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## Effects of estrogen receptor $\alpha$ and $\beta$ gene deletion on estrogenic induction of progesterone receptors in the locus coeruleus in female mice

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### Abstract

Locus coeruleus (LC) is involved in the LHRH regulation by gonadal steroids. We investigated the expression of progesterone and estrogen receptors (PR; ER) in LC neurons of ER $\alpha$  ( $\alpha$ ERKO) or ER $\beta$  ( $\beta$ ERKO) knockout mice, and their wild-type ( $\alpha$ WT and  $\beta$ WT). Immunocytochemical studies showed that LC expresses PR and both ERs, although ER $\beta$  was more abundant. Estradiol

benzoate (EB) decreased ER $\alpha$ -positive cells in WT and  $\beta$ ERKO mice, and progesterone caused a further reduction, whereas none of the steroids influenced ER $\beta$  expression. ER $\beta$  deletion increased ER $\alpha$  while ER $\alpha$  deletion did not alter ER $\beta$  expression. In both WT mice, EB increased PR expression, which was diminished by progesterone. These steroid effects were also observed in  $\alpha$ ERKO animals but to a lesser extent, suggesting that ER $\alpha$  is partially responsible for the estrogenic induction of PR in LC. Steroid effects on PR in  $\beta$ ERKO mice were similar to those in the  $\alpha$ ERKO but to a lesser extent, probably because PR expression was already high in the oil-treated group. This expression seems to be specific of LC neurons, since it was not observed in other areas studied, the preoptic area and ventromedial nucleus of hypothalamus. These findings show that LC in mice expresses  $\alpha$ ER,  $\beta$ ER, and PR, and that a balance between them may be critical for the physiological control of reproductive function.

## Keywords

Estradiol; Progesterone and estrogen receptors; Locus coeruleus; Knockout mice; LHRH

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## Introduction

It is well established that gonadal steroids, estrogen, and progesterone, control LHRH secretion and subsequent preovulatory LH surge in rodents. Although elevated levels of estradiol and progesterone are known to be essential for the synthesis and release of LHRH in the preoptic area (POA), it is not completely understood how they act on various parts of the brain to exert coordinately their regulatory action on LHRH and LH secretion.

Recent studies have shown that the locus coeruleus (LC) may be one of the key brain sites of estrogen action. Selective retrograde tracing showed that POA LHRH neurons receive direct projections from the LC, together with the brainstem A2 noradrenergic neurons [1]. LC, which is the main source of noradrenaline (NA) in the brain [2], has been demonstrated to play a critical role in facilitation of LHRH/LH surges. LC electrolytic lesion decreased NA content in the POA and ME and completely blocked the preovulatory LH surges as well as the surge induced by steroids in ovariectomized rats [3, 4]. It has also been shown that there is an increase in the number of FOS-immunoreactive (ir) neurons observed in the LC during the afternoon of proestrus [5] indicating an increase of neuronal activity in this area paralleled with LH release in response to LHRH surges.

LC neurons concentrate estradiol [6], express mRNA for estrogen receptor (ER) [7] as well as the two types of ERs, ER $\alpha$  and ER $\beta$  [8–11]. In addition, mRNA levels of NA synthesis enzyme, tyrosine hydroxylase, are increased by estrogen in the LC [12]. Therefore, it is hypothesized that noradrenergic LC neurons may be a target of estrogen actions, which are mediated by both types of ERs. The number of ER $\alpha$ -ir cells fluctuates during estrous cycle more profoundly in the LC than in the POA [13], however, it remains to be determined, how estrogen and progesterone regulate the levels of ER $\alpha$  and ER $\beta$  in the LC.

One of downstream effects of estrogen action is the induction of progesterone receptors (PR). Progesterone is known to work synergistically with estrogen to regulate LHRH/LH synthesis and release. For instance, progesterone administration to estradiol-primed

ovariectomized rats results in the amplification and anticipation of LHRH [14] and LH [15] surges, and administration of PR antagonist RU486 to proestrus rats blocks LH surge [16]. Since a clear fluctuation of the number of PR immunoreactive cells has been described in the LC during the estrous cycle [13], it is possible that these effects of progesterone on the LHRH system are potentially mediated by its action on the LC neurons. Therefore, in this study, we examined estrogen-inducible PR in the LC in estrogen or estrogen plus progesterone treated ovariectomized mice. To determine the differential roles of two types of ERs in the induction of PR, we used ER $\alpha$  and ER $\beta$  knockout mice. Since surprisingly, a high number of LC neurons expressed PR in the oil-treated ovariectomized  $\beta$ ERKO mice, we further examined the effects of ovarian steroids on PR expression in other brain regions related to reproduction, the POA and the ventromedial nucleus of hypothalamus.

## Results

It was found that ER $\alpha$ , ER $\beta$  and PR-ir cells were all present in the LC. Figure 1 shows the LC localization (Fig. 1a) and a typical staining for each receptor in the middle portion of the LC of an oil-treated (Fig. 1b, c) and an estradiol-treated (Fig. 1d)  $\alpha$ WT animal. All immunostaining was detected specifically within the cell nucleus. Overall, ER $\beta$ -ir neurons were more abundant than ER $\alpha$ -ir cells. In fact, nearly all TH-staining cells in LC express ER $\beta$  (Fig. 1c), while ER $\alpha$  expression was much more discreet and mainly localized ventrally (Fig. 1b). Interestingly, a strong staining for ER $\alpha$  was observed outside LC, in Barrington nucleus. Conversely, PR expression was scattered all over the LC nucleus (Fig. 1c).

The number of ER $\alpha$ -ir cells was significantly higher ( $P < 0.05$ ) in  $\beta$ ERKO oil-treated group, when compared to the oil-treated  $\beta$ WT animals (Fig. 2). Two days of EB treatment significantly reduced the number of ER $\alpha$ -ir cells in  $\alpha$ WT and  $\beta$ WT as well as in  $\beta$ ERKO ( $P < 0.001$  vs. OVX group) and progesterone treatment resulted in a further decrease in this number ( $P < 0.05$  vs. OVE group). Unlike its effects on ER $\alpha$ -ir cells, EB did not down-regulate the number of ER $\beta$ -ir cells in  $\alpha$ WT,  $\beta$ WT, or  $\alpha$ ERKO. Moreover, the number of ER $\beta$ -ir cells was similar in all groups studied, regardless the treatment or genotype (Fig. 3). It should also be noted that unlike an increase of ER $\alpha$  in  $\beta$ ERKO mice, ER $\alpha$  gene disruption did not affect ER $\beta$  expression.

A substantial number of PR-ir cells was found in the LC of all oil-treated groups, though it was significantly higher in  $\beta$ ERKO animals, when compared to the  $\beta$ WT mice ( $P < 0.001$ ) as shown in Fig. 4. In both  $\alpha$ WT and  $\beta$ WT mice, EB treatment significantly increased the number of PR-ir cells ( $P < 0.001$  vs. OVX group) while progesterone treatment decreased it ( $P < 0.001$  vs. OVE group) to the levels of the oil-treated groups. Although its effect was smaller than that seen in  $\alpha$ WT, EB also increased the number of PR-ir cells ( $P < 0.01$  vs. OVX group) in  $\alpha$ ERKO animals and progesterone treatment decreased it ( $P < 0.01$  vs. OVE group) to the level of the oil-treated group. In  $\beta$ ERKO mice, EB treatment induced a small, but still significant increase in the number of PR-ir cells ( $P < 0.05$  vs. OVX group) and progesterone reversed the effect of EB ( $P < 0.05$  vs. OVE group). It should be noted, however, that the number of PR-ir in  $\beta$ ERKO mice in the OVEP group was also significantly higher than that in  $\beta$ WT mice ( $P < 0.05$ ) as found in the oil-treated group.

Since an elevated level of PR expression was observed in the LC neurons of oil-treated  $\beta$ ERKO animals, we performed an additional experiment using separate groups of  $\beta$ ERKO and  $\beta$ WT mice ( $n = 6/\text{group}$  for a total of six groups) to examine whether the profile observed in the LC might also be found in other brain areas. In the LC, we could replicate our original findings, i.e., oil-treated  $\beta$ ERKO mice had significantly higher number of PR-ir than  $\beta$ WT mice. Unlike in the LC, PR expression in the POA and VMH of the oil-treated  $\beta$ ERKO mice was minimal and not different from that in  $\beta$ WT (Fig. 5). The number of PR-ir cells dramatically increased after EB treatment ( $P < 0.001$ ) and slightly decreased after progesterone injection ( $P < 0.05$ ) regardless the genotype. The increase in PR expression in the OVE and OVEP groups was larger in the VMH of  $\beta$ ERKO compared to  $\beta$ WT animals ( $P < 0.01$  and  $P < 0.001$ , respectively), while the genotype did not influence the PR expression in the POA. Representative photomicrographs showing PR-ir staining in the POA and VMH for each treatment group are shown in Fig. 6. To further test the possibility that a higher number of PR-ir cells in the oil-treated  $\beta$ ERKO mice may be due to an elevated level of residual estradiol in this group, we measured the plasma levels of estradiol and progesterone in these mice. We found that ovariectomy greatly decreased plasma levels of estradiol as well as progesterone almost equivalently in  $\beta$ WT and  $\beta$ ERKO (Table 1). Furthermore, there were no genotype differences in plasma levels of steroids in any other treatment groups, OVE and OVEP.

## Discussion

It was found in this study that LC neurons express ER $\alpha$ , ER $\beta$ , and PR, and that both estradiol and progesterone treatment affects the number of ER $\alpha$  and PR-ir expressing cells, while it has no effect on the number of ER $\beta$ -ir cells. The expression of ERs and PRs in LC neurons as well as the control exerted by gonadal steroids may be related to the female reproductive function, since there is a well-defined relationship between noradrenaline from LC neurons and LHRH secretion [1, 3, 4].

### Effects of estradiol and progesterone on ER $\alpha$ , ER $\beta$ , and PR expression

Although experimental evidence demonstrated the presence of low levels of ER $\beta$  mRNA [17] and protein [18] in LHRH neurons, there is no evidence of a role of ER $\beta$  in the regulation of GnRH neural physiology [19]. On the other hand, it is well known that ER $\alpha$  and PR are absolutely required for the generation of the preovulatory LH surge [20, 21]. As LHRH neurons do not express ER $\alpha$  [22] or PR [23] the positive steroid feedback upon LHRH neurons mainly occurs in an indirect manner, through neurotransmitter-producing neurons.

Similarly to the present results obtained in wild-type mice, previous studies using female rats demonstrated that LC neurons express ER $\alpha$  and PR and respond to ovarian steroid secretion [13], suggesting that these hormones could control LHRH secretion by acting on LC neurons. Although it is well known that estradiol increases TH mRNA synthesis [12] in the LC neurons, and presumably leads to increased capacity for catecholamine biosynthesis in this nucleus, it is not clear if there is a change in the number of TH-ir neurons. An attempt was made to calculate a percentage of TH neurons expressing each one of the steroid

receptors studied, but as the LC is a very compact nucleus, it was not possible to distinguish clearly the shape of each TH-ir neuron in the area. Apparently, there was no change in the boundaries of TH staining in LC, but we clearly demonstrated here that estradiol treatment decreased the number of ER $\alpha$ -ir cells of LC in WT and  $\beta$ ERKO animals, probably through down-regulatory mechanisms on its own receptor. The inhibitory effect of estrogen on ER $\alpha$ -expression was potentiated by progesterone treatment, which reduced the number of ER $\alpha$ -ir cells even more in both WT and  $\beta$ ERKO animals. Indeed, progesterone has been demonstrated to be a suppressor of the ER $\alpha$  expression in the brain, diminishing ER hypothalamic and adenohipophysis concentrations in ovariectomized rats [24].

ER $\beta$  was clearly more abundant in LC neurons than ER $\alpha$  of all studied animals. Whereas a high ER $\beta$ /ER $\alpha$  ratio in LC was also observed by Mitra et al. [9], another study performed by Merchenthaler et al. [10] demonstrated a higher number of ER $\alpha$ -ir cells compared to ER $\beta$ -ir cells in the LC. A possible reason for this discrepancy seems to be differences in the antibodies used. Since we used the same antibody as the one used in Mitra's study (80424), our results are in agreement with that study. Microscopic observation revealed that almost all TH immunostained cells express ER $\beta$ , whereas much smaller number of them express ER $\alpha$ . Interestingly, although ER $\beta$  expression is higher than ER $\alpha$ , neither estradiol nor progesterone affected ER $\beta$  expression, suggesting that ER $\beta$  has a minor contribution to the regulation of the HPG axis. It was previously shown that ovarian steroids replacement into ovariectomized monkeys also did not modify the expression of ER $\beta$  in several hypothalamic areas [25]. One possible role for the high expression of ER $\beta$  could be regulation of TH expression, since it has been demonstrated that ER $\beta$  plays a role in the transient sex difference in TH expression in the mouse LC [26]. However, ER $\beta$  has been shown to be required for several rapid, presumably nongenomic effects of estradiol on LHRH neurons such as CREB phosphorylation [27] and induction of galanin expression [28] that was related to the short-term negative feedback regulation of the LHRH levels. Recent studies reinforce that estradiol actions on LHRH control are exerted by ER $\alpha$  [29, 30] and that ER $\beta$  modulates these actions [31, 32].

As previously shown in other brain areas [33], estradiol treatment induced PR expression in the LC neurons in both WT mice. Although numbers of PR-ir cells were also increased by estrogen treatment in  $\alpha$ ERKO mice, this effect was smaller. This finding suggests that estradiol acts on LC neurons probably through ER $\alpha$ . Partial reduction of PR-induction by estradiol in  $\alpha$ ERKO mice was also found in the dorsal raphe nuclei (DRN) [34]. On the other hand, in hypothalamic regions, PR-induction by estradiol in  $\alpha$ ERKO mice was greatly reduced in comparison to WT control mice both at protein [35] and mRNA levels [36]. In this study, relative contribution of ER $\alpha$  and ER $\beta$  in the induction of PR by estrogen in the LC was not determined due to unexpectedly high levels of PR in oil-treated  $\beta$ ERKO mice. It is possible that these high levels of PR are a result of increased ER $\alpha$  expression in these animals. Furthermore, PR can be induced by estradiol in hypothalamic regions of  $\alpha$  $\beta$ ERKO female mice [37]. Therefore, it is likely that mechanisms other than those through ER $\beta$  and ER $\alpha$  may be involved in this estradiol-induced PR expression in the LC. Progesterone treatment diminished PR synthesis in the LC, POA and VMH neurons in WT animals, probably by this hormone down-regulating its own receptor. In all four genotypes studied, the number of PR-ir cells returned to its oil-treated levels after estrogen plus progesterone

treatment in LC neurons, but not in the POA and VMH. Though increases in progesterone levels clearly decrease the number of PR-ir cells in peripheral tissues [38], progesterone action in the brain is variable in different brain areas [39].

### **Effects of ER $\alpha$ and ER $\beta$ gene disruption on estrogen and progesterone receptor expression**

Interestingly, the number of cells expressing ER $\alpha$  in oil-treated  $\beta$ ERKO animals was higher compared to that in oil-treated WT mice, suggesting that ER $\beta$  has an inhibitory role in ER $\alpha$  expression. Indeed, a previous in vitro study has demonstrated that ER $\beta$  sometimes acts as a negative regulator of ER $\alpha$  activity [40]. On the other hand, ER $\alpha$  deletion did not change the number of ER $\beta$  containing cells in the LC neurons, consistent with the finding in several tissues that regulation of ER $\beta$  expression is not dependent on ER $\alpha$  [10, 41]. Conversely, a previous study demonstrated that ER $\beta$  protein levels were greatly reduced by ER $\alpha$  gene disruption site specifically in the POA [42]. These findings suggest that expression of one type of ER in certain brain region may be influenced by the lack of the other type of ER.

It has been shown that ER $\beta$  has biological roles that are distinct from those of ER $\alpha$ . Both ER $\alpha$  and ER $\beta$  activation is able to stimulate the transcription of the estrogen response element (ERE) through homo or heterodimeric complexes [43]. Thus, the activity of estradiol may depend on whether a cell contains ER $\alpha$ , ER $\beta$  or both. Indeed, there are differences in the rapid effects of estradiol on MAPK/CREB signaling pathways within the cells containing ER $\alpha$  or ER $\beta$  [44] that also suggests that maybe some of the biological functions of the ER $\beta$  may be dependent on the presence of ER $\alpha$ . Since we did not analyze ER $\alpha$  and ER $\beta$  expression in the same sections we cannot say whether they are colocalized in the same neurons. However, as most of the LC neurons express ER $\beta$ , it is likely that at least part of them express also ER $\alpha$ . Therefore, it is possible that interaction between ER $\alpha$  and ER $\beta$  plays a role in gonadal steroid regulation of various biological functions in the LC.

In this study, a considerable number of PR-ir cells were found in the LC of oil-treated mice from all four genotype groups. Actual numbers of PR-ir cells were not different among oil-treated animals except in the  $\beta$ ERKO group, which showed a significantly higher number of PR-ir cells compared to oil-treated  $\beta$ WT group. These data suggest that ER $\beta$  may inhibit PR expression specifically in the LC neurons since in the other brain areas studied such as VMH and POA, the number of PR-ir cells was low in the oil-treated  $\beta$ ERKO mice as seen as in other genotype groups. These results corroborate with previous findings that suggests that estradiol can regulate the expression of PR via multiple mechanisms, based upon brain region [34].

It is worth noting that the regulation of ERs and PR expression by ovarian steroids as well by other receptors on LC may also affect a wide variety of other functions. It is well known that LC is related to the autonomic and behavioral responses to stress challenges [45]; stress is also known to suppress HPG axis-activity [46] and estradiol decreases stress-induced Fos-expression in the LC neurons [47]. In addition, LC is involved with cognitive and behavioral functions, including arousal [48, 49]. For instance, arousal responses are greatly reduced by the ER $\alpha$ , but not ER $\beta$  disruption [50], but the mechanisms involved in such controls are still unknown.

Regarding reproductive function, findings in this study allow us to make advancements in the area of gonadal steroids action on LC neurons. Although the number of ER $\alpha$  expressing cells was much less than that of ER $\beta$ , only ER $\alpha$  seems to be regulated by ovarian steroids. As estradiol increases PR expression in the LC, ER $\alpha$  may play an important role on the positive feedback mechanisms in the LC for the LHRH release. The suppressive effect of ER $\beta$  on ER $\alpha$  expression could be relevant to the maintenance of low plasma LH levels during most of the cycle. However, during the LHRH and gonadotropin surges, this suppressive effect may be disrupted or superseded by other mechanisms, allowing an increase of ER $\alpha$  and PR expression, and consequently triggering the surges. Finally, since progesterone inhibits ER $\alpha$  expression, its release with the LH preovulatory surge could exert an important role in terminating the surge.

In summary, findings in this study demonstrate that LC neurons do express ER $\alpha$ , ER $\beta$  and PR and that the expression of these receptors is modulated not only by ovarian steroids but also by their own interdependence. Thus, a balance between these receptors in the LC may be critical for the physiological control of reproductive function.

## Materials and methods

### Mice

A total of 98 adult female  $\alpha$ ERKO,  $\beta$ ERKO and their respective wild-type (WT) littermate mice were used. They were obtained from the  $\alpha$ ERKO and  $\beta$ ERKO breeding colonies maintained at the Rockefeller University by mating heterozygous male and female mice. Original breeding pairs, which had been completely backcrossed to C57BL/6J, were obtained from the National Institute of Environmental Health Sciences. Throughout the study, mice were group housed under controlled temperature (22°C) on a 12-h light, 12-h dark cycle (lights off at noon) with food and water supplied ad libitum. All procedures were approved by the IACUC at the Rockefeller University.

### Hormonal treatment

All mice were ovariectomized under methoxyflurane anesthesia at the age of 9–14 weeks and housed with the mice from the same genotype and treatment group (3 mice/cage). After 10 days ( $\beta$ ERKO) or 14 days ( $\alpha$ ERKO), the animals received two subcutaneous injections of 0.1 ml of sesame oil (OVX) or 10  $\mu$ g  $\beta$ -estradiol 3-benzoate (EB: Sigma Chemical Co., St. Louis, MO) in 0.1 ml oil at 48 and 24 h before perfusion (OVE). The third group of mice (OVEP) received two EB injections followed by a single injection of 0.5  $\mu$ g progesterone (Sigma) in 0.1 ml oil at 5 h prior to perfusion, which was performed at 4 pm.

### Preparation of brain samples

Mice were deeply anesthetized with Nembutal (75 mg/kg; i.p.). They were then perfused through the left ventricle, with 30 ml of 100 mM phosphate-buffered saline (PBS, pH 7.2) containing heparin, followed by 40 ml of 2% paraformaldehyde and 2.5% acrolein in 100 mM phosphate buffer (PB, pH 7.4). Blood samples were collected from the left ventricle prior to perfusion in some mice. Brain tissues were removed and post-fixed in the same fixative for 2 h at room temperature. After overnight washing in PB 100 mM at 4°C, the

brains were incubated in a 30% sucrose solution in PB until it sank, frozen in cold 2-methylbutane (Fisher Scientific, Pittsburgh, PA) and sectioned using a sliding freezing microtome. The sections were stored in a cryoprotectant solution (30% glycerol, 30% ethylene glycol in 100 mM PB, pH 7.4) at  $-20^{\circ}\text{C}$  until processed.

### Single and dual label immunocytochemistry

Free-floating sections (30  $\mu\text{m}$ ) were washed several times with 50 mM Tris-buffered saline (TBS, pH 7.2) in order to thoroughly remove cryoprotectant. They were then treated with three kinds of blocking solutions. To remove residual aldehyde in acrolein fixed tissue and reduce non-specific staining, the sections were first incubated in a 0.5% sodium borohydrate ( $\text{NaBH}_4$ ) solution in TBS for 10 min followed by 0.1% hydrogen peroxide in TBS for 20 min to block endogenous peroxidase. They were rinsed three times (10 min each) in TBS between different blocking solutions. The sections were then incubated for 60 min in TBS containing 0.3% Triton X-100, 3% bovine serum albumin (BSA) and 3% normal goat serum (NGS). Without TBS rinse, the LC containing sections were incubated in one of three different primary antibodies, i. e., rabbit polyclonal anti ER $\alpha$  (C1355, Upstate Biotechnology, Lake Placid, NY, 1:2500), ER $\beta$  (80424, a gift of Merck Research Laboratories, 1:16000), and PR (SP2, LabVision Corporation, Fremont, CA, 1:600) for 72 h at  $4^{\circ}\text{C}$ . After washing with TBS (three times, 10 min each), the sections were incubated for 120 min at room temperature in a biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, Inc., Burlingame, CA, 1:400). All primary and secondary antibodies were diluted in the same buffer as used in the last blocking step. After TBS washes the tissues were incubated with avidin–biotin complex (ABC; Vectastain ABC Elite Kit, Vector Laboratories, 1:250) diluted in TBS for 60 min at room temperature, and washed again with TBS (three times, 10 min each). They were then developed using 0.02% diaminobenzidine (DAB; Sigma) and 0.01% hydrogen peroxide with 2.5% nickel sulfate in TBS. No staining was observed after incubation of sections obtained from  $\alpha\text{ERKO}$  animals with the  $\alpha\text{ER}$  antibody and from  $\beta\text{ERKO}$  animals with the  $\beta\text{ER}$  antibody (data not shown). Further ICC in POA and ventromedial nucleus of hypothalamus (VMH) sections from another group of  $\beta\text{ERKO}$  and  $\beta\text{WT}$  animals were similarly stained only for PR. After stringent washing with TBS, only the LC-containing sections were incubated with a rabbit polyclonal anti-tyrosine hydroxylase antibody (CA-101, Protos Biotech Corp, New York, NY, 1:4000) overnight at  $4^{\circ}\text{C}$ . After washing with TBS, the sections were incubated for 60 min in a biotinylated goat anti-rabbit secondary antibody (1:400). After TBS washes, the tissues were incubated for 30 min with ABC, washed again with TBS and developed using DAB (0.02%) with 0.01% hydrogen peroxide. All sections were mounted on gelatin-coated slides, air-dried, dehydrated in ascending ethanol concentrations, cleared with xylene and coverslipped with DPX mounting medium (BDH, Poole, England).

### Quantitative analysis

For quantification, three sections (120  $\mu\text{m}$  apart) containing anterior, middle or posterior part of the LC were selected for each animal and the number of cells stained for each of ER $\alpha$ , ER $\beta$ , and PR was counted bilaterally at a magnification of 200 $\times$  under a Nikon light microscope (Nikon, Japan). Since the majority of the LC cells express TH [51], we used TH immunostaining to identify the exact boundary of the LC regions. In the POA and VMH,



PR-ir cells in an area of 500  $\mu\text{m}^2$  in two sections for each brain region were quantified bilaterally. The sum of immunopositive cells in each brain area was calculated for each mouse and averaged for each genotype and treatment group.

### Radioimmunoassay

Blood samples collected from the further experiments with  $\beta\text{ERKO}$  and  $\beta\text{WT}$  mice were used to measure plasma levels of estradiol and progesterone by double antibody radioimmunoassay (RIA) using the Estradiol and Progesterone Maia kits (Biochem Immunosystems, Serotec, Italy). The lower limit of detection was 6.71 pg/ml for estradiol and 0.43 ng/ml for progesterone. The intra-assay coefficient of variation was 2.1 and 3.2%, for estradiol and progesterone, respectively. In order to prevent inter-assay variation, all samples were measured together in a single assay.

### Statistics

Data were analyzed first using a two-way analysis of variance (ANOVA) for main effects of genotype, treatment group and their interaction, followed by post-hoc one-way ANOVAs with Newman-Keuls for multiple comparisons or *t*-test. The level of significance was set at  $P < 0.05$ .

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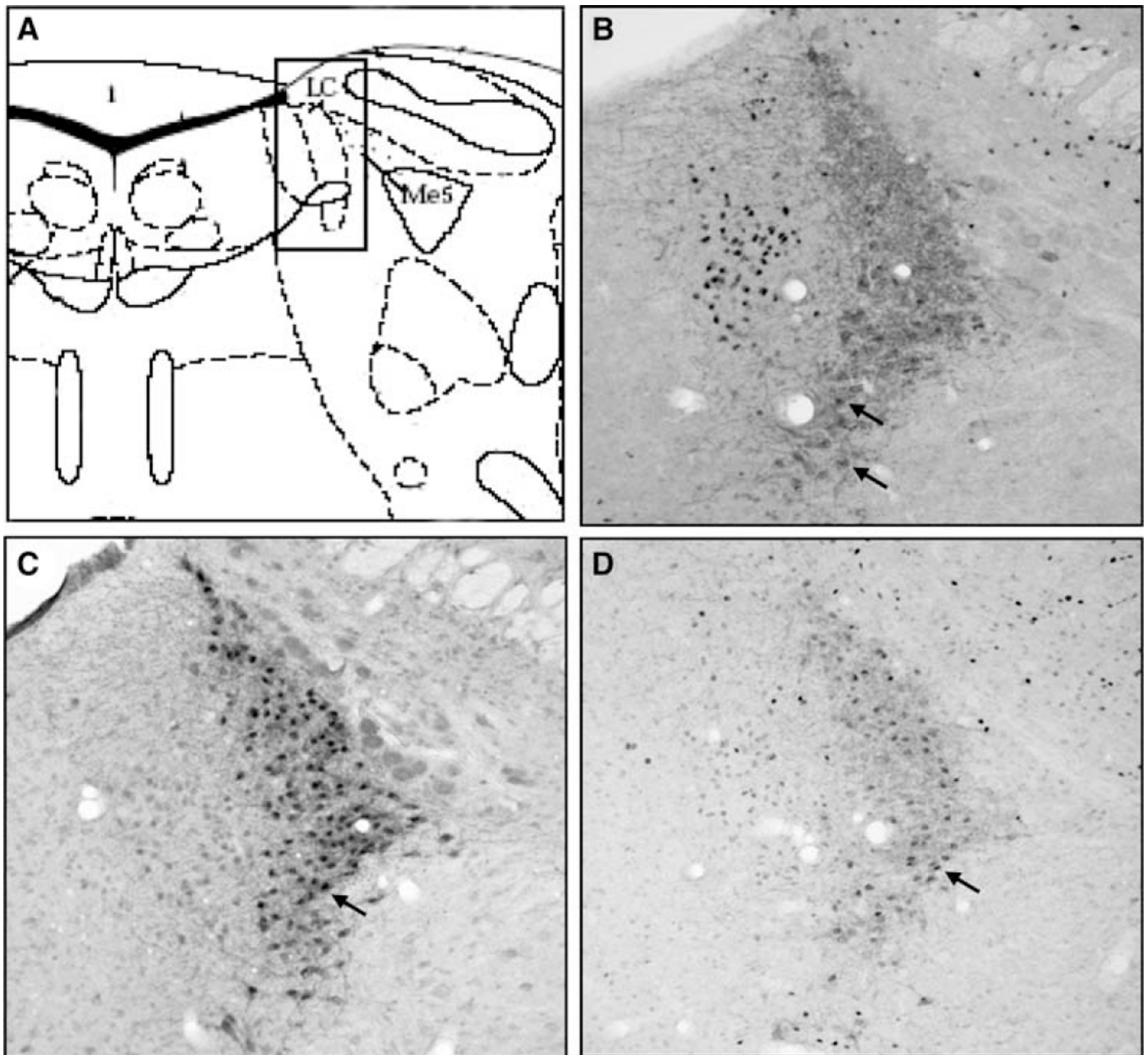
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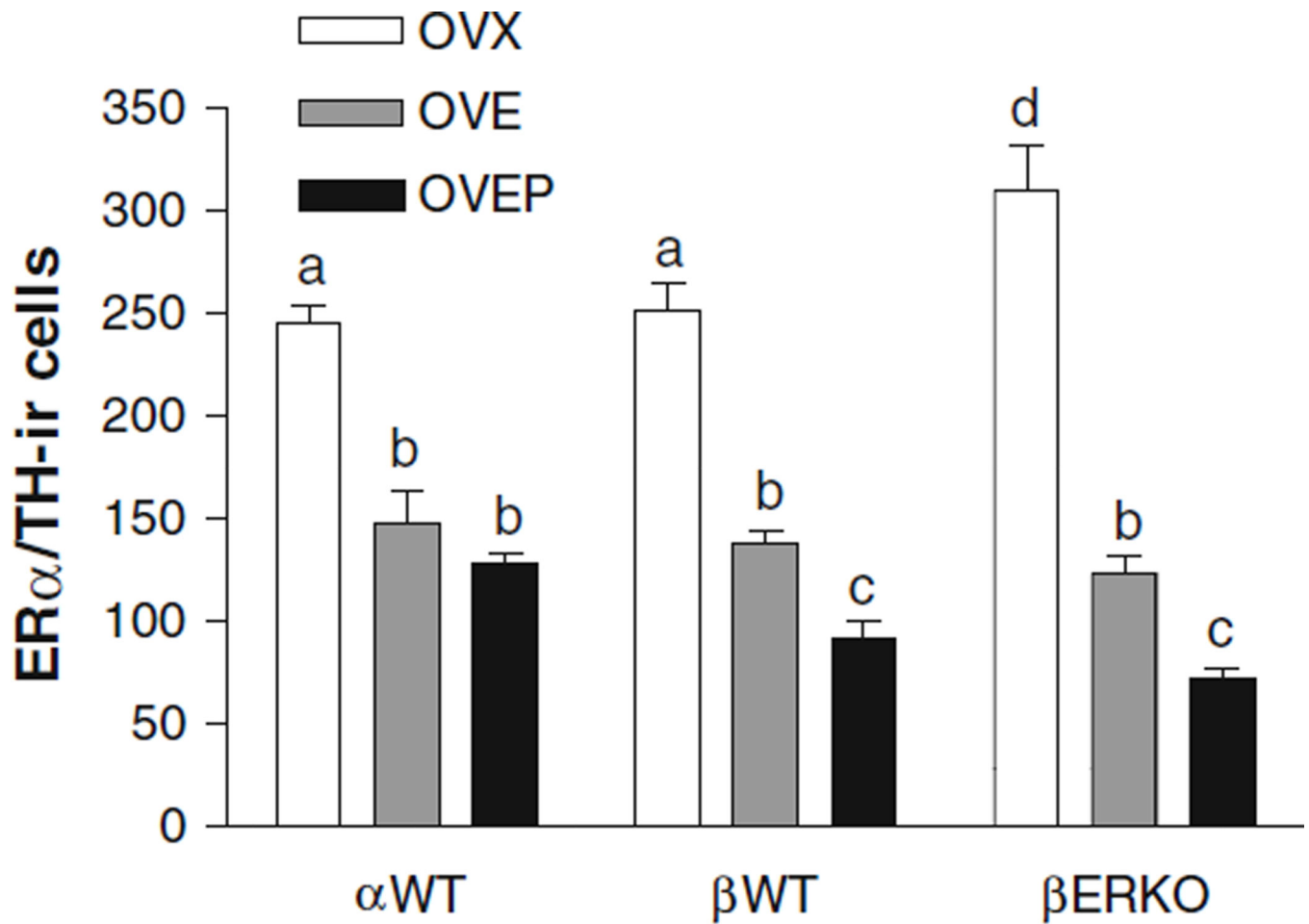
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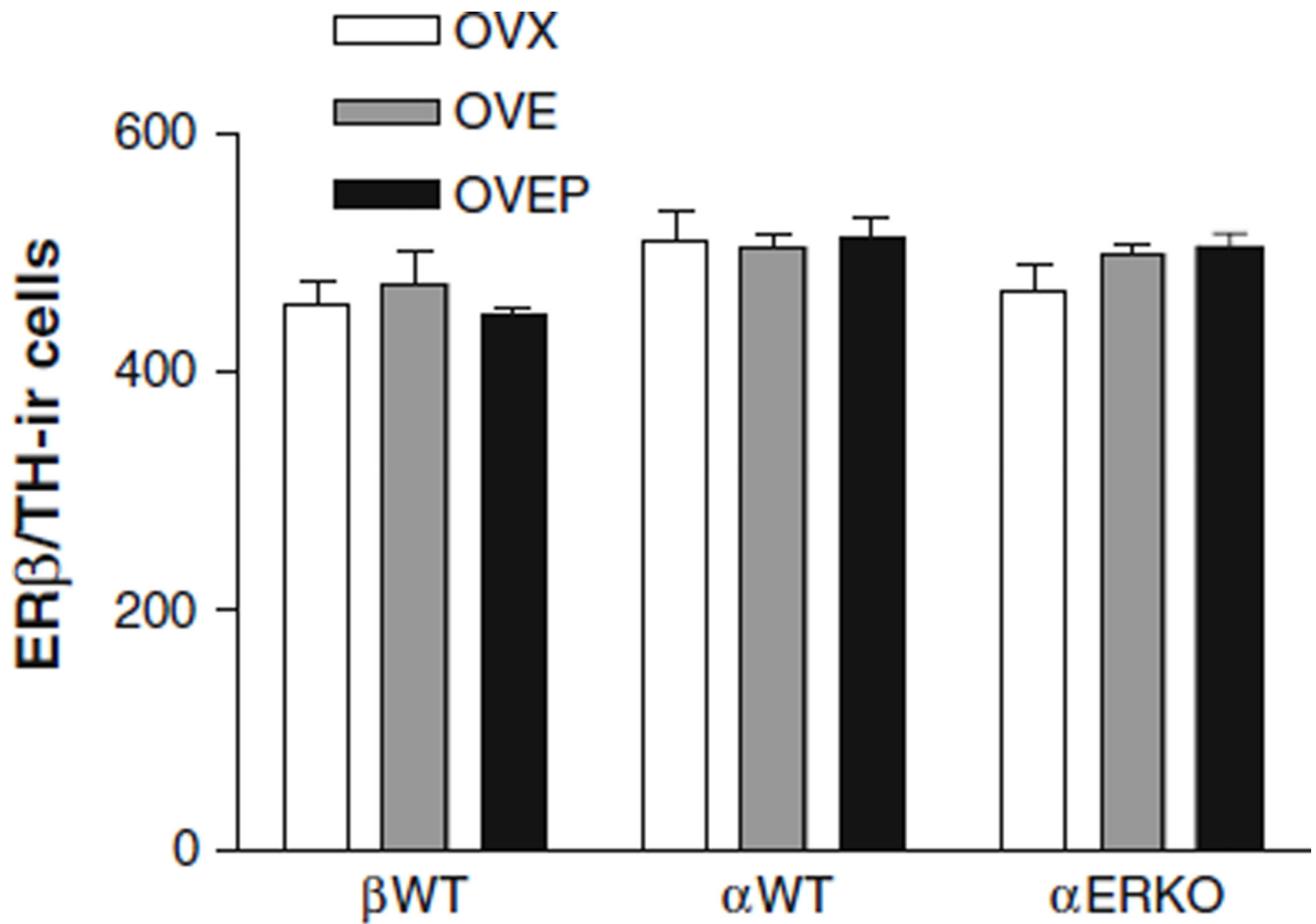
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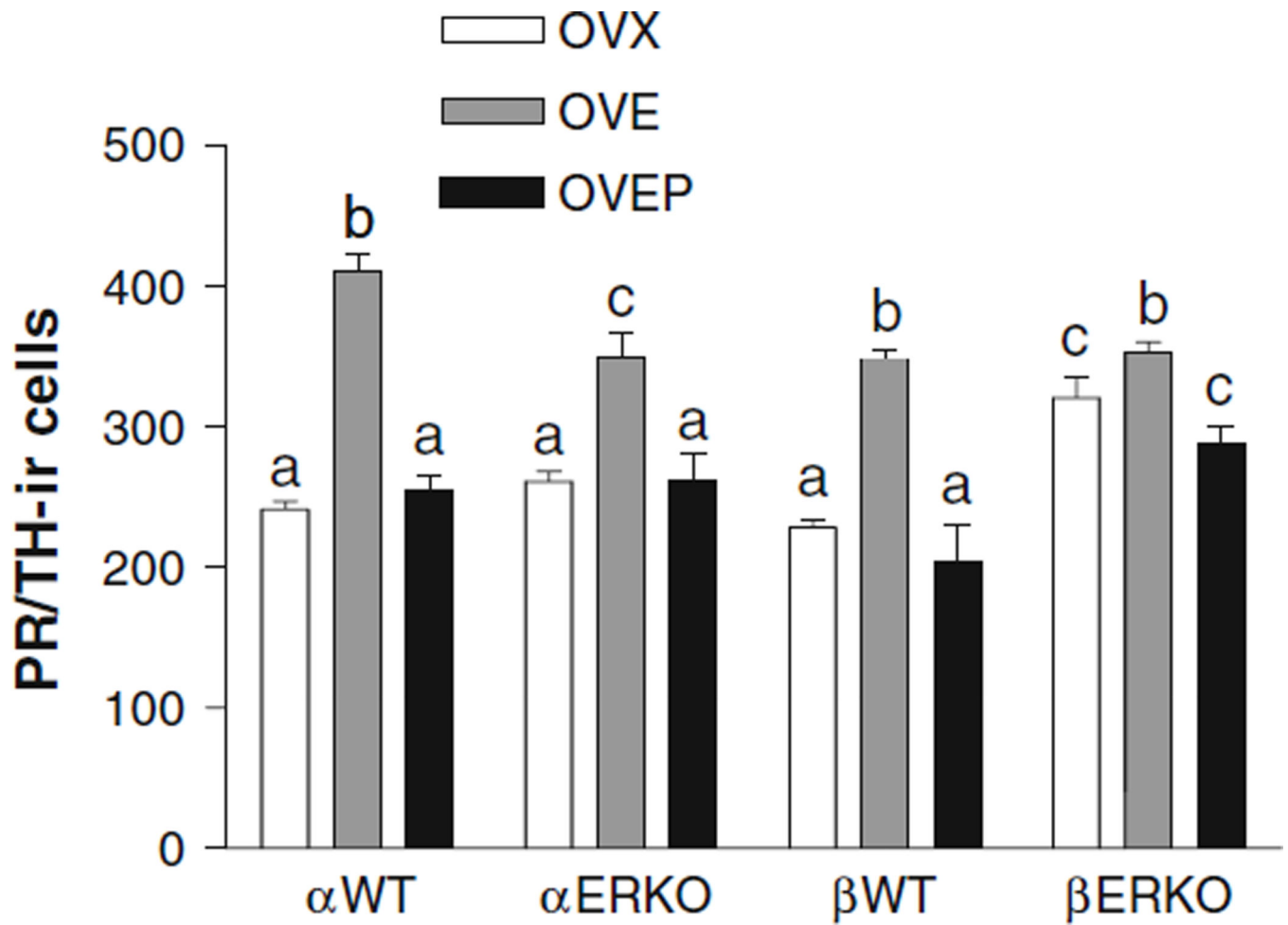
**Fig. 1.** Schematic view of the locus coeruleus (a) and representative photomicrographs (200 $\times$ ) showing nuclear immuno staining for ER $\alpha$  (b), ER $\beta$  (c) and PR (d) and cytoplasmatic staining for tyrosine hydroxylase in the locus coeruleus. The *arrows* indicate examples of double-labeled neurons. (Color figure online)



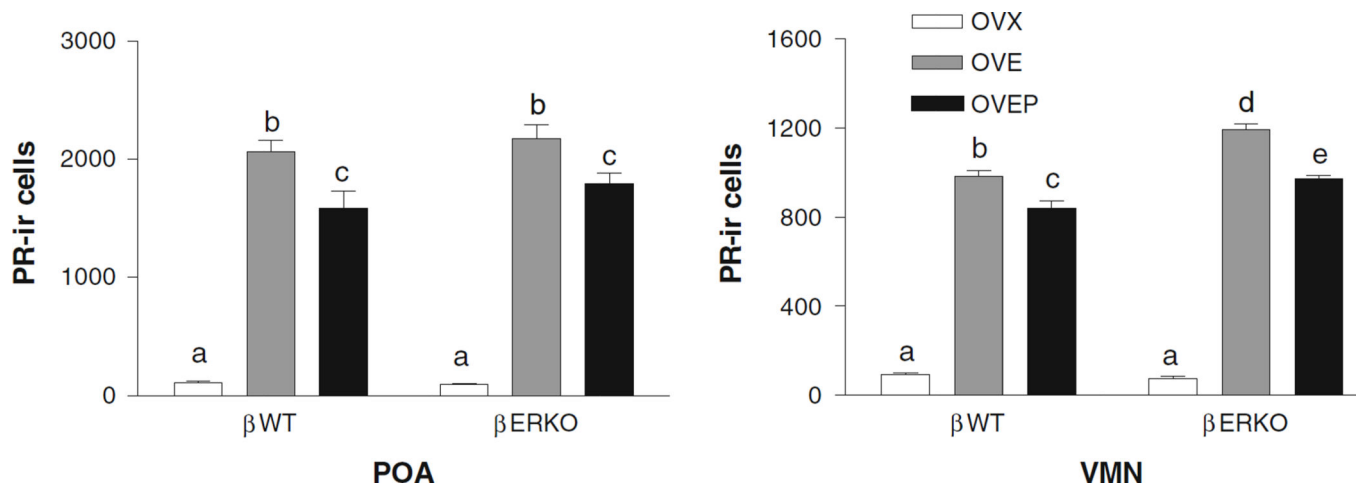
**Fig. 2.** Histogram showing the effects of estradiol and progesterone treatment on the mean number ( $\pm$ SEM) of TH-immunoreactive cells expressing ER $\alpha$  immunoreactivity in the locus coeruleus of ovariectomized  $\alpha$ WT,  $\beta$ WT, and  $\beta$ ERKO mice treated with either oil (OVX), EB (OVE) or EB plus progesterone (OVEP). Different letters indicate statistical significance ( $n = 6-11$ /group)



**Fig. 3.** Histogram showing the effects of estradiol and progesterone treatment on the mean number ( $\pm$ SEM) of TH-immunoreactive cells expressing ER $\beta$  immunoreactivity in the locus coeruleus of ovariectomized  $\alpha$ WT,  $\beta$ WT and  $\alpha$ ERKO mice treated with either oil (OVX), EB (OVE) or EB plus progesterone (OVEP). ( $n = 5-7$ /group)



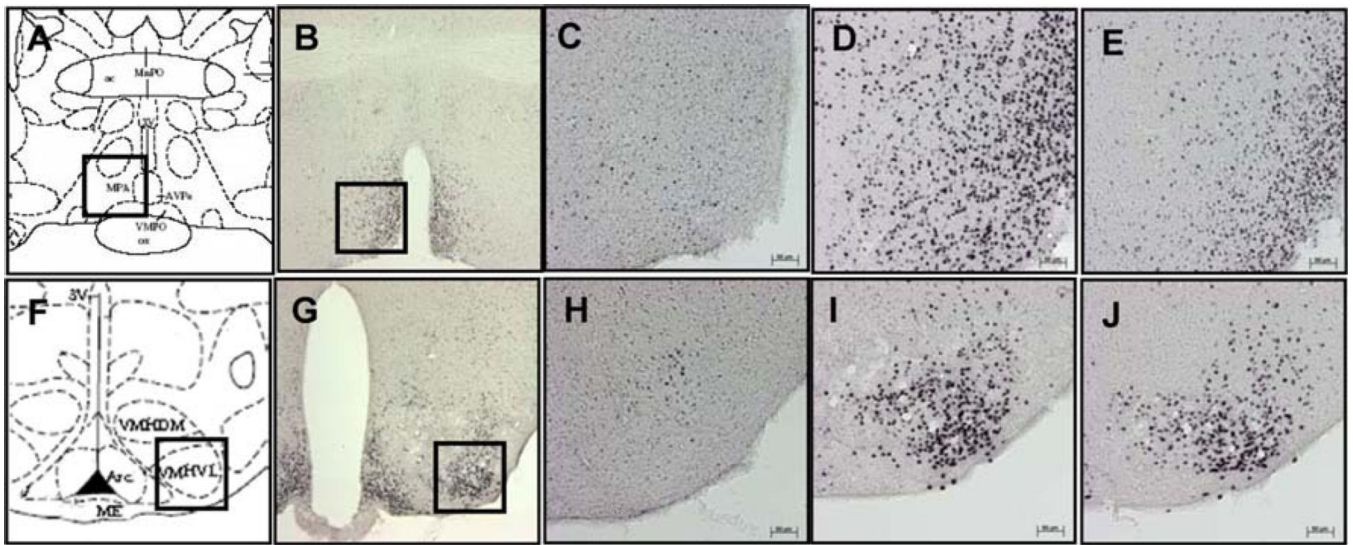
**Fig. 4.** Histogram showing the effects of estradiol and progesterone treatment on the mean number ( $\pm$ SEM) of TH-immunoreactive cells expressing PR immunoreactivity in the locus coeruleus of ovariectomized  $\alpha$ WT,  $\alpha$ ERKO,  $\beta$ WT and  $\beta$ ERKO mice treated with either oil (OVX), EB (OVE) or EB plus progesterone (OVEP). Different letters indicate statistical significance ( $n = 5-11$ /group)



**Fig. 5.**

Histogram showing the effects of estradiol treatment on the mean number ( $\pm$ SEM) of PR immunoreactive cells in the POA (a) and the VMN (b) of ovariectomized  $\beta$ WT and  $\beta$ ERKO mice treated with either oil (OVX), EB (OVE) or EB plus progesterone (OVEP). Different letters indicate statistical significance ( $n = 6$ /group)





**Fig. 6.** Schematic view and representative photomicrographs of POA (**a–e**) and VMN (**f–j**). *Squares* in the lower magnification (50×; photos **b** and **g**) indicate the same areas shown in the higher magnification (200×; photos **c–e** and **h–j**) where the PR immunoreactive cells were quantified in the POA (**c–e**) and VMN (**h–j**) of ovariectomized  $\beta$ WT mice treated with either oil (**c** and **h**), EB (**d** and **i**) or EB plus progesterone (**e** and **j**)

Mean ( $\pm$ SEM) plasma estradiol and progesterone concentrations of ovariectomized mice treated with oil (OVX), estradiol (OVE) or estradiol plus progesterone (OVEP)

**Table 1**

	$\beta$ WT					
	$\beta$ ERKO			$\beta$ ERKO		
	OVX	OVE	OVEP	OVX	OVE	OVEP
Estradiol (pg/ml)	11.0 $\pm$ 1.1	231.6 $\pm$ 59.9**	274.9 $\pm$ 46.8**	9.2 $\pm$ 0.9	241.7 $\pm$ 36.4**	263.8 $\pm$ 73.1**
Progesterone (ng/ml)	0.9 $\pm$ 0.2	1.1 $\pm$ 0.2	66.3 $\pm$ 11.4***	1.2 $\pm$ 0.2	1.1 $\pm$ 0.2	66.7 $\pm$ 16.3***

Different letters indicate statistical significance (n = 6/group)

\*\*  $P < 0.05$ ;

\*\*\*  $P < 0.001$