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Electroacupuncture stimulates the expression of prolactin-releasing peptide (PrRP) in the medulla oblongata of ovariectomized rats

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Abstract

Electroacupuncture (EA) in reproductive medicine has become established in Western medicine as a therapy over the last decade. EA performs a variety of neuromodulatory functions in the central nervous system (CNS). Prolactin-releasing peptide (PrRP) is a neuropeptide identified as an endogenous ligand for the orphan G protein-coupled receptor hGR3. PrRP can affect the function of hypothalamus-pituitary-ovary axis (HPOA) and hypothalamus-pituitary-adrenal axis (HPAA). The present study was undertaken to characterize the effect of EA on the expression of PrRP in the medulla oblongata in ovariectomized (OVX) rats by immunohistochemistry and reverse transcription-polymerase chain reaction (RT-PCR). In addition, estrogen (E_2) levels were detected by radioimmunoassay (RIA). The results suggest that EA significantly increase the blood level of E_2 and the expression of PrRP in the medulla oblongata of OVX rats. The number of PrRP immunoreactive (ir) neurons was higher in the group ovariectomized with EA than that in the OVX group. The numbers of PrRP-ir neurons in intact (INT) and intact with EA (INT + EA) were not significantly different between the two groups. The expression of PrRP mRNA was increased in the OVX + EA group than that in the OVX group. These results suggest that the mechanism that EA improved reproductive disorders induced by ovariectomy in rats is related to the modulation of the blood E_2 level and the expression of PrRP in the medulla oblongata.

Keywords: Electroacupuncture; Prolactin-releasing peptide (PrRP); Medulla oblongata; Ovariectomy; Rat

Traditionally, acupuncture therapy has been applied in the treatment of various diseases in Oriental medicine [19]. Acupuncture in reproductive medicine today, as a therapy has become established in Western medicine over the last decade [17]. It is known that electroacupuncture (EA) treatment exerts 'normalizing' effects on endocrinological and neuroendocrinological disturbances, and EA performs a variety of neuromodulatory functions in the central nervous system (CNS), such as triggering the release of neuropeptides, regulating neuronal gene expression, and enhancing neurogenesis. It has been shown that EA may significantly increase the corticotrophin-releasing hormone (CRH) concentrations in the median eminence in rats with polycystic ovaries [16]. Our laboratory has studied the mechanism of acupuncture using reproductive disorders as the model system for the past decade. We have observed that repeated EA in specific acupoints significantly reduces the elevated plasma luteinizing hormone (LH) of rats due to ovariectomy [1]. In addition, EA also increases the number of gonadotropin-releasing hormone (GnRH) neurons, CRH cells [21] and the releasing of CRH [22] in the brain of ovariectomized (OVX) rats. These results suggest that EA effectively enhance the function of the hypothalamus-pituitary-ovary axis (HPOA) and hypothalamus-pituitary-adrenal axis (HPAA) of ovariectomized rats.

Prolactin-releasing peptide (PrRP) is a neuropeptide identified as an endogenous ligand for the orphan G protein-coupled receptor hGR3 [4]. PrRP shows specific prolactin (PRL)-releasing activity in vitro [4] and in vivo [18,20]. However, in addition to this function, PrRP also affects the funtion of HPOA and HPAA. It is reported that PrRP stimulates luteinizing hormone and follicle-stimulating hormone (FSH) release via a hypothalamic mechanism involving the release of gonadotropin-releasing hormone [13]. The administration of PrRP into the hypothalamic paraventricular nucleus (PVN) increased the plasma adrenocorticotropic hormone (ACTH)

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level and the release of CRH from hypothalamic explants [14].

Above results led us to hypothesize that the expression of PrRP in the brain of OVX rats might be affected with the treatment of EA. To test the hypothesis, we detected the expression of PrRP in the medulla oblongata and blood estrogen (E_2) level in the OVX rats that treated with EA.

Fifty female Sprague–Dawley rats (180–200 g), with regular 4-day estrus cycles were purchased from Medical Experimental Animals Center of Fudan University (Shanghai, China). The animals were housed under laminar flow in an isolated room with controlled temperature and at 12/12 (light/dark) schedule. Half of the rats underwent ovariectomy with ether anesthesia, which were then divided randomly into three groups: ovariectomized, ovariectomized with EA in specific points (OVX + EA) and ovariectomized with EA in non-specific points (OVX + C). The rest were treated as controls, which were divided into two groups: intact (INT) and intact with EA in specific points (INT + EA). Four weeks after the ovariectomy, OVX + EA ($n = 8$), INT + EA ($n = 8$) and OVX + C ($n = 8$) received EA treatment. Thirty minutes before the EA treatment, all the animals were bound as comfortably as possible, and during the EA procedure, the rats were conscious without anaesthesia. Electrical stimulation was administered via three stainless steel needles of 0.3 mm diameter inserted 5 mm in four acupoints in the belly: "Guanyuan" acupoint (RN4), in the middle of abdomen (15 mm bellow the umbilicus); "Zhongji" acupoint (RN3), 5 mm bellow "Guanyuan" acupoint (one needle was flatly punctured in the RN4 which penetrated into the RN3); bilateral "Zigongxue" acupoints (EXTRA22), 7.5 mm lateral to the "Zhongji" acupoint, and one needle inserted 3 mm at one acupoint in the hind leg, "Sanyinjiao" (SP6), near ankle joint (at the concentration of the superior border of the medial melleolus, between the posterior border of the tibia and anterior border of the Achilles tendon). These acupoints are widely applied in Oriental medicine for the treatment of gynaecological diseases in women [15] and for the acupuncture mechanism research in the ovaricetomized rats [22,2]. The control acupoints were "Waiguan" (SJ5), between the radius and ulna (5 mm above the dorsal transverse crease of the wrist) and "Huatuojiaji" (EXTRA15), in the back (5 mm lateral to the lower border of each spinous process from the first thoracic vertebra to the fifth lumbar vertebra). The stimulation was generated by an EA apparatus (Model G6805-2, SMIF, Shanghai, China) and lasted for 30 min (8:00–10:00 a.m.), QD, for 3 days altogether. The stimulation parameters were 2 mA of density and a low-burst frequency of 3 Hz. Individual pulses within the burst frequency were square wave pulses with alternating polarities and pulse duration of 0.2 ms, 80 pulses/s. The intensity was adjusted to produce a slight twitch of the limbs. At the time of sacrifice (6 h after the last EA), the vaginal cytology of each OVX + EA rat was examined. The animals whose epithelial cells reappeared were adopted for the following experiments, which could be a validity indicator of the EA. All experimental procedures involving the use of animals were conducted in accordance with NIH Guidelines and were reviewed and approved by the Animal Use and Care Committee of the Fudan University.

At the time of sacrifice, the blood samples of the OVX, OVX + EA rats were collected (6 h after the last EA treatment) from left ventricle, and the blood samples of the INT and INT + EA rats were collected during the period of proestrus to avoid spontaneous estrogen surges that occur during estrus. The plasma was separated by centrifugation and stored at −70 °C until assayed. Concentrations of blood estrogen were determined using the kit purchased from the National Atomic Energy Research Institute (Beijing, China). The samples were assayed in duplicate, and all the subjects' samples were assayed together. The sensitivity of the kit was 1.4 pg/ml; the intra- and interassay coefficients of variation were 6.5% and 8.3%.

With the blood samples, half subjects in each group were exsanguinated with normal saline and then a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). Perfusion done, the medulla oblongatas were removed and post-fixed for 48 h in 4% paraformaldehyde with 30% sucrose, and their sections were sliced at 35 μm thickness on a vibratome microslicer and stored at −20 °C in tissue culture wells containing cryoprotectant solution (0.05 M PB/30% sucrose/30% ethylene glycol) for further process.

Free-floating sections were washed with 0.01 M phosphate buffered saline (PBS) for 15 min at room temperature. After extensive rinsing, the sections were quenched with 0.3% H_2O_2 in PBS for 10 min and blocked with 0.01 M PBS. Thereafter, sections were incubated with the rabbit anti-PrRP antiserum (Phoenix Pharmaceuticals) at a dilution of 1:500 in PBS containing 10% goat serum at 4 °C for 48 h, the sections were rinsed three times in PBS and incubated for 30 min with biotinylated anti-rabbit IgG (Vector Lab) at 1:50 dilution in PBS. The sections were rinsed and incubated with avidin-biotinylated peroxidase complex (Vector Lab) in PBS for 30 min. The peroxidase-substrate reaction was performed for 5 min using 3,3′-diaminobenzidine (DAB) (0.5 mg/ml DAB, 0.01% H_2O_2) prepared in PBS. The sections were mounted on gelatin-coated glass slides and dried in air, then dehydrated with ethanol, cleared with xylene and covered with coverslips. Neural structures were identified according to the rat brain atlas [10]. Specificity of PrRP staining was determined by preincubation of antiserum for 24 h at 4 °C with varying concentrations of PrRP, with primary antibody omitted to identify non-specific staining as well.

PrRP positive neurons were quantified by a video camera and a computer-aided densitometric image-analysing software program. Sections were included for the statistical evaluation if the anatomical structure could easily be identified.

With the blood samples, half subjects in each group were sacrificed by decapitation followed by immediate removal of the brains and dissection of the medulla oblongatas, extending from the rostrocaudal level of the most rostral part of the cervical cord (CC) to that of the middle part of the fourth ventricle in the medulla. The above tissue samples were snap-frozen in liquid nitrogen and then stored at −80 °C for further process. Total RNA was isolated from the medulla oblongatas and stored in liquid nitrogen by using Trizol reagent (Invitrogen) according to the manufacture's instructions. The yield and quality of the RNA were assessed by measuring absorbance at 260 nm

Table 1
The percent of mature vaginal epithelia of the INT, INT + EA, OVX, OVX + C, and OVX + EA rats

The percent of mature vaginal epithelia	
INT ($n = 12$)	18.7 ± 0.45
INT + EA ($n = 12$)	17.9 ± 0.59
OVX ($n = 8$)	0.45 ± 0.02*
OVX + C ($n = 8$)	0.5 ± 0.021*
OVX + EA ($n = 8$)	3.85 ± 0.03*,#

* $P < 0.05$ compared with INT and INT + EA.
# $P < 0.05$ compared with OVX.

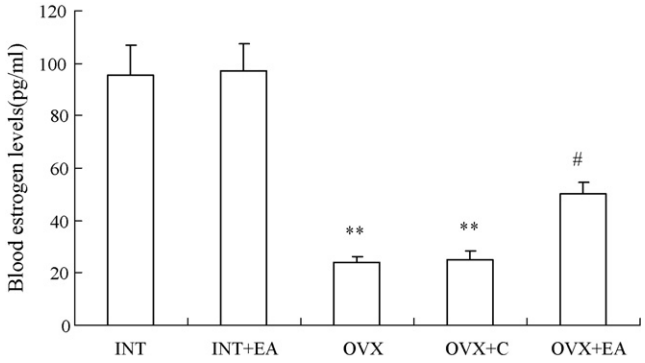

Fig. 1. Effects of EA on the blood estrogen concentrations in INT, INT + EA, OVX, OVX + C and OVX + EA groups. The levels of estrogen in blood were measured by RIA kit. Values are expressed as the mean ± S.D. **$P < 0.01$ compared with INT and INT + EA; #$P < 0.05$, compared with OVX and OVX + C.

and 280 nm followed by electrophoresis on 1.5% agarose gels. One microgram of RNA was reverse-transcribed into cDNA by using oligo(dT)$_{18}$ primers and M-MLV reverse transcriptase at 42 °C for 1 h in standard buffer. Sequence-specific primers for PrRP and the house-keeping gene β-actin were used for cDNA amplification. Primers used to amplify PrRP cDNA were 5′-GACGTGGCTTCTGTGCTTGCTGCTG-3′ (forward) and 5′-GCAGCACTGTCTTCTCGAGCTG-3′ (reverse) and yielded a PCR product of 268 bp. The primers for β-actin were 5′-TGAACCCTAAGGCCAA CCGTG-3′ (forward) and 5′-GCTCATAGCTCTTCTCCAGGG-3′ (reverse) and the size of PCR product was 420 bp. cDNA was amplified using the following parameters: 94 °C for 30 s, 60 °C for 30 s and 72 °Cfor 30 s, in a total of 30 cycles with a final extension step at 72 °C for 10 min. These PCR profiles were determined by the amplification for PrRP mRNA and β-actin to ensure that PCR was performed within the linear part of the amplification curves. PCR products were electrophoresed through 1.5% agarose gel, stained with ethidium bromide and visualized under ultraviolet illumination. Band intensity was calculated densitometrically using Quantity One-4.4.1 imaging software (Bio-Rad Laboratories). Levels of mRNA were expressed as the ratio of band intensity of PrRP relative to that for β-actin.

All data are presented as mean ± S.D. Statistical analysis was performed on raw data using one-way analysis of variance (ANOVA). For multiple comparisons, the Bonferroni *t*-test was performed. The value of $P < 0.05$ was considered significant.

The epithelial cells were stained by haematoxylin-eosin (HE). The INT and INT + EA rats showed regular 4-day estrus cycle change, and the cyclic changes disappeared in the OVX, OVX + EA and OVX + C rats. Few mature vaginal epithelia were observed in the smears of the OVX and OVX + C rats, and the percent of mature epithelia increased significantly in the OVX + EA rats ($P < 0.05$) (Table 1).

The blood E_2 concentration decreased significantly in OVX ($P < 0.01$) compared with that in INT and INT + EA, and was higher in OVX + EA ($P < 0.05$) than in OVX and OVX + C. The E_2 level was lower in OVX + EA ($P < 0.05$) than that in INT. There were no disparities between INT and INT + EA (Fig. 1).

Immunostaining of the medulla oblongata sections revealed that PrRP-ir neurons were present exclusively in a small region in the caudal part of the solitary tract nucleus (NTS) and the lateral reticular nucleus (LRN). The number of PrRP cells at NTS and LRN was calculated. At NTS, the number of PrRP neurons was less in OVX group than that in INT and INT + EA groups ($P < 0.01$). There were no disparities between INT and INT + EA groups. However, EA administration can increase the number of PrRP neurons of OVX compared with OVX group and OVX + C group ($P < 0.05$). At LRN, there was no significant difference among the five groups (Fig. 2).

To confirm that reverse transcription-polymerase chain reaction (RT-PCR) yields were not affected by the efficiency of the RT reaction, different amounts of total RNA from the medulla oblongata were reverse transcribed and amplified by PCR. When the amount of total RNA was increased from 0.25 μg to 2 μg, there was an exponential increase in yields for both PrRP mRNA and β-actin mRNA, indicating that no saturation of the RT reaction has occurred. Experiments with fixed total RNA and varied PCR cycles were also carried out to ensure that the PCR reaction was within the linear part of the amplification curve. Amplification of cDNA from β-actin and PrRP gene were linear up to approximately 35 cycles. As a matter of result, amplifications of cDNA from β-actin and PrRP mRNA were carried out using 30 cycles.

RT-PCR analysis showed a decreased expression of PrRP mRNA in the medulla oblongata from OVX rats compared to that from INT and INT + EA rats ($P < 0.01$). EA treatment increased PrRP mRNA levels in OVX rats ($P < 0.05$). However, there were no disparities between INT and INT + EA, the OVX and OVX + C as well (Fig. 3).

The interesting finding in the present study is that EA significantly increases the blood E_2 level in OVX rats. The biosynthesis of estrogens from androgens is catalyzed by the key enzyme aromatase. In our previous work, we found the aromatase expression in adipose and liver tissues increased significantly after given the treatment of EA in ovariectomized rats [23]. The effects of EA on the extragonadal aromatization may contribute to promote the blood concentrations of estrogen in the ovariectomized rats. Above all, we hypothesize that the sources of the high E_2 levels produced in ovariectomized rats treated with EA may be the adipose and liver tissues that convert androgens mainly from adrenal cortex to estrogens. Our results, however, did not show the same effects in the INT + EA rats as in the OVX + EA rats, suggesting that the EA may play a normalizing role. It is known that maturation of vaginal epithelia cells is a

reaction dependent on estrogen level, and few mature epithelia cell was observed in the smears of the OVX + C rats, which verified the specificity of the EA effects.

The significant decrease of PrRP in the medulla oblongata induced by estrogen depletion in OVX rats shown in the current study was consistent with previous studies [18]. We showed that the number of PrRP-ir neurons in the NTS of the medulla oblongata of OVX rats was most decreased compared with that of INT rats. EA treatment increased the number of PrRP-ir neurons in the NTS and the expression of PrRP mRNA of the medulla oblongata of OVX rats. Because the results obtained by immunohistochemistry can only show the quantity of certain immunoreactive (ir) substances in situ, we also detected the release of PrRP. Our unpublished data on the release of PrRP from medial preoptic area (MPOA) by push–pull perfusion suggest that the level of PrRP release in OVX + EA is higher than that in OVX. Therefore, we hypothesize that EA may enhance both the release and synthesis of PrRP in ovariectomized rats.

Our findings raise the question as to how EA increases the expression of PrRP in the medulla oblongata of OVX rats. Kataoka et al. [5] found that PrRP gene expression is regulated by gonadal steroid hormones in the medulla oblongata, and parts of PrRP synthesizing neurons are considered to be directly influenced by estrogen in the NTS. The increase of PrRP expression in OVX + EA may be due to the increased level of blood E_2 induced by EA. It is reported that administration of estrogen after ovariectomy induces an increase in PrRP mRNA expression both in NTS and LRN. But EA has no effect on the number of PrRP in LRN in the present study. Differences in the results may be attributable to species, treatments and degree of deprived estrogen, as each of these potentially confounding variables may influence the feedback of estrogen on PrRP.

The largest concentration of PrRP peptide is found in the medulla oblongata where it is associated with noradrenergic neurons that project to and activate GnRH neurons in the hypothalamus [3]. Within the hypothalamus, PrRP-ir neurons are found in the bed nucleus of stria terminalis (BST) [7], in close proximity to the preoptic area that contains the cell bodies of the GnRH neurons [3]. It is reported that PrRP following intracerebroventricular (ICV) injection stimulates LH and FSH via a

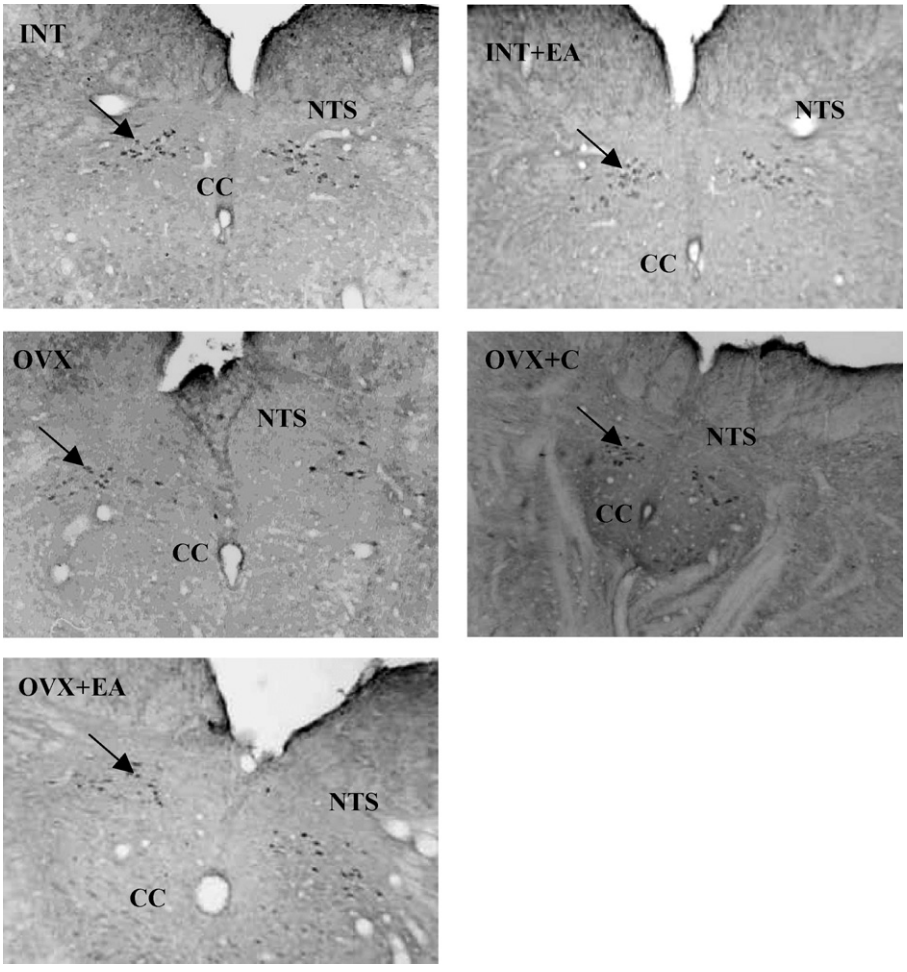

Fig. 2. Effects of EA on the number of PrRP positive neurons in NTS and LRN of INT, INT + EA, OVX, OVX + C and OVX + EA groups by immunohistochemistry analysis. Upper panel: PrRP-ir neurons in NTS; middle panel: PrRP-ir neurons in LRN. Positive signals are indicated by arrows. Lower panel: the number of PrRP immunoreactive neurons in NTS and LRN. Comparison among INT (open bars), INT + EA (stippled bars), OVX (black bars) OVX + C (grey bars) and OVX + EA (streaked bars) groups. Values are presented as the mean ± S.D. $^{**}P < 0.01$, compared with INT and INT + EA; $^{\#\#}P < 0.01$, compared with OVX and OVX + C group. NTS, solitary tract nucleus; LRN, lateral reticular nucleus; CC, central canal. Scale bar = 500 μm.

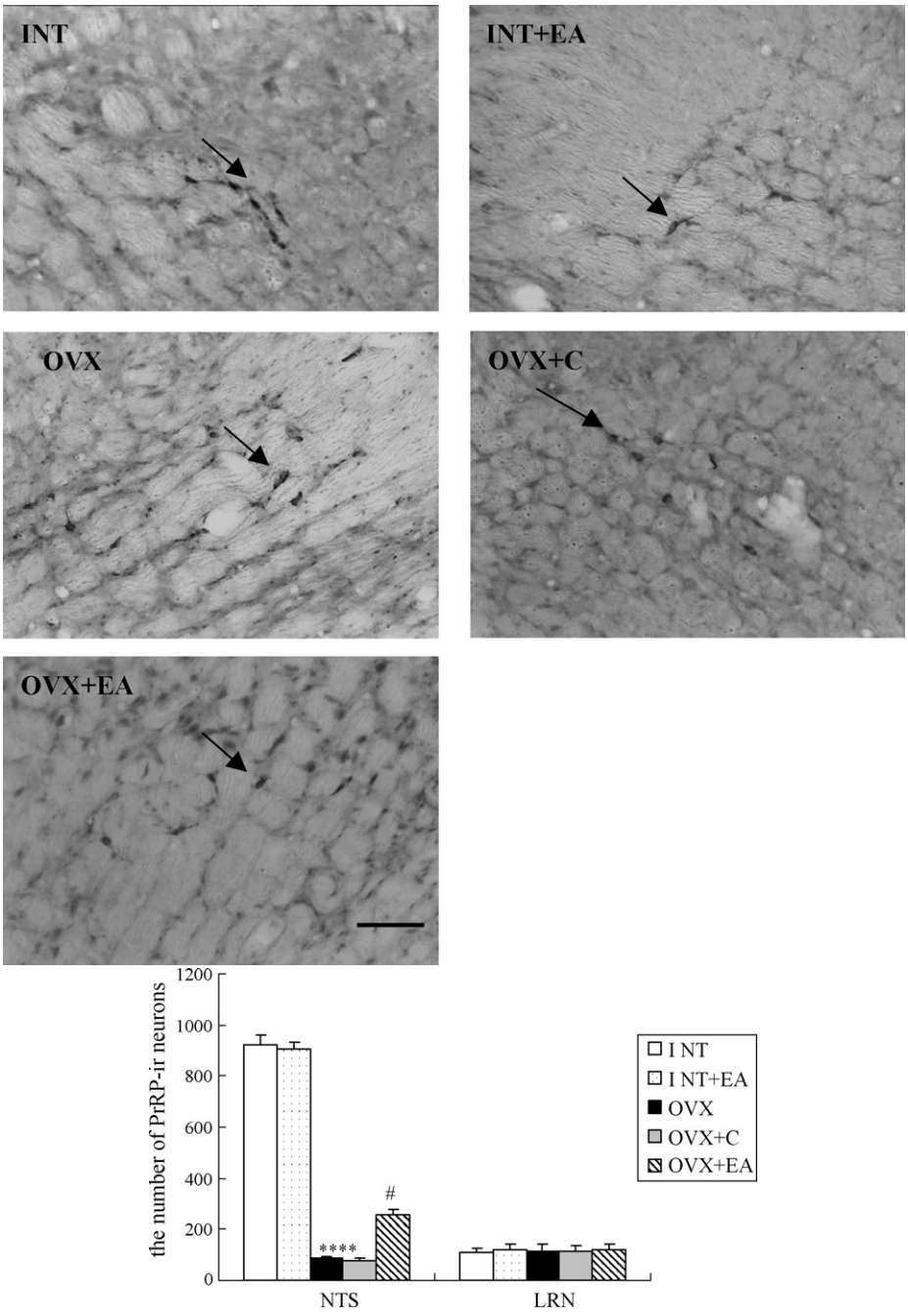

Fig. 2 (*Continued*).

hypothalamic mechanism [13]. Our previous study showed that EA can increase the expression of GnRH-ir in ovariectomized rats' brains [21]. All these above data suggest that the expression of PrRP increased in OVX + EA may affect the activities of GnRH neurons.

It is known that PrRP neurons project to the paraventricular hypothalamic nucleus (PVN) and make contact with corticotropin-releasing hormone cell bodies in the PVN [6–9] in which PrRP receptors exist [11]. Administration of PrRP into the lateral ventricle dramatically induced the expression of c-Fos protein in the CRH neurons in the PVN [8]. And central administration of PrRP increased the corticosterone level [12], the plasma ACTH level and the release of CRH [14]. These data suggest that PrRP regulate the function of HPAA. Our results of push–pull perfusion technique have also shown that EA can increase the release of CRH from PVN in ovariectomized rats [22]. So we hypothesize that the expression of PrRP increased in OVX + EA may also affect the activities of CRH neurons.

Put the results above together, EA may have a stimulatory effect on the expression of PrRP and the level of blood E_2 in ovariectomized rats. The present study evaluated the effect of EA on the expression of PrRP in ovariectomized rats, which is valuable in research for the mechanism of EA on reproductive disorders.

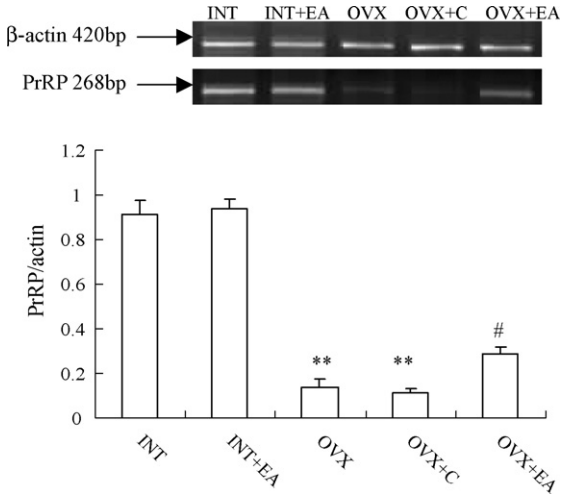

Fig. 3. Effects of EA on the expression of PrRP mRNA in the medulla oblongatas from INT, INT + EA, OVX, OVX + C and OVX + EA rats by RT-PCR analysis. Upper panel: ethidium bromide staining gels of RT-PCR products for PrRP mRNA and β-actin mRNA from the medulla oblongatas of INT, INT + EA, OVX, OVX + C and OVX + EA groups. Arrows indicate the lengths of RT-PCR products. Lower panel: relative optical density of PrRP mRNA normalized against β-actin mRNA from the medulla oblongatas of INT, INT + EA, OVX, OVX + C and OVX + EA groups. $^{**}P < 0.01$, compared with INT and INT + EA; $^{\#}P < 0.05$, compared with OVX and OVX + C.

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