

# EFFECT OF ELECTRO-ACUPUNCTURE ON THE EXPRESSION OF OREXIN-A AND C-FOS IN HYPOTHALAMUS OF SLEEP DEPRIVATION RATS AFTER CEREBRAL ISCHEMIA-REPERFUSION INJURY

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**Abstract:** Objective: To discuss the effect of Electro-acupuncture (EA) on the expression of Orexin-A and C-fos in hypothalamus of sleep deprivation rats after cerebral ischemia-reperfusion injury. Methods: The cerebral ischemia-reperfusion injury (MCAO/R) models were established with reference to the modified longa thread bolt method. All MCAO/R models were divided into the MCAO/R model group, the control group and the experimental group. The sleep deprivation models were made by the method of soft stimulation combined with cage replacement. The rats in the experimental group were given EA intervention for 7 days, and the acupoints were Shenmen and Sanyinjiao. After the experiment, the content of Orexin-A and C-fos in hypothalamus was detected by the method of ELISA. Results: Compared with the blank group, the content of Orexin-A and C-fos of the other four groups increased significantly ( $P < 0.05$ ). Compared with the control group, the content of Orexin-A and C-fos of the experimental group, the MCAO/R model group and sleep deprivation model group decreased significantly ( $P < 0.05$ ). The content of Orexin-A of the MCAO/R model group was significantly lower than the sleep deprivation model group ( $P < 0.05$ ). The content of C-fos of the experimental group was significantly lower than the sleep deprivation model group ( $P < 0.05$ ). Conclusion: The cerebral ischemia-reperfusion injury and sleep deprivation all can activate the expression of Orexin-A neurons and C-fos gene to a certain extent and affect the sleep-wake cycle of rat. EA may regulate the disordered sleep-wake cycle of sleep deprivation rat after cerebral ischemia-reperfusion injury by inhibiting the expression of Orexin-A neurons and C-fos gene.

**Keywords:** Electro-acupuncture (EA); cerebral ischemia-reperfusion injury; Sleep Deprivation; Orexin-A; C-fos.

## 1. INTRODUCTION

Sleep is one of the advanced functions of brain. Long-term sleep disorder can lead to insulin resistance, obesity, cardiovascular and cerebrovascular diseases, etc. However, sleep disorder is also a common complication of ischemic stroke. Previous research have showed that more than 1/2 of patients with ischemic

stroke have sleep disorder. Sleep disorder not only affects the functional recovery of patients, but also may increase the risk of stroke recurrence.

At present, the pathogenesis of sleep disorder after ischemic stroke is not clear. Sedative and hypnotic drugs are widely used to treat such disease, but the drug addiction and side effects are obvious. Therefore, it is urgent to strengthen the

research on the pathogenesis of the disease, and actively explore the intervention measures with reliable curative effect, clear mechanism of action and less side effects. Through long-term clinical research, it is cleared that EA has a positive effect on improving the sleep of patients with ischemic stroke. Previous studies have also confirmed that EA can regulate sleep- wake cycle by regulating 5-HT, NA, DA, Glu, GABA and other neurotransmitters [1]. EA can reduce NO, NOS, SOD and increase CCK-8 in the brain of sleep deprivation rat, which suggest that EA can reduce the oxidative stress and neurotoxicity by restoring the balance between CCK-8 and NO pathway, enhance the activity of SOD in brain tissue, thus delay the atrophy of immune organs and enhance the immune function<sup>[2, 3]</sup>. EA may also can protect the brain and improve sleep by increasing the content of nerve growth factor and reducing the expression of C-fos and C-jun gene in the brain of sleep deprivation rat[4].

This study will discuss the mechanism of EA on the sleep-wake cycle of sleep deprivation rat after cerebral ischemia-reperfusion injury from the perspective of Orexin-A and C-fos.

## 2 EXPERIMENTAL

### MATERIALS

#### 2.1 Experimental Animals

50 healthy Wistar rats of SPF-grade, male, 3~4 months of age, weight 220 ~ 280g (provided by Guangdong Province Medical Laboratory Animal Center). All experiments were carried out in the animal center of Medical department of Jinan University. The experiment operation were in accordance with the regulations of management. The feeding followed the principle of humanity.

#### 2.2 Main Reagents and Instruments

10% chloral hydrate solution, Mouse Orexin-A ELISA Kit, Mouse C-fos ELISA

#### 3.2 The evaluation criteria of MCAO/R model

When the rats waked up, the neural function scores were assessed according to the following evaluation criteria.

Kit(Shanghai Jianglai Biotechnology Co., Ltd), 0.24mm diameter MCAO Monofilaments(Beijing Sadong Biotechnology Co., Ltd), Disposable acupuncture needle(0.22 \* 25MM, Suzhou Hualun medical supplies Co., Ltd), KWD-808-I Acupuncture Instrument(Changzhou Yingdi electronic medical equipment Co., Ltd) etc.

## 3 METHODS

### 3.1 MCAO/R Model Rats

The MCAO/R model rats were established according to the modified suture-occluded method of Zea Longa. Rats were anesthetized with 10% chloral hydrate (3.5ml/kg) by intraperitoneal injection. Neck disinfection and the right side incision were performed. The cervical vessels were separated gently. The right external carotid artery(ECA) and the common carotid artery(CCA) were ligated in turn. A "V" small incision was cut in the right CCA, and then a 0.24mm diameter MCAO monofilament was inserted into the skull. When there was resistance, it meant the monofilament had arrived at the middle cerebral artery(MCA). The length of insertion of the monofilament was about 1.8~2.0cm. After the thread was well fixed, the incision was stitched by layer. 2 hours later, the monofilament was pulled out of 1cm gently and the ends were cut off. (Figure.1)

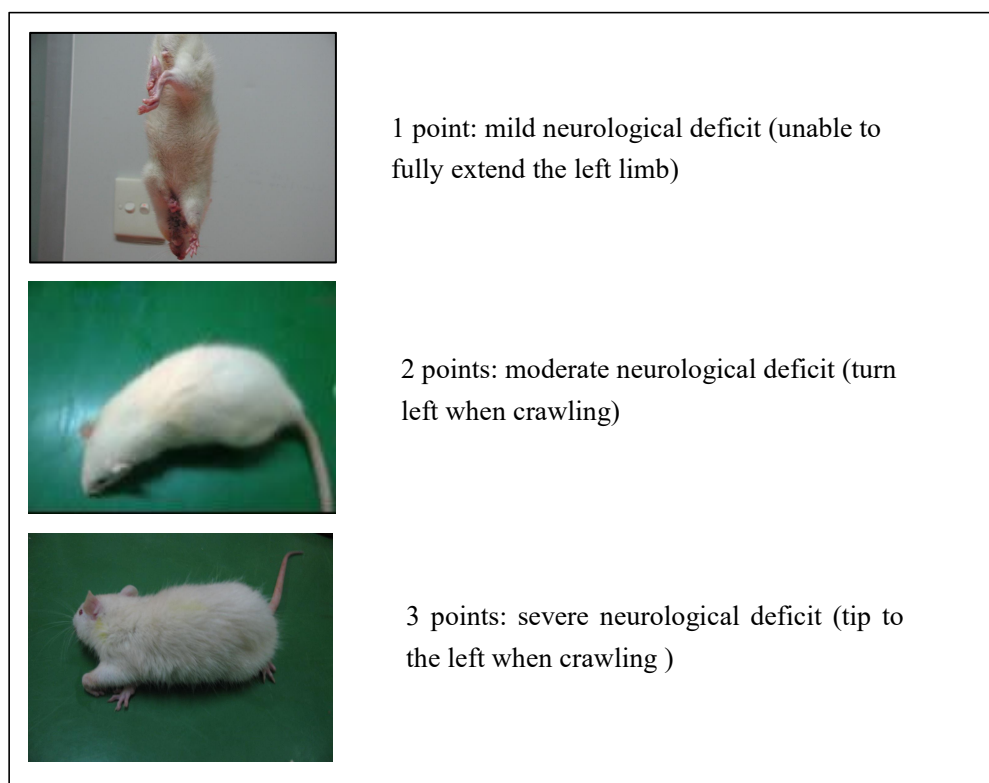


**Fig. 1** The operation picture of MCAO/R model rat

0: no neurological deficit ;  
1 point: mild neurological deficit (unable to fully extend the left limb);  
2 points: moderate neurological deficit (turn left when crawling) ;

3 points: severe neurological deficit (tip to the left when crawling );  
4 points: loss of consciousness and inability to act autonomously

Rats with a score of 1-3 were included in this study. (Figure.2)



**Fig. 2** The evaluation criteria of MCAO/R model

### 3.3 Groups

All MCAO/R model rats were randomly divided into three groups: the experimental group, the control group, the MCAO/R model group, 10 rats in each group. Another 20 rats were randomly divided into two groups: the blank group and the sleep deprivation model group, 10 rats in each group, 5 groups in total.

### 3.4 Intervention

(1)The experimental group: After 48 hours of continuous sleep deprivation, EA intervention lasted for 7 days.

(2)The control group: 48 hours of continuous sleep deprivation without any intervention;

(3)The MCAO/R model group: without any intervention.

(4)The sleep deprivation model group: 48 hours of continuous sleep deprivation without any intervention;

(5)The blank group: without any intervention.

### 3.5 Sleep deprivation

The method of sleep deprivation is soft stimulation combined with cage replacement. When the rats are about to go to sleep, the experimenter will gently pat the cage of the rats or use the stimulation of sound or light to make the rats stay awake. If necessary, the experimenter can also touch the rats directly with paper roll, pencil or hand. The rats were forced to leave their original living environment and enter a new one once an hour, so that they could not enter the sleep state. During the experiment, the rats were continuously illuminated, the indoor temperature was controlled at 18.0-22.0 °C. The above measures lasted for 48 hours.

### 3.6 EA

The manipulation was: 0.22mm diameter needles were inserted into the acupoints of Shenmen and Sanyinjiao to 5mm depth, then connected with the electric-acupuncture apparatus with the

frequency of 2/15HZ and the electrical current stimulation intensity of 2mA for 20min,once a day for 7 consecutive days.

### 3.7 Sampling

After the experiment, the rats were anesthetized by intraperitoneal injection of 10% chloral hydrate. After perfusion, the brain was cut off and the right hypothalamus was separated. After weighing, the supernatant was taken after homogenization and centrifugation.

### 3.8 ELISA

The content of Orexin-A and C-fos in hypothalamus were measured by enzyme-linked immunosorbent assay (ELISA).The specific operation should be carried out in accordance with the instructions. Record the OD value of each sample. In Excel, take the standard concentration as the abscissa and the OD value as the ordinate, draw the linear regression curve of the standard, calculate the concentration value of each sample according to the curve equation.

### 3.9 Statistical Analysis

Statistical analysis was performed using SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA). The measurement data were represented as the mean values± standard deviation ( $\bar{X} \pm s$ ). The experimental results were evaluated using single factor analysis of variance (One-way ANOVA), a P value of less than 0.05 was considered to indicate statistical significance.

## 4. RESULTS

### 4.1 Comparisons of the content of Orexin-A in hypothalamus among groups

Compared with the blank group, the content of Orexin-A of the other four groups increased significantly ( $P < 0.05$ ). Compared with the control group, the content of Orexin-A of the experimental group, the MCAO/R model group and the sleep deprivation model group decreased significantly ( $P < 0.05$ ). The content of Orexin-A of the MCAO/R model group was significantly lower than the sleep deprivation model group ( $P < 0.05$ ). (Table 1, Figure 3)

**Table 1** Comparisons of the content of Orexin-A in hypothalamus among groups(ng/ml)

Group	the content of Orexin-A
The experimental group	27.56±4.38 # *
The control group	34.12±5.27 #
The MCAO/R model group	25.37±3.66 # * □
The sleep deprivation model group	29.19±3.45 # *
The blank group	12.41±2.32

Notes: # indicates  $P < 0.05$ (vs.The blank group); \* indicates  $P < 0.05$ (vs.The control group); □ indicates  $P < 0.05$ (vs.The sleep deprivation model group)

### 4.2 Comparisons of the content of C-fos in hypothalamus among groups

Compared with the blank group, the content of C-fos of the other four groups increased significantly ( $P < 0.05$ ). Compared with the control group, the content of C-fos of the experimental group, the

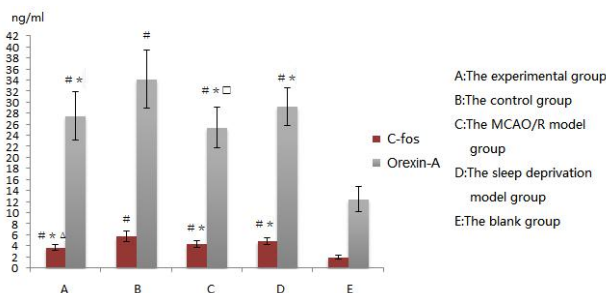
MCAO/R model group and the sleep deprivation model group decreased significantly ( $P < 0.05$ ). The content of C-fos of the experimental group was significantly lower than the sleep deprivation model group ( $P < 0.05$ ). (Table 2, Figure 3)

**Table 2** Comparisons of the content of C-fos in hypothalamus among groups(ng/ml)

Group	the content of C-fos
The experimental group	3.71±0.48 # * Δ
The control group	5.69±0.97 #
The MCAO/R model group	4.28±0.66 # *
The sleep deprivation model group	4.83±0.62 # *
The blank group	1.94±0.32

Notes: # indicates  $P < 0.05$ (vs.The blank group); \* indicates  $P < 0.05$ (vs.The control group);

Δ indicates  $P < 0.05$  (vs. The sleep deprivation model group)



**Fig. 3** Comparisons of the content of C-fos and Orexin-A in hypothalamus

Notes: # indicates  $P < 0.05$  (vs. The blank group); \* indicates  $P < 0.05$  (vs. The control group);

Δ indicates  $P < 0.05$  (vs. The sleep deprivation model group); □ indicates  $P < 0.05$  (vs. The sleep deprivation model group)

## 5 DISCUSSION

Sleep-wake cycle is a physiological process involving multiple systems, which has a complex neural regulatory mechanism. Orexin neurons are located in the lateral hypothalamus (LH), which is not only closely related to sleep-wake regulation, but also involved in regulating mood, energy balance, and reward and drug addiction. Orexin-A is a neuropeptide specifically synthesized and secreted by orexin neurons in the lateral hypothalamus [5].

Estabrooke [6] found that the expression of Fos gene of Orexin neurons in the fornix area of rats was positively correlated with arousal and negatively correlated with sleep, so activation of Orexin neurons may promote and maintain arousal. When Lee [7] recorded the EEG of rats, it was found that orexin neurons in the LH area continued to discharge during the active period of arousal (accompanied by movement), decreased during the quiet period of arousal (not accompanied by movement), and almost stopped discharging during the sleep period. Three hours before microinjection of Orexin-A into the rat's LH area, it was found that the rat's wakefulness increased and sleep decreased by measuring the rat's EEG and EMG [8]. The double OX1R/OXR2 antagonist (ACT-078573) can inhibit the awakening of rats in a dose-dependent manner and increase sleep, especially rapid eye movement (REM) sleep [9]. In the light genetic experiment, it was found that after the expression of OPN4 in the Orexin neurons of the double transgenic

mice (Orexin-tTA, Bitet-O hOPN4/mCherry), short-term blue light irradiation could activate the Orexin neurons and promote the awakening of the mice from the non-rapid eye movement (NREM) sleep [10]. After over-expression of a green light induced protein archt in Orexin neurons of transgenic mice (Orexin-tTA; TetO ArchT), the mouse NREM sleep time was significantly increased and the wake-up time was reduced after exposure of Orexin neurons to green light for one hour during the active period [11]. These experiments all showed that orexin neurons can promote and maintain arousal and regulate sleep-wake cycle.

Immediate early gene (IEG) refers to the genes that are activated first after receiving external information, including C-fos, C-jun, C-myc, C-Myb, etc., which participate in the physiological processes of cell growth, development, differentiation, learning, and memory and information transmission. A large number of data show that C-fos and C-jun proteins are expressed at a low level in nerve cells under normal conditions. In the insomnia activities caused by various chemical and electrical stimulation, C-fos and its protein products can act as the third messenger of neuronal excitation and intracellular information transmission, forming heterodimer acting on the AP-1 site in the target gene regulatory sequence, thus activating LRG. The expression products of LRG change the excitability of neural network and the intrinsic components of

neurons, such as the decrease of GABA release, the increase of glutamate release and the sprouting of neurons, which eventually leads to the destruction of the excitation inhibition balance of neural network and insomnia. Therefore, c-fos is closely related to sleep-wake state [12].

The results of this study confirmed that cerebral ischemia-reperfusion injury and sleep deprivation can activate the expression of Orexin-A neurons and C-fos gene to a certain extent and affect the sleep-wake cycle of rat. At the same time, compared with the control group, the expression of Orexin-A and C-fos protein of the experimental group decreased significantly. EA may regulate the disordered sleep-wake cycle of rats after cerebral ischemia-reperfusion injury by inhibiting the expression of Orexin-A neurons and C-fos gene. However, the mechanism of EA has the characteristics of multi-level and multi-target, so more researches on the mechanism of EA on sleep disorders after cerebral ischemia-reperfusion injury need to be further studied.

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#### REFERENCES

- [1] Zhou P, Zhao CH, Ma XM. Effect of laser acupoint irradiation on monoamine transmitters in hypothalamus of insomnia rats[J]. Chinese J of Clin Healthcare , 2012,15(1):50-52.
- [2] Zhao CH, Lin ZM, Jiang I. Effect of different amount of moxibustion on the content of no and NOS in hypothalamus of insomnia rats[C]. China Association of Acupuncture-Moxibustion. Proceedings of 2011 Annual Meeting of Chinese Acupuncture Association (Abstract), 2011:449-454.
- [3] Li YH, Zhang LT. Effect of acupuncture on the content of 5-HT and NGF in rats with exercise-induced insomnia [J]. Zhejiang J Tradit Chin Med, 2009,44(6):402-403.
- [4] Zhang LT, Li YH. Effect of acupuncture on the content of C-fos and C-jun in rats with exercise-induced insomnia[J]. Lishizhen Med Mat Med Res, 2009, 20(9):2233-2234.
- [5] Sakurai T, Amemiya A, Ishii M, et al. Orexins and orexin receptors : a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior[J]. Cell, 1998,92(4):573-585.
- [6] Estabrooke IV, McCarthy MT, Ko E, et al. Fos expression in orexin neurons varies with behavioral state [J]. J Neurosci, 2001,21(5):1656-1662.
- [7] Lee MG, Hassani OK, Jones BE. Discharge of identified orexin/hypocretin neurons across the sleep-waking cycle [J]. Neurosci, 2005,25(28):6716-6720.
- [8] Cun Y, Tang L, Yan J, et al. Orexin A attenuates the sleep-promoting effect of adenosine in the lateral hypothalamus of rats [J]. Neurosci Bull, 2014,30(5):877-886.
- [9] Brisbare-Roch C, Dingemans J, Koberstein R, et al. Promotion of sleep by targeting the orexin system in rats, dogs and humans [J]. Nat Med, 2007, 13(2):150-15.
- [10] Tsunematsu T, Tanaka KF, Yamanaka A, et al. Ectopic expression of melanopsin in orexin/hypocretin neurons enables control of wakefulness of mice in vivo by blue light[J]. Neurosci Res, 2013,75(1):23-28.
- [11] Tsunematsu T, Tabuchi S, Tanaka KF, et al. Long-lasting silencing of orexin/hypocretin neurons using archaerhodopsin induces slow-wave sleep in mice [J]. Behav Brain Res, 2013, 255:64-74.
- [12] Song GP, Miao DM, Huang PE, et al. Expression of c-fos protein in brain stem of rats after 96 hours of continuous and partial sleep deprivation[J]. Chin J Clin Psychol , 2003,11(1):9.