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Elevated estrogen receptor expression in hypothalamic preoptic area decreased by electroacupuncture in ovariectomized rats

Shulan Ma[a,b], Jihong Wu[c], Yi Feng[a,*], Boying Chen[a,*]

[a] WHO Collaborating Center for Traditional Medicine, Institutes of Brain Science, Department of Integrative Medicine and Neurobiology, State Key Lab of Medical Neurobiology, Shanghai Medical College, Fudan University, Shanghai, China
[b] Teaching Center of Experimental Medicine, Shanghai Medical College. Fudan University, Shanghai, China
[c] Experimental Research Center, EYE & ENT Hospital of Fudan University, Shanghai, China

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ABSTRACT

In the present study, effects of electroacupuncture (EA) on estrogen receptor alpha (ERα) and beta (ERβ) mRNA and protein expression in the hypothalamus of ovariectomized (OVX) rats were detected by quantitative real-time reverse transcription PCR (qRT-PCR) and western blot analysis. Gonadotropin-releasing hormone (GnRH) release and GnRH mRNA level in hypothalamic preoptic area (POA) were evaluated by push–pull perfusion and qRT-PCR. Our results showed that elevated mRNA and protein expression of ERα and ERβ in hypothalamus were restrained following EA treatment in OVX rats. EA treatment also inhibited GnRH release and GnRH mRNA levels in OVX rats. These results provide novel evidence that EA treatment down regulates the expression of hypothalamic estrogen receptors (ERs), thus restores the response of GnRH neurons to estrogen depression, and partially resets the negative feedback of estrogen to hypothalamus–pituitary–ovary axis (HPOA) in OVX rats, which may be a critical mechanism for EA on female reproductive disorders.

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Acupuncture therapy has been used to treat female reproductive disorders for more than 2000 years in oriental medicine. Over the last decade, acupuncture has also become an established therapy in Western reproductive medicine [15,21]. Electroacupuncture (EA) treatment exerts beneficial neuroendocrine effects on reproductive dysfunction by modulating neuroendocrine system [22]. Our group has been studying the mechanism of acupuncture using ovariectomized (OVX) rats as a model system for hypothalamus–pituitary–ovary axis (HPOA) dysfunction [3,24]. Bilateral ovariectomy results in loss of ovarian-derived estrogen and unrestrained elevated levels of hypothalamic gonadotropin-releasing hormone (GnRH) and circulating luteinizing hormone (LH) in female rats [3]. Our previous studies showed that after repeated EA treatment at a group specific acupoints in OVX rats, plasma estradiol (E_2) increased [3,24], elevated plasma LH was reduced and hypersecretion of GnRH was inhibited [3], suggesting that EA might beneficially regulate HPOA. However, the mechanisms remain unclear.

We have reported that EA stimulates hypothalamic aromatization and increases local estrogen synthesis in OVX rats [26]. EA increases the release of neuroactive substances, such as beta-endorphin (βEND) [3] and GABA (unpublished data) in the medial preoptic area of OVX rats. Neurons that synthesize and release βEND and GABA are commonly believed to be estrogen-sensitive neurons to regulate GnRH neuronal function [7]. Since the effects of estrogen are local and are mediated by estrogen receptors (ERs), study of the expression of ERs of OVX rat may be very useful in understanding EA mechanism. We hypothesized that EA treatment might be able to partially restore the function of HPOA in OVX rats by regulation of the expression of ERs in hypothalamus to recover the response of GnRH neurons to estrogen negative feedback, and to inhibit the hypersecretion of GnRH. In present study, the effects of EA on the expression of ER-alpha (ERα) and ER-beta (ERβ), as well as GnRH release and its mRNA expression in hypothalamus, were observed.

Sixty-five female Sprague–Dawley rats (200–220 g), with regular 4-day estrus cycles, were purchased from Sino-British Sippr/BK Lab Animal Ltd., Shanghai, China. The rats were housed under laminar flow in an isolated room with controlled temperature and at a 12/12 (light/dark) schedule. Food and water were available ad libitum. Rats underwent either bilateral ovariectomy (OVX, $n = 45$) or surgery but no ovariectomy (Sham, $n = 20$) with isoflurane. OVX rats were randomly divided into ovariectomized (OVX; $n = 20$) and OVX with EA treatment (OVX + EA; $n = 25$) groups. Four weeks after ovariectomy, rats in OVX + EA group received EA treatment. For easy handling of the rats, all rats were lightly anesthetized with isoflurane for 2–3 min before being fixed in a fabric harness suspended

* Corresponding authors. Tel.: +86 21 54237693; fax: +86 21 54237526.
E-mail addresses: fengyi17@fudan.edu.cn (Y. Feng), chen-bo-ying@hotmail.com (B. Chen).

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above a desk. During EA treatment, the rats were conscious and alert. Stainless steel needles of 0.3 mm diameter were inserted into four acupoints in the belly as follows: the "Guanyuan" (CV4), the "Zhongji" (CV3), with one needle flatly punctured from CV4 to CV3, and the bilateral "Zigong" (EXTRA22, 5 mm deep). The above three needles were jointly connected with the positive pole of the Han's Acupoint and Nerve Stimulator (Model LH202H, Huawei Co. Beijing, China). The negative pole was connected with the other needle, which was inserted 3 mm at the "Sanyinjiao" (SP6) in the hind leg. This group specific acupoints have been widely applied in both acupuncture clinic and basic research in the treatment of gynecological disease [5,13]. The electrical stimulation was maintained for 30 min (during 09: 00–11: 00 am). The stimulating current was applied at a frequency of 2 Hz with pulse width of 0.6 ms for a total duration of 3 s. The intensity was adjusted to produce local muscle contractions and varied from 0.8 mA to 1.4 mA. Rats in Sham and OVX were also anesthetized, suspended in a harness, and handled in the same manner as the rats in OVX + EA but without needle insertion and electrical stimulation. Treatments were applied Q.D. for 3 days. All experimental procedures involving the use of animals were in accordance with the guidelines of the national institutes for the Care and Use of Laboratory Animals.

At the time of sacrifice (6 h after the last EA treatment), the vaginal cytology of each rat was first examined. The rats with epithelial cells reappeared in OVX + EA were adopted for further treatment and tissue collection. The reappearance of epithelial cells could be used as an indicator of EA treatment. The blood samples of OVX, OVX + EA and Sham rats were collected from tail incision. The plasma was separated by centrifugation and stored at −80 °C. Plasma E_2 levels were measured by double-antibody radioimmunoassay (RIA) kits purchased from the National Atomic Energy Research Institute (Beijing, China.). The assay sensitivity of the kit was 1.4 pg/ml and the intra- and inter-assay coefficients of variation were 4.74% and 7.7%, respectively. With the blood sampled, the brains of all the animals were removed. The hypothalamic preoptic area (POA) was scraped and subjected to RNA and protein extraction. The POA was defined as follows: the initiation of the third ventricle was determined as the rostral limit, the site where the entry point of the optic chiasm as the caudal limit, bottom of the anterior commissure as the dorsal limit, the upper side of the optic chiasm as the ventral limit, and an extension of both lateral ventricles as the side limit.

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) was employed for gene expression analyses. The rat POA fragments (five for each group) were used for total RNA isolation and reverse transcription. Amplification of individual genes was performed on the iCycler iQTM real-time PCR detection system (Bio-Rad, Richmond, CA) using SYBR Green RealMasterMix (TIANGEN, China) and a standard thermal cycler protocol. Sense and antisense primers used for amplification in this study were as follows: 5′-GCGGCTGCCACTGACCATG-3′ and 5′-CCTCGGGGTAGTTGAACACGG-3′ for ERα; 5′-AAAGCCAAGAGAAACGGTGGGC-3′ and 5′-GCCAATCATGTGCACCAGTTCC-3′ for ERβ; 5′-AGGAGGATCAAATGGCAGAACC-3′ and 5′-TCTTCAATCAGACGTTCCAGAGC-3′ for GnRH; 5′-AATTCCGATAACGAACGAGA-3′ and 5′-ATCTAAGGGCATCACAGACC-3′ for 18S ribosomal RNA. All real-time experiments were run in triplicate and a mean value was used for the determination of mRNA levels. Relative mRNA expression levels for ERα, ERβ and GnRH were determined using the $2^{-\Delta\Delta Ct}$ method and normalized to the 18S rRNA.

Each of six rats in Sham, OVX and OVX + EA was used to investigate ERβ and ERα protein expression by western blot with a standard procedure. The POA fragments were homogenized in RIPA buffer and protein concentration were determined with BCA protein assay (Pierce, Rockford, IL). Protein were loaded, separated on 10% SDS polyacrylamide gels and electro-transferred on polyvinyldifluoride membranes, which were then incubated overnight with primary antibody (Rabbit anti-ERβ polyclonal antibody, 1:500, Upstate Biotechnology, Lake Placid, NY; Rabbit anti-ERα monoclonal antibody (MC-20), 1:200, Santa Cruz Biotechnology, Inc., Santa Cruz, CA; mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody, 1:5000, Sigma,USA) followed by SuperSignal® West Pico Chemiluminescent Substrate (Pierce, USA). The developed film signals were quantified using Image J software. The results were expressed as intensity of the signals in arbitrary densitometry units after normalization to GAPDH as an internal standard.

Three weeks after OVX, push–pull cannulas (PPC) were implanted in the rest of rats of Sham ($n = 9$), OVX ($n = 9$) and OVX + EA ($n = 12$). A stainless steel outer PPC [0.7 mm outer diameter (OD)] fitted with a removable stylette (0.35 mm OD) was stereotaxically inserted into the rat brain under pentobarbital anesthetization. The tip of the cannula was directed toward the medial preoptic area (A, 0.6 mm posterior to the bregma; L, 0.6 mm from the midline; H, 8.0 mm ventral from the dura) [16]. The cannula was fixed onto the skull with dental acrylic cement. After one week recovery, rats in OVX + EA received EA treatment for three consecutive days. On the day of perfusion, the inner stylette was removed and replaced with the inner cannula perfusion assembly. After an hour equilibration period, the push–pull perfusion (PPP) was collected for 30 min while OVX + EA rats were treated with EA for the third time. Artificial cerebrospinal fluid (aCSF, pH 7.4) made up of 127.6 mM NaCl, 2.5 mM KCl, 1.4 mM $CaCl_2$, 1.0 mM $MgSO_4$, 12 mM sodium phosphate, and 0.1 mM bacitracin was pumped through the inner cannula and pulled up between the inner and outer cannulae by two peristaltic slow-speed pumps (HL-2, Shanghai, China) at a constant flow rate of 20 μl/min. Perfusate fractions were collected continuously on ice and were then rapidly frozen at −80 °C for subsequent GnRH RIA. After perfusion, only rats confirmed with the correct cannula placement were considered suitable for further measurement. The GnRH level in push–pull perfusates was measured using double-antibody RIA kits purchased from Sinouk Institute of Biological Technology (Being, China). The sensitivity for the GnRH RIA was 4 pg/ml and the intra- and inter-assay coefficients were 4.1% and 6.2%, respectively.

Data were analyzed using one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls (S.N.K) test for multiple comparison. All data were presented as means ± SEM. p-value less than 0.05 were considered significant.

The percentage of mature vaginal epithelia and the concentration of E_2 are presented in Table 1. A regular 4-day estrus cycle was observed in Sham whereas the cyclic change disappeared in OVX and OVX + EA. Few mature vaginal epithelia were observed in the vaginal smears of the OVX, and the percentage of mature epithelia increased significantly in the OVX + EA ($p < 0.001$). The blood E_2 concentration decreased significantly in the OVX ($p < 0.01$, $p < 0.05$) compared with that in the Sham and OVX + EA, and was higher in OVX + EA ($p < 0.05$) than in the OVX, but still lower compared to the Sham ($p < 0.05$).

Table 1
Percentage of mature vaginal epithelia and concentration of blood estradiol of Sham, OVX and OVX + EA rats.

Group	Percentage of mature vaginal epithelia (%)	Blood E_2 level (pg/ml)
Sham ($n = 6$)	19.48 ± 1.06	103.57 ± 7.27
OVX ($n = 6$)	0.42 ± 0.14**	53.45 ± 6.77**
OVX + EA ($n = 6$)	4.41 ± 0.32$^{\#\#,**}$	77.57 ± 4.26$^{\#,*}$

$^{\#}$ $p < 0.05$ vs. OVX.
$^{\#\#}$ $p < 0.01$ vs. OVX.
* $p < 0.01$ vs. Sham.
** $p < 0.01$ vs. Sham.

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