuN SVZ and expression in SGZ area was less. After 7 d, positive cells was increased significantly, and the expression was peaked at 14d. The expression in the EA+NGF group was obviously increased, with difference to EA group and NGF group, while the model group showed the least increase. This indicated that EA and NGF at any time after cerebral infarction can transfer the migration of NSCs.

The results showed that the dynamic observation of cerebral ischemic side of endogenous SVZ and SGZ region NSC proliferation growth factor under the intervention of EA combined with NGF can enhance nerve regeneration and functional recovery after nerve injury. But the mechanism underlying the activation of endogenous neural regeneration after cerebral ischemia by EA combined with NGF remains unclear and needs further study.

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