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

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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.

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

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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.

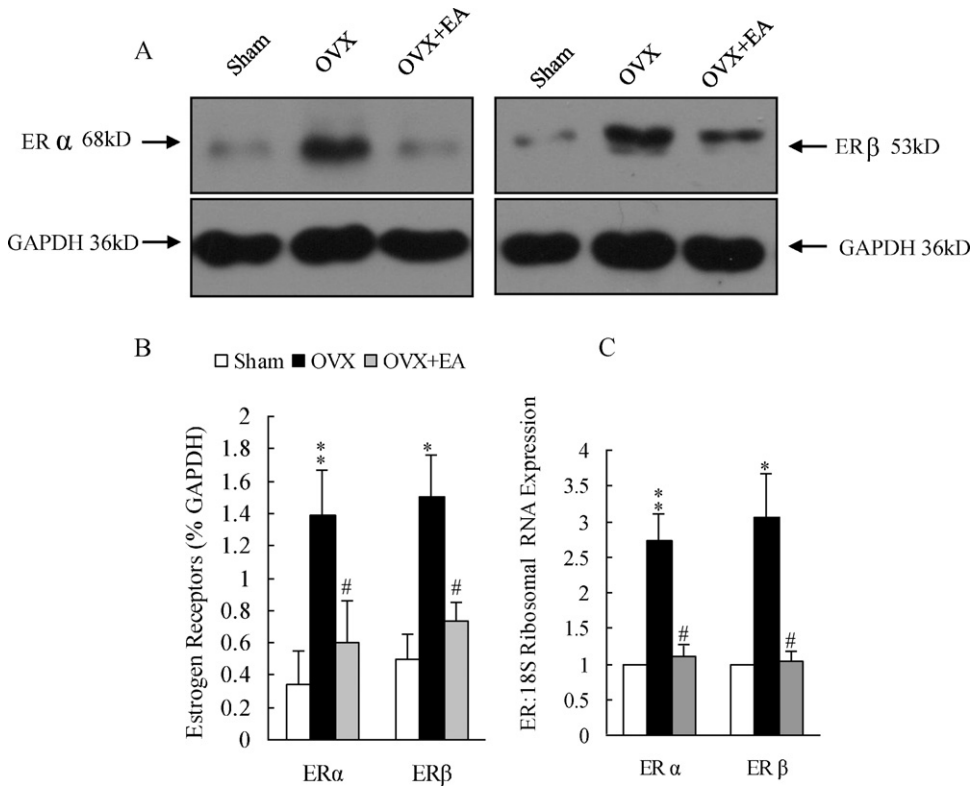

Fig. 1. Effects of EA and ovariectomy on expression of ERα and ERβ in hypothalamic preoptic area. ERα and ERβ expression at mRNA and protein level were measured by qRT-PCR and western blotting, respectively. (A) Pictures showed the western blot analysis of ERα and ERβ. The blot is representative of each run with independent samples. (B) Densitometric analysis the level of ERα and ERβ protein expression. Relative levels of ERα and ERβ were expressed as a ratio of densitometric value to GAPDH. Values are mean ± SEM of two independent experiments ($n = 3$ pools/group). (C) Relative mRNA expression levels were determined by qRT-PCR and normalized to the 18S rRNA. Sham values were assigned to a reference level of 1.00. Values are given as mean ± SEM ($n_{sham} = 5$; $n_{OVX} = 5$; $n_{OVX+EA} = 5$). $^{*}p < 0.05$, $^{**}p < 0.01$ compared to Sham. $^{\#}p < 0.05$ compared to OVX.

The effects of EA and ovariectomy on expression of ERα and ERβ at mRNA and protein level were presented in Fig. 1. Relative mRNA expression levels for ERα and ERβ were determined using qRT-PCR and normalized to the 18S rRNA. Briefly, the mean ΔCt of the sham samples was used as an internal calibrator when the mRNA quantities of ERα and ERβ in OVX or OVX + EA were compared. The relative linear quantity of the target gene was calculated using the formula $2^{-\Delta\Delta CT}$. Therefore, the data expressed an *n*-fold relative change in gene expression normalized to a reference gene (18S rRNA). qRT-PCR analysis showed an enhanced mRNA expression of ERα ($p < 0.01$) and ERβ ($p < 0.05$) in POA from OVX rats compared to that from Sham rats. Interestingly, elevated ERα and ERβ mRNA expression in OVX rats was decreased significantly ($p < 0.05$) by EA treatment. There were no disparities in ERs mRNA expression between Sham and OVX + EA. The protein expression of both ERβ and ERα, as indicated by western blot analysis of POA, increased significantly ($p < 0.01$, $p < 0.05$) in the OVX compared with the Sham. EA treatment decreased the elevated protein expression of ERα ($p < 0.05$) and ERβ ($p < 0.05$) in OVX rats. No significant difference was detected in ERα and ERβ protein between the Sham and OVX + EA.

The use of PPP technique to measure neuropeptide release in unanesthetized rats has received validation for many years. In the present study, the average GnRH output of OVX ($n = 7$) rats was significantly higher ($p < 0.01$) than that of Sham ($n = 7$), but showed a noticeable decrease when combined with EA ($n = 8$, $p < 0.01$). Relative mRNA expression levels of GnRH exhibited reflected changes in hormone release. Enhanced expression of GnRH mRNA ($p < 0.01$) in POA from OVX rats was observed, but when treated with EA, GnRH mRNA expression decreased significantly below that of OVX rats ($p < 0.01$). There was no statistical difference in GnRH mRNA level between sham and OVX + EA rats (Fig. 2).

The restraint, and sometimes EA stimulation can be considered a stressor for a conscious rat, i.e., strong electrical stimulation. In our

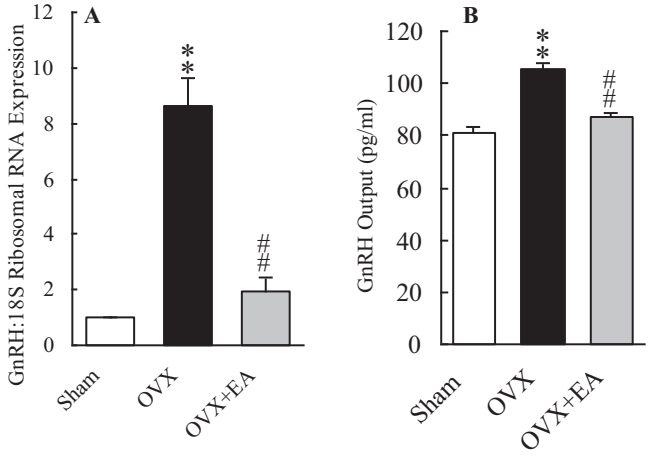

Fig. 2. Effects of EA and ovariectomy on GnRH mRNA level and output in hypothalamus by qRT-PCR and push–pull perfusion. (A) Relative mRNA expression levels were determined by qRT-PCR and normalized to the 18S rRNA. Sham values were assigned to a reference level of 1.00. Values were given as mean ± SEM ($n_{sham} = 5$; $n_{OVX} = 5$; $n_{OVX+EA} = 5$). (B) Push–pull cannulae were implanted in the hypothalamic medial preoptic area of rats and perfusion was collected for GnRH RIA assay. Values were given as mean ± SEM ($n_{sham} = 7$; $n_{OVX} = 7$; $n_{OVX+EA} = 8$). $^{**}p < 0.01$ compared to Sham. $^{\#\#}p < 0.01$ compared to OVX.

present study, the animals were fixed as comfortably as possible under no anesthesia. While treated with EA, they did not show any violent reactions. There was also no significance change of blood levels of corticosterone, one of the most reliable indicators of stress, in SD rats with or without EA treatment when they were handled as previously reported [3], suggesting restraint did not appreciably influence our results.

The most interesting result in the present study is that EA treatment inhibited the expression of ERs in POA of OVX rats. Estrogen is known to be a principal regulator of GnRH neuronal function in the female brain. The medial preoptic area contains a large population of estrogen-concentrating neurons [17]. The majority of the GnRH neurons which project to the median eminence are also found in the medial preoptic area [10,23], and are known to express ERs [1,8,9,20]. Within the medial preoptic area, regions with high ERs expression in rats include anteroventral periventricular nucleus (AVPv), periventricular preoptic nucleus, and medial preoptic nucleus (MPN) [2,8]. Therefore, the medial preoptic area is believed to be one of the most important locations within the brain where estrogen can influence GnRH neuronal activity. During the most rodent estrous cycle, estrogen exerts negative feedback upon the release of GnRH [19]. Upon exposure to sustained elevated estrogen levels, however, the negative feedback loop switches to a positive feedback loop, resulting in induction of a surge in the release of GnRH that serves as a neuroendocrine signal to trigger ovulation [12]. The mechanisms by which estrogen exerts a feedback influence on GnRH neurons are beginning to be elucidated. Recently, a detailed investigation from the Moenter laboratory has documented two different effects of E_2 on GnRH neuron cell firing. nM levels of E_2 were found to rapidly and directly activate firing in most GnRH neurons through a mechanism involving ERβ, protein kinase A and the suppression of calcium-activated potassium channels underlying the after-hyperpolarization potential. However, low pM levels of E_2 have no direct effects on GnRH neurons, but instead, inhibit firing using a complex, indirect pathway likely to involve both ERα and ERβ modulation of GABA and glutamate inputs to GnRH neurons [4]. For the greater part of the estrous cycle, estrogen helps restrain LH secretion through negative feedback action. This has been shown to occur, in part, through the inhibition of GnRH secretion in rats [19].

In the present study, elevated GnRH mRNA expression and increased output of GnRH in OVX rats suggested that ovariectomy (4 weeks later) was associated with increased GnRH synthesis and release. Additionally, our previous study had shown that the pituitary LH content of OVX rats was decreased while the plasma LH was increase [3]. Therefore, it is highly likely that estrogen deprivation (due to ovariectomy) in plasma failed to exert negative feedback on HOPA and inhibit hypothalamic GnRH neurons, which resulted a secretion increase of GnRH in OVX rats and drived the increase in mean LH levels. The dysfunction of HPOA is induced by ovariectomy.

Regarding the principal role of estrogen in GnRH release, it is crucial to explore the ERs expression to study the mechanism of EA treatment of HPOA dysfunction. Expression of functional ERs in GnRH neurons, from studies of both in vivo [9] and GnRH-expressing neuronal cells lines [18], suggests that estrogen may exert a direct effect at the level of the GnRH neuron. Furthermore, ERs are highly expressed in other neurons [6] and glial-cells [11] which afferent to GnRH neurons rather than in GnRH neurons in hypothalamus, suggesting that there is a strong indirect effect of estrogen on the regulation of GnRH neurons. In consistent with the evidence that the expression of ERα and ERβ in hypothalamus was modulated by estrogen [9,14], we also detected increased expression of ERα and ERβ in POA at both mRNA and protein levels in OVX rats. Interestingly, EA treatment inhibited the increased expression of ERα and ERβ induced by ovariectomy. We previously found that aromatase expression increased significantly in the hypothalamus including the AVPv and MPN after EA treatment in OVX rats. The up-regulation of hypothalamic aromatization by EA may drive local E_2 concentrations to higher levels in hypothalamic regions in OVX rat [26]. Similarly, the elevated local E_2 concentration by EA treatment in OVX rats may facilitate the role of EA on ERs in POA. Moreover, the restoration of hypothalamic ERs by EA treatment contributed to increasing sensitivity of GnRH neurons to estrogen feedback inhibition in OVX rats, and in turn resulted in suppression of GnRH neuronal activity, as monitored by GnRH release and transcription in hypothalamus. These results are consistent with previous reports that EA treatment suppresses the hypersecretion of GnRH and leads to an increase in the number of immunoreactive GnRH cells in hypothalamus [25]. EA treatment may also modulate release of neurotransmitter in hypothalamus, such as βEND [3] and GABA (unpublished data). Additionally, no colocalizations of ERs with GnRH neurons or other GnRH regulating neurons were detected in this study. In this manner, we could not determine whether estrogen acted on GnRH neurons directly, and/or indirectly through estrogen-sensitive neurons contacting the GnRH neurons. Further research may resolve these questions.

Our results, previous and present, have shown that EA treatment increases the local estrogen level and restores the expression of estrogen receptors in hypothalamuses of OVX rats. Recovery of the response of GnRH neurons to estrogen might in turn reset the negative feedback of estrogen to HPOA, thereby inhibiting the hypersecretion of GnRH due to ovariectomy. These results may help understand the mechanisms of EA effects on female reproductive disorders.

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