Front. Agric. China 2007, 1(2): 224–228
DOI 10.1007/s11703-007-0039-2

RESEARCH ARTICLE

LUO Qihui, CHEN Zhengli, ZHU Chunmei, FAN Guangli, HUANG Yidan

Effects of Shenpang acupoint-stimulation on estrogen receptor immunoreactive neurons in thalamus of rabbits

Abstract To investigate the effects of Shenpang acupoint-stimulation in reproductive endocrinology, the changes in estrogen receptor immunoreactive (ER-IR) neurons after Shenpang acupoint-stimulation were studied by using immunohistochemistry. ER-IR positive reactions were detected in most nuclei of the thalamus. In the acupuncture-treated group, a great number of ER-IR positive neurons with clear dendrites existed in the nucleus, paraventricular nucleus, ventrolateral nucleus, ventromedial nucleus, ventro-principal nucleus, centromedian nucleus, reticular nucleus, and periventricular nucleus of thalamus, and they were strongly stained. In addition, the ER-IR positive neurons were mainly located in the cytoplasm, nucleus and neutrite, and some also existed in the cytoplasmic membrane. In contrast, a few neurons existed in the above-mentioned nuclei in the control group, but they were slightly stained. It is concluded that Shenpang acupoint-stimulation can promote the expression of estrogen receptors in the above nuclei.

Keywords rabbit, electrical acupuncture, Shenpang acupoint, estrogen receptor, thalamus

1 Introduction

Modern researches in Traditional Chinese Medicine have proven that the application of acupuncture stimulation at some acupoints can change the level of estrogen, and can regulate the feminine abilities in reproduction and internal hormones (Liu and Zhu, 1998; Lang et al., 2000; Chen et al., 2004). However, all those researches were not supported by morphological evidence. We discovered that the expression of estrogen receptors in most nuclei of the hypothalamus was improved by Shenpang acupoint-stimulation (Chen et al., 2004). In addition, relay nuclei for visual sensation and acoustic sensibility in the thalamus play a role in the ability and activity of reproduction; such as when animals change sounds with the estrous cycle so as to attract the opposite sex whose sounds can likewise stimulate sexual behaviors. In the present study, healthy rabbits were employed as experimental animals, with the pars thalamica in the diencephalon as the observed parts. Anti-rat ER polyclonal antibodies were used as the first antibody to investigate the effects of Shenpang acupoint-stimulation on the estrogen receptors in the thalamus, and the effects and significance of estrogen receptor immunoreactive (ER-IR) neurons post-Shenpang acupuncture. The experiment was designed to provide more morphological evidences for understanding the regulative mechanism of reproduction and internal hormones by Shenpang acupoint-stimulation.

Translated from *Acta Veterinaria et Zootechnica Sinica*, 2006, 37(1): 71–75 [译自: 畜牧兽医学报]

LUO Qihui, CHEN Zhengli (✉), ZHU Chunmei, HUANG Yidan
College of Animal Science and Technology, Sichuan Agricultural University, Yaan 625014, China
E-mail: chzhli75@163.com

FAN Guangli
College of Animal Science and Technology, Northwestern Agriculture and Forestry University, Yangling 712100, China

2 Materials and methods

2.1 Experimental animals

After identification of empathema, eight young normotrophic female rabbits, identified conventionally to be in estrus, were used as experimental animals.

2.2 Experimental methods

2.2.1 Method of the acupuncture point and electro-acupuncture

The Shenpang acupoint was chosen according to Chen et al. (2004). Eight young female rabbits in estrus were randomly divided into two groups. One group was the control and the other was the electro-acupuncture treated group. Animals in the electro-acupuncture treated group were electrically-treated at Shenpang acupoints (Chen et al., 2004) for 30 min.

The control group received a false stimulation, i.e. no electro-acupuncture was carried out on the control animals.

2.2.2 Preparation of tissue sections

Sample collection and processing were carried out as described by Chen et al. (2004), and serial frontal cryostat sections (30 μm thick) of the brain with thalamus were prepared. Two sets of the sections were stained by immunohistochemical method, Nissl's staining and negative control. Brain regions were located according to Alvin L Berman.

2.2.3 Methods of immunohistochemistry

The sections for immunocytochemistry were rinsed thrice, each for 10 min with 0.01 mol phosphate buffered saline (PBS, pH 7.4), and then incubated in endogenous peroxidase blocking solution and in calf serum for 30 min. The sections were then reacted with ER polyclonal antibody (Boster) at a dilution of 1:150 for 72 h at 4°C and then incubated in the second antibody and in streptavidin-peroxidase each for 1 h at room temperature. The sections were washed thrice for 10 min in PBS after each step, stained in ammonium nickel sulfate-DAB for no more than 30 min then washed twice (10 s each) in PBS. The solution was prepared as follows: 0.03 g DAB, dissolved and filtered in 50 mL double distilled water, was added to 50 mL nickel sulfate amine acetic acid buffer, followed by 0.2 g glucose, 0.04 g ammonium chloride and 0.001 g glucose oxidase. Finally, the sections were immersed, dehydrated, and observed under a microscope. 0.01 mol/L PBS (pH 7.4) was used in the negative control group instead of ER polyclonal antibody. The other steps were the same as above.

2.2.4 Microphotograph, image analysis and data processing

The sections were observed under a light microscope and data were recorded. Motic digital microscope was used to take photos. Analysis of the positive cells was done using a Jiangsu Jieda 801 series morphological analysis system. Five pieces of the sections were chosen randomly from among the different materials in every group. The number of positive cells and the value of the gray scale were measured. Data were expressed in $\overline{X} \pm \mathrm{SD}$. The results were saved in the computer and statistical analysis was done using the *t* test.

3 Results

3.1 Distribution of ER in thalamus

Because of the ammonium nickel sulfate used in the experiment, the color of the positive product was atroceruleous, but the same section in the negative control showed no atroceruleous-positive products. The distribution of ER-IR was widespread in the thalamus, particularly in the nuclear zone near the hypothalamus and in the periventricular zone of the third ventricle. There were more positive products and strong positive chromatosis. However, the distribution of ER-IR in the positive cells was complicated, mainly located in the plasma, nucleus and neurite, and also in the cytoplasmic membrane.

3.2 Effects of Shenpang acupoint-stimulation on ER expression in thalamus

Table 1 illustrates the exact effects of Shenpang acupoint-stimulation on ER-IR expression in the thalamus in rabbits. Compared with the control group, the general tendency was that the number of ER-IR positive products in the acupuncture group was significantly higher with stronger positive chromatosis.

In observing the ventrolateral nucleus in the control group, weakly positive neurons could be seen occasionally. The neurite was indistinct and cell circumsciption was indefinite. There was no chromatosis in the nuclei; in the acupuncture group, a great number of strongly-stained, positive, large-sized cells could be observed, with definite neurites that interlaced like a net. There also existed a few positive nuclei. In addition, the number and intensity of the stained neurons were significantly higher than the control group ($P < 0.01$) (Plate I 1–2). The same changes could also be seen in the ventromedial nucleus. In the ventroprincipal nucleus, no significant ($P > 0.05$) changes in ER-IR products were seen in

Table 1 The effects of Shenpang acupoint-stimulation on ER-IR production in the thalamus in rabbits

Nuclei	Group	Neuron number	Gray scale	Nuclei	Group	Neuron number	Gray scale
Ventrolateral nucleus (TVL)	Control	10.21 ± 3.26	127.19 ± 6.57	Paraventrcular nucleus (TPAV)	Control	70.28 ± 8.54	105.18 ± 6.26
	Acupuncture	$25.61 \pm 4.47^{**}$	$81.91 \pm 6.53^{**}$		Acupuncture	$110.73 \pm 8.25^{**}$	$85.75 \pm 6.62^{**}$
Ventromedial nucleus (TVM)	Control	9.38 ± 4.27	118.61 ± 5.36	Centromedian nucleus (TCM)	Control	73.62 ± 9.46	108.58 ± 6.34
	Acupuncture	$25.66 \pm 4.13^{**}$	$86.27 \pm 5.06^{**}$		Acupuncture	$125.24 \pm 10.23^{**}$	$88.73 \pm 6.09^{**}$
Ventroprincipal nucleus (TVP)	Control	45.83 ± 7.54	98.46 ± 5.24	Reticular nucleus (TR)	Control	76.83 ± 9.15	115.84 ± 6.43
	Acupuncture	51.04 ± 6.73	96.49 ± 4.86		Acupuncture	$165.77 \pm 11.54^{**}$	$81.64 \pm 5.51^{**}$
Periventrcular nucleus (TPEV)	Control	68.05 ± 7.24	115.09 ± 5.84	dorsomedial nucleus (TDM)	Control	18.27 ± 4.15	115.46 ± 7.24
	Acupuncture	$97.16 \pm 7.91^{**}$	$92.46 \pm 5.57^{**}$		Acupuncture	20.42 ± 4.31	111.54 ± 6.13
Mamillothalamic tract (MTT)	Control	23.17 ± 5.74	108.06 ± 5.47	dorsolateral nucleus (TDL)	Control	19.51 ± 5.60	117.54 ± 5.94
	Acupuncture	22.64 ± 4.39	106.35 ± 5.33		Acupuncture	22.48 ± 4.63	114.07 ± 6.25

Notes: Compared with the control group, ** stands for $P < 0.01$; the lower the gray scale, the deeper the color, which showed more positive expressions.

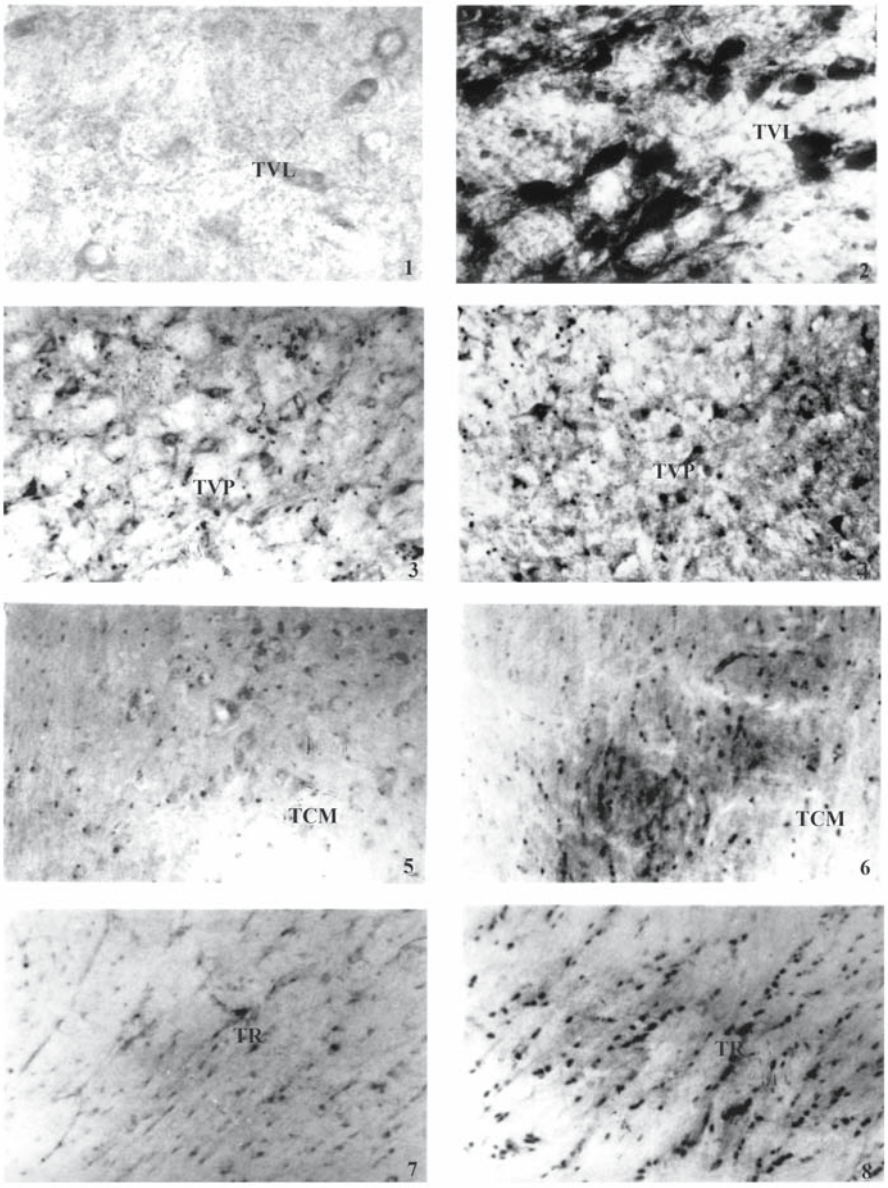

Plate I Comparison of ER-IR cells between the control group and the acupuncture group
1: ER-IR cells in the TVL of control group ×400; 2: ER-IR cells in the VL of acupuncture group ×400; 3: ER-IR cells in the TVP of control group ×400; 4: ER-IR cells in the TVP of acupuncture group ×400; 5: ER-IR cells in the TCM of control group ×400; 6: ER-IR cells in the TCM of acupuncture group ×400; 7: ER-IR cells in the TR of control group ×400; 8: ER-IR cells in the TR of acupuncture group ×400.

the acupuncture group (Plate I 3–4). In the centromedian nucleus (Plate I 5–6), ER-IR products were mainly located in the nucleus zone, though a few were seen in the cytoplasm. The positive products in the control group were fewer and weakly stained. In the acupuncture group, there were more strongly stained neurons. The difference between the two groups was significant ($P < 0.01$). However, in the central zone, like the paraventricular nucleus and periventricular nucleus, the location and change tendency of nuclei were the same as the above. In the central part of the reticular nucleus, positive products were mainly located in the nuclei and some were in the neurites, distributed declivently like a girdle. It can be seen that in this part, the number and stain intensity of positive products in the control group decreased significantly ($P < 0.01$) (Plate I 7–8).

Moreover, there were ER-IR products in the dorsomedial nucleus, dorsolateral nucleus and mamillothalamic tract. They were weak-positive neurons with low density and unequal size. The difference between the two groups was not significant.

4 Discussion

Recent researches have discovered that the brain is one of the important target organs of estrogen. Many nuclei in the brain

can produce estrogen synthetase and express ER. In our experiment, we observed that ER-IR products were widely distributed in the thalamus, mainly located in cytoplasma, nucleus and neurite, and also existed in the cytoplasmic membrane. These results proved that estrogen plays an important part in the thalamus in a very complex manner. Perhaps one mechanism is that estrogen combines with the cytosol receptor and goes into the nuclei or it combines directly with the nuclear receptor to modulate the transcription and expression of the gene; another mechanism could be that estrogen mediates the secondary messengers in the cells through membrane receptors to accommodate the nongenomic mechanism, and to control the animal's advanced behaviors.

The thalamus is a very important part of the diencephalon. It is an extremely complicated integration center for aesthesia. Every kind of sense impulse is translated in the thalamus before reaching the cerebral cortex. The thalamus has an important relationship with all kinds of feelings, movement, reproduction and behavior control. It has already received the general attention of scholars (Zeng et al., 1999; Zuo, 1998; Chen et al., 2001). The wide distribution of ER in the thalamus proves that estrogen plays an important role in the transmission and regulation of all kinds of feelings. It was observed that there was a greater number and deeper color of positive products in the nuclear zone near the thalamus and in the periventricular nucleus of thalamocele. This means that in the process of the animal's evolution towards advanced movement, perhaps the thalamus was related to the hypothalamus and needed the neurotransmitters in the cerebrospinal fluid of the thalamocele. On the other hand, in comparison with the control group, it was discovered that the number of ER-IR products in the acupuncture group rapidly increased. This proved that Shenpang acupoint-stimulation can promote the expression of ER-IR products in the thalamus of rabbit and can adjust the more advanced function of the thalamus, like the sense of sight, sense of hearing and reproduction. Researches on reptiles, birds and mammals have shown that the voice of animals during mating season affects the changes in the function of the genitical gland (McComb, 1987; Cheng and Zuo, 1994). There are reports that the centrifugal fiber projects to the hypothalamus from the acoustic area in the thalamus of reptiles and mammals. Durand et al. (1992) reported for the first time that the ovoid-shaped demilune nucleus, which is in the ventral part of oval nucleus, has the centrifugal fiber to project to ventromedial nucleus of the ring dove's thalamus and the ventromedial nucleus was proved to be the important nucleus of reproduction of birds (Bernstein et al., 1993). It also proved that the sound and hearing of birds have connection to and has an effect on the endocrine encephalic region. These results are in conformity with our study. Zuo (1998) discovered the locations of the centrifugal projection in the L zone near the neostriatum of the telencephalon, the oval nucleus and the ventromedial nucleus at the same time after injection of biotin-labeled glucosan amine to the oval nucleus in the caudal part of the dove's thalamus; and for the first time, Zuo gained anatomical evidence that the relay nucleus of acouesthesia has a direct nerve connection with some endocrine nuclei. Based on all these researches and our result, it can be inferred that in the mammal's thalamus, there are many nuclei related to the movement of nerve reproduction and endocrine secretion of the hypothalamus. Shenpang acupoint-stimulation can promote the expression of ER-IR products in these nuclei and enhance a mammal's reproduction function and endocrine secretion.

On the other hand, the ventromedial nucleus was discovered to be the main projection target site of the basal nucleus in the rat's thalamus and the last relay link before basal nucleus's message projects to sensory motor cortex (Craig et al., 1982). It can also receive the projection of the nucleus in the deep part of the cerebellum (Yoshida et al., 1992). That means the two subcortex circuit loops, which adjust body movement, converge considerably in the level of the thalamus. Basal forebrain is known to be the main area of the central regulation for learning and memory. Our study discovered that ER-IR products appeared in the ventromedial nucleus, and was promoted in its expression after acupoint-stimulation. It proved that the nucleus of the thalamus plays an important part in adjusting body movement, learning and memory, and that it can be regulated by Shenpang acupoint-stimulation. Accordingly, Shenpang acupoint-stimulation can delay the occurrence of gerontal nerve degenerative disease like senile dementia and Parkinson's disease in the lab in some ways.

These results indicate that the sensory information in the acupoint can go into the spinal cord through the posterior root ganglion after Shenpang acupoint-stimulation, then go up into the brain through the spinal cord (directly or indirectly), and is transmitted to the encephalic region like the hypothalamus and the telencephalamus directly or after relaying, resulting in the activation of the function and movement of nerve cells in the central nerve system. Estrogen in the nuclear zone of the thalamus binds with ER to adjust the related gene expression of nerve cells, so as to function in information transfer, and reproductive regulation of domestic animals through neurotransmitter transportation by the hypothalamic-pituitary-gonadal axis or cerebrospinal fluid in the thalamocele. It can also promote reproduction and the endocrine, empathema, follicular development, ovulation and androlepsis. Besides, it can also promote the function of nerve cells in the thalamus which relates to advanced movement such as body movements, learning and memory, emotion and so on.

Shenpang acupoint-stimulation regulates reproductive and endocrinal hormones and this study is a breakthrough in exploring the ways to adjust these hormones in Traditional Chinese medicine. Acupuncture is an economical and safe method without side effects and easy to spread and apply.

Acknowledgments The project was supported by the National Natural Science Foundation of China (No. 39670550), the Sichuan Agricultural University Introducing Wisdom Foundation, China (No. 00223600), and the Sichuan Agricultural University Youthful Science & Technology Innovation Foundation, China (No. 00230800).

References

Bernstein P L, Zuo M X, Cheng M F (1993). Social condition affects the courtship behavior of male ring doves with posterior medial hypothalamic lesions. Behav Neurol Biology, 59: 120–125

Chen Z L, Fan G L, Lang Z W, Zhang M, Zheng X M, Niu J R, Yang G L, Zhang G W (2004). The effects of Shenpang acupoint-stimulation on estrogen receptors immunoreactive neurons in hypothalamus in rabbits. Acta Veterinaria et Zootechnica Sinica, 35(2): 222–226 (in Chinese)

Chen Z L, Fan G L, Xu Y P, Wu J Y, Xu X M (2001). Distribution of ChAT in thalamic optical and auditory nuclei in 1 d chicken. Chinese Journal of Veterinary Science, 21(6): 581–582 (in Chinese)

Cheng M F, Zuo M X (1994). Proposed pathways for vocal self-stimulation: Met-enkephalinergic projections linking the midbrain vocal nucleus, auditory-responsive thalamic regions and neurosecretory hypothalamus. J Neurobio, 25: 361–379

Craig A D, Wiegand S J, Price J L (1982). The thalamo-cortical projection of the nucleus submedius in the cat. Comp Neurol, 206(1): 28–48

Durand S E, Tepper J M, Cheng M F (1992). The shell region of the nucleus ovoidalis: A subdivision of the avian auditory thalamus. J Comp Neurol, 323: 495–518

Lang Z W, Yang G L, Zhang G W, Niu J R (2000). Functional regulation of acupuncture acupoint on reproductive-endocrine of cow. Study and Application of Traditional Chinese Veterinary Medicine, 189–192 (in Chinese)

Liu Q, Zhu D N (1998). The effects of acipoint-stimulation and parachlorophenylalanine on ER level in brain stem and cardiac muscle of reproductive period. Shannxi Traditional Chinese Medicine, 18(2): 90–91 (in Chinese)

McComb K (1987). Roaring in red deer stags advances the date of oestrus in hinds. Nature, 330: 648–649

Yoshida A, Dostrovsky J O, Chiang C Y (1992). The afferent and efferent connections of the nucleus submedius in the rat. Comp Neurol, 324(1): 115–133

Zeng Q H, Zhang J L, Lan S C (1999). A comparative study of auditory centre and neural pathway in anura amphibian Bufo Gargarizans and Songbird. Chinese Journal of Neuroanatomy, 15(1): 75–78 (in Chinese)

Zuo M X (1998). Efferent projections of thalamical auditory nuclei in ring dove. Acta Zoologica S inica, 44 (1): 35–40 (in Chinese)