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ORIGINAL RESEARCH

Estrogen Receptors Alpha and Beta in POA-AHA Region Regulate Asymmetrically Ovulation

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Abstract

We examined the role of the estrogen receptors alpha (ERα) and beta (ERβ) in of the preoptic-anterior hypothalamic area (POA-AHA) in the regulation of ovulation in rats. The number of ERα- and ERβ-immunoreactive (-ir) cells was determined at 09:00, 13:00, and 17:00 h of each stage of the estrous cycle in intact rats. Additionally, the effects of blocking ERα and ERβ on ovulation rate at 09:00 h on diestrus-2 or proestrus day through the microinjection of methyl-piperidino-pyrazole (MPP) or cyclofenil in either side of POA-AHA were evaluated. The number of ERα-ir and ERβ-ir cells in POA-AHA varied in each phase of estrous cycle. Either MPP or cyclofenil in the right side of POA-AHA on diestrus-2 day reduced the ovulation rate, while at proestrus day it was decreased in rats treated in either side with MPP, and in those treated with cyclofenil in the left side. MPP or cyclofenil produced a decrease in the surge of luteinizing hormone levels (LH) and an increase in progesterone and follicle stimulating hormone (FSH). Replacement with synthetic luteinizing hormone-releasing hormone in non-ovulating rats treated with MPP or cyclofenil restored ovulation. These results suggest that activation of estrogen receptors on the morning of diestrus-2 and proestrus day asymmetrically regulates ovulation and appropriately regulates the secretion of FSH and progesterone in the morning and afternoon of proestrus day. This ensures that both, the preovulatory secretion of LH and ovulation, occur at the right time.

Keywords MPP · Cyclofenil · POA-AHA · Ovulation · Asymmetry

Background

Estrogens, especially 17β-estradiol (E_2), play a crucial role in coordinating the neuroendocrine events that control reproduction. Estrogen effects are mediated mainly by two intracellular estrogen receptors (ERs), alpha (ERα) and beta (ERβ) (Green et al. 1986; Kuiper et al. 1996; Mosselman et al. 1996; White et al. 1987). Both ERs have been detected in neurons and glia in the brain (Chaban et al. 2004; Donahue et al. 2000).

In spontaneously ovulating species, the rising plasma estrogen levels of the mid to late follicular phase of the cycle unleashes a positive feedback action on the gonadotropin-releasing hormone (GnRH) neuronal network to induce a gonadotropin surge. At other times of the cycle, E_2 is responsible for exerting a suppressive effect on gonadotropin secretion (Herbison 2006). ERα protein or mRNA in GnRH neurons have not been detected mainly by immunocytochemical and in situ hybridization studies (Herbison 1998) in the case of ERβ protein or mRNA in GnRH neurons have been

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characterized in several stages of the development, such as embryonic, prepuberal and adult stages in mice (Kallo et al. 2001; Sharifi et al. 2002) as well as in adult rats (Hrabovszky et al. 2000, 2001; Legan and Tsai 2003; Shivers et al. 1983). These facts suggest that GnRH neurons express more ERβ even though estrogens levels may be quite low, demonstrating that E_2 is activating approximately 10% of GnRH neurons (Hrabovszky et al. 2001).

In primates, sheep and rodents the luteinizing hormone (LH)/GnRH surge is induced only if a sufficiently high signal or increasing levels of E_2 lasts for several hours (Bronson 1981; Caraty et al. 1989; Legan et al. 1975; Moenter et al. 1990; Xia et al. 1992; Yamaji et al. 1971). There is still discussion on the neuroendocrine control of ovarian cycle between whether the rising levels of E_2 through diestrus-2 day or the surge of progesterone (P_4) secreted on proestrus day act in the gonadotroph cell to enhance its responsiveness to GnRH and induce the ovulation in the rat (Freeman 2006).

In order to assess the participation of ERα and ERβ in the right or left side of preoptic-anterior hypothalamic area (POA-AHA) on rat spontaneous ovulation, in the current study we set the following goals: (1) to determine the number of ERα or ERβ immunoreactive (-ir) cells in POA-AHA region at three different time points for each phase of the estrous cycle; (2) to analyze the effects of blocking ERα or ERβ in either side of POA-AHA region on spontaneous ovulation specifically on diestrus-2 and proestrus day of the estrous cycle; (3) to examine whether ERα or ERβ in POA-AHA region is involved in generating one of the multiple signals that modulates the preovulatory secretion of GnRH and LH, as well as secretion of FSH, E_2 and P_4 on proestrus day.

Materials and Methods

All experiments were carried out in strict accordance with the Mexican Law of Animal Treatment and Protection Guidelines, and the specifications of the Mexican Official Standard, NOM-062-ZOO-1999. The Committee of the Facultad de Estudios Superiores Zaragoza approved the experimental protocol (FES/DEPUCI/236/14). This study tried to minimize the number of animals used, and all procedures were undertaken in a humane manner.

Animals

Adult virgin female rats (90 days of age), 195–225 g of body weight, of strain CIIZ-V from our own stock were used. The animals were kept under controlled light/dark conditions (05:00–19:00 h), with free access to feed (Teklad, 2018S, 18% protein rodent diet, ENVIGO, RMS, INC, USA) and water. The estrus cycle of the animals was followed by daily vaginal smears (09:00–10:00 h) and only animals undergoing at least two consecutive 4-day cycles were used in the experiments. Once the animals have reached 120 days of age they are no longer used in the studies. All treatments were performed at 09:00 h.

Experimental Procedures

ERα or ERβ Immunoreactive Neurons in POA-AHA During the Estrous Cycle

Immunohistochemistry Animals from each phase of the estrous cycle were anesthetized intraperitoneally and sacrificed at 09:00, 13:00 or 17:00 h ($n=3$ rats per group). The brains were dissected and fixed in 4% paraformaldehyde solution overnight, and then processed to be embedded in paraffin. Brain sections (10 µm thick) were used to detect ERα or ERβ immunoreactive neurons (ERα-ir or ERβ-ir) using a standard avidin–biotin immune peroxidase protocol. Brain slices were incubated at 4 °C for 72 h with either ERα or ERβ, a rabbit polyclonal antibody each one (sc-542 or sc-8974, Santa Cruz Biotechnology Inc., Dallas, TX, USA); the antibody solution was used to 1:100 dilution. Later, secondary polyclonal antibodies (1:200 dilution) were used to incubate the brain sections for 3 h at room temperature (pk-6101, Vectastain Elite ABC Kit, Vector Laboratories, Inc., Burlingame, CA, USA), and immunoreactivity was detected with the avidin–biotin horseradish peroxidise complex. To perform the negative control, we omitted the primary antibody. The number of ERα-ir or ERβ-ir was determined by counting positive cells as was previously published (Mendoza-Garces et al. 2011). Briefly, ERα-ir or ERβ-ir cells were counted in the left or right side from medial preoptic nucleus that includes the central, lateral, and medial portions (Fig. 1) using a light microscope (Nikon Eclipse E400, Mountain View, CA, USA); the counts were restricted to rostral-caudal from −0.6 to −0.68 mm relative to the bregma of the left or right side from POA-AHA region (Paxinos and Watson 2005). Between six to eight brain slices per animal were used for counting positive cells.

Blocking ERα or ERβ in the Left or Right POA-AHA on Spontaneous Ovulation

To assess if blocking ERα or ERβ in the left or right POA-AHA prevents ovulation, at 09:00 h of the diestrus-2 or proestrus day, groups of 8 to 10 rats were microinjected on the left or right side of POA-AHA as follows: (a) tween 20, 1/10 v/v (control group); (b) 25 µg/µL MPP (Sigma-Aldrich Corp. St. Louis, MO, USA) an ERα specific antagonist, or (c) 25 µg/µL cyclophenyl (Sigma-Aldrich Corp. St. Louis, Mo, USA) a selective estrogen receptor modulator (SERM)

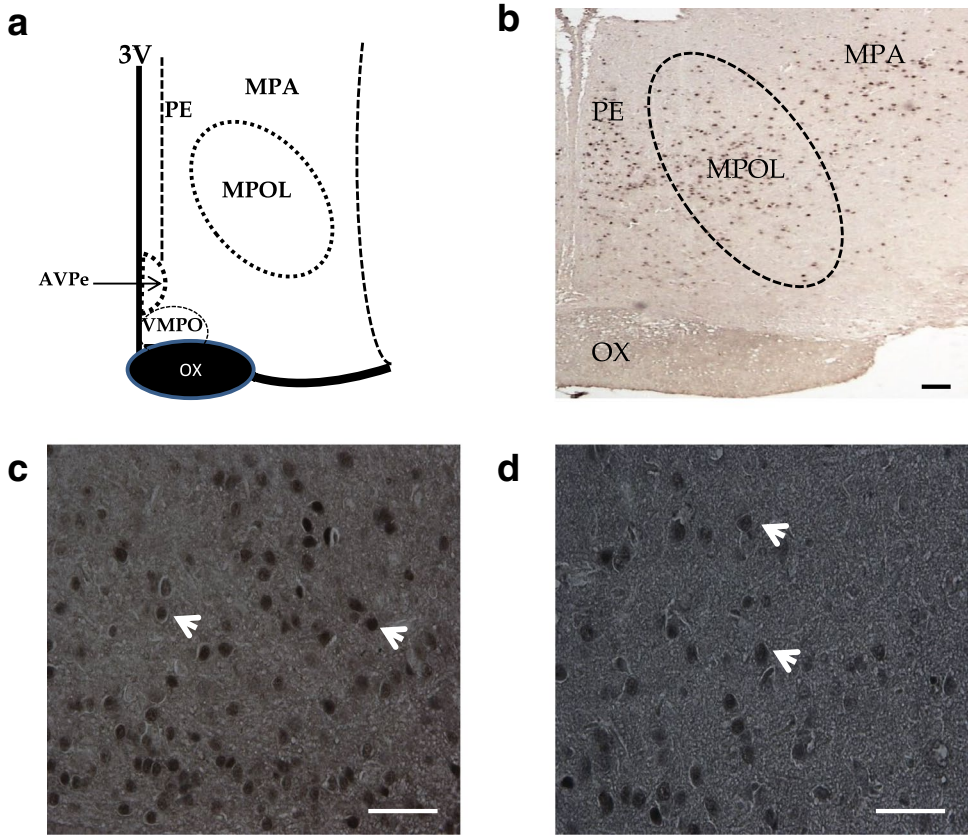

Fig. 1 Diagram of the POA-AHA region showing the site of microinjections and location where cell counting was performed. The diagram is based in the Paxinos and Watson's stereotaxic atlas for the rat brain **a** Representative photomicrograph showing the immunoreactive neurons (brown) for ERα of intact rats on the left side of POA-AHA, where cells were counted within the central, lateral, and medial portions of the medial preoptic nucleus, magnification 10× (**b**) and 40× (**c**), arrowheads indicate the immunostained (nuclear) neurons. ERβ, magnification 40× (**d**). Scale bar = 50 µm. See materials and methods section for details. MPA, medial preoptic area; MPOL, lateral part of medial preoptic nucleus; VMPO, ventromedial preoptic nucleus; PE, periventricular nucleus; 3 V, third ventricle; AVPe, anteroventral periventricular nucleus; ox, optic chiasm

and has been shown to be an inhibitor of gonadotropin secretion in different experimental models and infections (Bowman et al. 1982; Nencioni et al. 1982; Taubert et al. 1970), and is considered as ERβ selective ligand (Muthyala et al. 2003; Seo et al. 2006).

All solutions were injected in a total volume of 1 µL delivered over 1 min. The rats were anesthetized with 25 mg/kg weight of pentobarbital (Pisabental, Mexico City) intraperitoneally. The animals were mounted on a model 900 stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). The scalp was washed with antiseptic soap and then shaved. The skin was cut with a scalpel and the left or right side of the skull was drilled (1 mm diameter), following the coordinates of the rat brain atlas (Paxinos and Watson 2005), a stainless steel needle was lowered until reach the POA-AHA center.

The brain region was located using coordinates from Bregma as follows: A-P, 0.679 to 0.628 mm; Lateral, 0.06 mm; Vertical, 0.86 mm) (Espinosa-Valdez et al. 2016; Lopez-Ramirez et al. 2017) based on the Paxinos and Watson (2005) atlas. Using a Teflon tube (0.65 mm OD, 0.12 mm OI, Bioanalytical Systems Inc., West Lafayette, IN), the needle was connected to a 20 µL Hamilton syringe, placed in a microinjection pump (CMA/100; BAS, Stockholm, Sweden) to deliver the treatments. To verify the accuracy of the microinjection site, the brains were cut coronally every 100-µm sections with a vibratome (Technical Products International Inc, St. Louis, MO, USA) and examined under a stereoscopic microscope. The animals were euthanized by decapitation 48 h (for animals treated in diestrus-2 day) or 24 h (for animals treated in proestrus day) after the treatment. Rats were not anesthetized prior to euthanasia because it is well documented that pentobarbital, nembutal or ether anesthesia inhibits LH secretion and ovulation (Daane and Parlow 1971; Blake 1974; Domínguez and Smith 1974). At euthanasia, the oviducts were dissected, and the number of oocytes released was counted with a stereomicroscope (Olympus SZ51-LGB, Tokyo, Japan).

To verify the accuracy of the microinjection site, the brain of microinjected rats was fixed in 4% paraformaldehyde and, with the aid of a vibratome (Technical Products International Inc., St. Louis, MO, USA), was cut coronally in sections of 100 µm in the POA-AHA region. The sections were mounted on slides and examined immediately under a stereoscopic microscope. Only the data obtained from animals with verified microinjection in the POA-AHA were included in the analysis.

Replacement of LH-Releasing Hormone in Rats with ERα or ERβ Blockade in the Left or Right POA-AHA on Spontaneous Ovulation

To check whether blocking ERα or ERβ affects the surge GnRH secretion on the day of the proestro, at 14:00 h of the

proestrus day, other groups of animals treated with MPP or cyclophenyl (n = 5-8) in the left or right POA-AHA were injected with 3.7 µg/Kg of synthetic LH-releasing hormone (LHRH-Gly-OH) (Sigma Chemical Corp. St. Louis, Mo, USA) subcutaneously (Humphrey et al. 1973), and then those animals were sacrificed between 09:00 and 10:00 h the following day.

Hormones Serum Levels

To analyze the endocrine mechanisms that occur on the day of proestrus were affected by the blockade of the ERα or the ERβ, a separate group of animals ($n = 5$) that received microinjections of MPP or cyclofenil at diestrus-2 or proestrus day, that did not ovulate were sacrificed at 11:00 or 17:00 h of predicted proestrus day. These hours were selected according to the secretion profile previously reported in this rat strain (Domínguez et al. 1998). P_4 and E_2 serum levels were measured using enzyme-linked immunoassays (ELISAs) with kits obtained from AccuBind (Monobind Inc., Lake Forest, CA, USA) and were performed according to the manufacturer's instructions. The sensitivity of each assay was as follows: P_4, 0.105 ng/ml and E_2, 6.5 pg/ml.

The concentration of Luteinizing Hormone (rLH) and Follicle Stimulating Hormone (rFSH) was measured by radioimmunoassay (RIA) of double antibody in liquid phase, using reagents and methods of the National Hormone and Pituitary Program (NIDDK, Baltimore, MD, USA). The first antibody was used at an initial dilution of 1: 252,000 and 1: 62,500, for rLH (NIDDK-anti-rLH-S-11) and rFSH (NIDDK-anti-rFSH-S-11), respectively, incubated 24 h at room temperature, and the second antibody (sheep serum anti-gamma globulin rabbit) was used at an initial dilution 1:10 with PBS + 8% Polyethylene glycol (PEG). rLH-I125 (NIDDK-rLH-I-10), 12,000 counts per minute, cpm) or rFSH-I125 (NIDDK-rFSH-I-9), 12,000 cpm, were added to each tube in a volume of 100 µl. The tubes were incubated at room temperature for 2 h and centrifuged at 3000 rpm for 60 min. The intra-assay and inter-assay coefficients of variation for FSH and LH were 12.1% and 14.6%, respectively, and the sensitivity of the tests for both FSH and LH was 0.1 ng/ml. The hormone serum levels were measured only in non-ovulating rats.

Statistical Analyses

The statistical analyses were performed using the GraphPad InStat3 Software, Inc. (San Diego, CA, USA). The results from ERα-ir or ERβ-ir cells and hormones serum levels were expressed as the mean ± SEM and analyzed with one-way analysis of variance (ANOVA) followed by Tukey test when assessing the effects of treatments. The ovulation rates (the number of ovulating animals/the number of treated animals) were analyzed using either the Fisher's exact probability test. For data on the number of ova shed, we used the Kruskal–Wallis test followed by Mann–Whitney U test. A probability value (p) ≤ 0.05% was considered statistically significant.

Results

Changes in the Number of ERα-ir or ERβ-ir Neurons in POA-AHA Region Throughout the Estrous Cycle

In diestrus-2 and proestrus, the total percentage of ERα-ir is greater than the ERβ-ir cells. On the same days, the percentage of ERα-ir cell is greater on the right side than on the left side. In contrast, the percentage of ERβ-ir cells is greater on the left side on diestrus-2 (Table 1). Clear differences were observed between the right and left side of POA-AHA region at the same time points: the number of ERα-ir cells was higher in the right side (right side: 1785 ± 56 vs. left side: 865 ± 44, $p < 0.001$) while ERβ-ir cells increased in the left side (right side: 1690 ± 166 vs. left side: 3890 ± 362, $p < 0.001$) of the diestrus-2 day at 17:00 h. In contrast, the number of ERα-ir or ERβ-ir cells increased in the right side of the proestrus day at 9:00 h (ERα-ir cells; right side: 1085 ± 138 vs. left side: 578 ± 78, $p < 0.05$; ERβ-ir cells; right side: 1070 ± 85 vs. left side: 626 ± 115, $p < 0.05$).

The number of ERα-ir or ERβ-ir cells in the POA-AHA region changed throughout the estrous cycle at each time point measured (Fig. 2). While the number of ERα-ir cells

Table 1 Total percentage of ERα or ERβ immunoreactive neurons in POA-AHA region in each phase of estrous cycle

% cells	Diestrus-1			Diestrus-2			Proestrus			Estrus		
	POA-AHA			POA-AHA			POA-AHA			POA-AHA		
ER	Total	Left	Right	Total	Left	Right	Total	Left	Right	Total	Left	Right
α	51	44	56	66[b]	33	57[a]	69[b]	39	61[a]	55	51	49
β	49	53	47	34	61	39[a]	31	45	55	49	45	55

The numbers represent the sum of immunoreactive neurons counted at 9:00, 13:00, and 17:00 h in the left or right side of the POA-AHA region for each phase of the estrous cycle

[a] $p < 0.01$ versus left POA-AHA; [b] < 0.01 versus ERα

was similar in diestrus-1, diestrus-2, and estrus day at 9:00 and 13:00 h, such number increased significantly at diestrus-1 and proestrus day at 17:00 h (Fig. 3a). In contrast, the number ERβ-ir cells was similar in all days of the oestrus cycle at 9:00 and 13:00 h, such number increased significantly at diestrus-1, diestrus-2, and estrus day at 17:00 h in comparison with proestrus day (Fig. 3b).

Effects of Treatment with an ERα or ERβ Antagonist in the POA-AHA Region on Ovulation Rate and Number of Ova Shed

The ovulation rate of rats treated with MPP or cyclofenil in the right side of POA-AHA was lower than in the control group at diestrus-2 day (MPP: 3/10, cyclofenil: 2/10 vs. control: 10/10, $p < 0.0031$) while MPP in either side of POA-AHA decreased ovulation rate at proestrus day (left: 2/10, right: 1/10 vs. control: 10/10, $p < 0.0007$). However, cyclofenil only in the left side reduced the ovulation rate (4/10 vs. 10/10, $p < 0.01$) (Fig. 4). The number of ova shed by ovulating rats treated with MPP or cyclofenil at diestrus-2 or proestrus day was similar to that of control rats (Table 2).

Effects of the Treatment with ERα or ERβ Antagonist in POA-AHA Region on Hormone Serum Levels

Rats treated either ER antagonist in the right POA-AHA at diestrus-2 day and sacrificed at 11:00 h on predicted proestrus day showed similar serum levels of all hormones compared to the control group. The animals sacrificed at 17:00 h showed elevated E_2 serum levels for the MPP group and decreased LH serum levels with either treatment compared to the control group (Table 3).

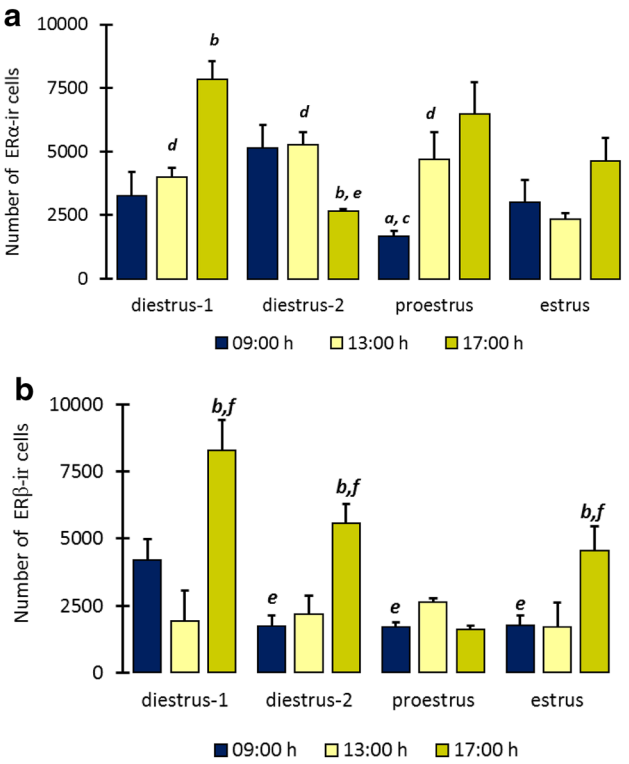

Fig. 3 Number of ERα and ERβ, immunostained neurons (ERα- and ERβ-ir) in the POA-AHA region throughout the estrous cycle in the rat. ERα-ir (**a**) and ERβ-ir (**b**) neurons were counted on both sides (left plus right side) of the POA-AHA region at 9:00, 13:00, or 17:00 h for each phase of the estrous cycle. The results are expressed as the mean ± SEM. **a**: $p < 0.01$ versus 9:00 h and 17:00 h, for its respective phase of the estrous cycle. **b**: $p < 0.001$ versus 09:00 and 13:00 h, for its respective phase of the estrous cycle. **c**: $p = 0.0404$ versus diestrus-2 at 09:00 h. **d**: $p = 0.0002$ versus estrus at 13:00 h. **e**: $p = 0.005$ versus diestrus-1 at 09:00 h. **f**: $p = 0.0001$ versus proestrus

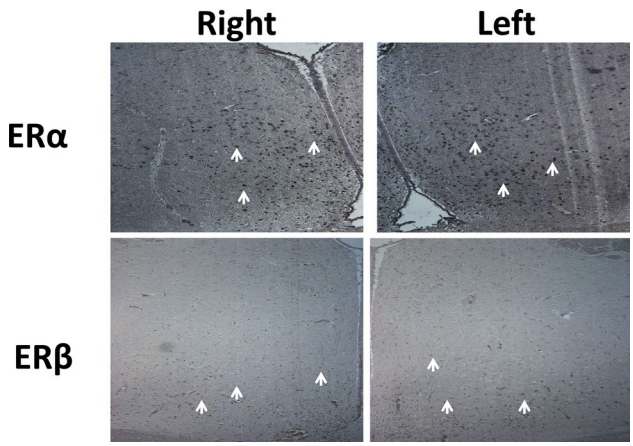

Fig. 2 ERα or ERβ immunoreactive neurons in POA-AHA region. Representative photomicrographs showing the immunoreactive neurons for ERα (upper panel) or ERβ (lower panel) from right or left side of the medial preoptic nucleus in the proestrus day, magnification 10×. Arrowheads indicate the immunostained (nuclear) neurons

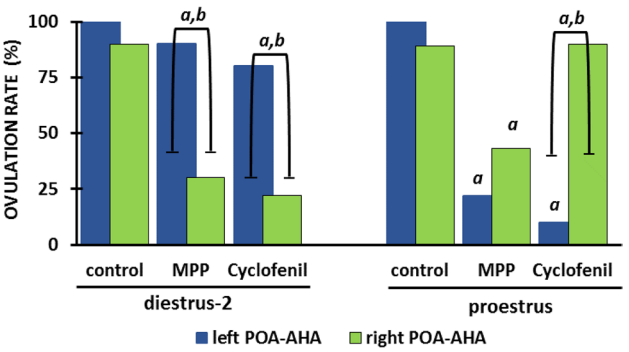

Fig. 4 Effects of the treatment with ERα or ERβ antagonist in POA-AHA region on ovulation rate. The ovulation rate (number of ovulating animals/total number of the treatment group) was calculated in rats treated with MPP or cyclofenil in the left or right POA-AHA at 09:00 h of the diestrus-2 or proestrus day. **a**: $p < 0.05$ versus the respective control group, **b**: $p < 0.05$ versus between left or right POA-AHA

Regarding the animals treated with either ER antagonist in the left POA-AHA region at proestrus day, these showed P_4 and FSH serum levels significantly higher than the control group measured at 11:00 h of the same proestrus day. However, at 17:00 h the animals treated only with MPP showed E_2 serum levels higher than the control group. Meanwhile, the treatment with either ER antagonist decreased the serum level of LH in comparison with the control group (Table 4).

Animals treated with either ER antagonist in the right POA-AHA region at proestrus day showed P_4 and FSH serum levels significantly higher than the control group measured at 11:00 h. In the animals sacrificed at 17:00 h, the serum levels of E_2 were higher and those of LH were lower in contrast with the control group (Table 4).

Effects of Replacing LHRH-Gly-OH on the Ovulation Rate of Rats Previously Treated with ERα or ERβ Antagonist in Either Side the POA-AHA

Treatment with synthetic LHRH-Gly-OH in non-ovulating rats unilaterally microinjected with MPP or cyclofenil on right POA-AHA at diestrus-2 day, or in either side of the POA-AHA at proestrus day, restored ovulation in all treated rats (diestrus-2-day, right side: 12/12 vs. 4/12,

Table 2 Number of ova shed from rats treated with ERα or ERβ antagonist in POA-AHA region

Day of the cycle	Control		MPP		Cyclofenil	
	POA-AHA		POA-AHA		POA-AHA	
	Left	Right	Left	Right	Left	Right
Diestrus-2	13.3 ± 1.1	14.5 ± 0.3	14.4 ± 0.8	11.0 ± 4.0	14.5 ± 0.3	11.0 ± 3.0
Proestrus	13.4 ± 0.6	13.0 ± 1.8	12.0 ± 1.0	16.5 ± 0.5	11.4 ± 1.1	9.7 ± 0.3

The number of ova shed of ovulating rats treated with MPP or cyclofenil in the POA-AHA at diestrus-2 or proestrus day was measured next predicted day of estrus. The results are expressed as mean ± SEM

Table 3 Progesterone, estradiol, FSH and LH Serum levels at 11:00 or 17:00 h of the predicted proestrus in groups of rats treated with MPP or cyclofenil in the right POA-AHA at 09:00 h of diestrus-2

Proestrus	Group	Progesterone (ng/mL)	17β-estradiol (pg/mL)	FSH (ng/mL)	LH (ng/mL)
11:00 h	Control	8.9 ± 0.81	127.1 ± 6.64	0.7 ± 0.18	1.01 ± 0.08
	MPP	10.4 ± 3.13	112.0 ± 6.15	0.9 ± 0.37	0.65 ± 0.31
	Cyclofenil	9.25 ± 2.423	130.7 ± 10.56	0.8 ± 0.14	1.26 ± 0.78
17:00 h	Control	22.8 ± 3.38	58.3 ± 5.04	5.1 ± 0.56	65.0 ± 5.74
	MPP	21.2 ± 2.87	97.9 ± 6.4[a]	5.9 ± 1.34	0.85 ± 0.18[a]
	Cyclofenil	27.2 ± 2.26	56.9 ± 7.26	7.9 ± 0.98	16.9 ± 7.67[a]

The results are expressed as mean ± SEM

[a] $p < 0.01$ versus control sacrificed at same hour (ANOVA followed Tukey test)

Table 4 Effects of the blocking ERα or ERβ in right or left POA-AHA performed at 09:00 h of the proestrus, on the progesterone, estradiol, FSH and LH serum levels assess 2 h (11:00 h) or 8 h (17:00 h) after the treatment

Side of POA-AHA	Proestrus hour	Group	Progesterone (ng/mL)	17β-estradiol (pg/mL)	FSH (ng/mL)	LH (ng/mL)
Left	11:00	Control	8.9 ± 0.81	127.1 ± 6.64	0.7 ± 0.18	1.0 ± 0.08
		MPP	46.0 ± 2.24[a]	118.9 ± 12.6	2.8 ± 0.14[a]	0.8 ± 0.06
		Cyclofenil	26.1 ± 1.20[a]	110.3 ± 5.89	3.9 ± 0.07[a]	1.2 ± 0.57
	17:00	Control	22.8 ± 3.38	58.3 ± 5.04	5.1 ± 0.56	65.0 ± 5.74
		MPP	21.0 ± 4.86	158.5 ± 8.46[a]	5.4 ± 0.53	11.3 ± 8.97[a]
		Cyclofenil	23.5 ± 3.52	73.1 ± 10.7	6.4 ± 1.02	16.9 ± 7.66[a]
Right	11:00	Control	8.9 ± 0.81	127.1 ± 6.64	0.7 ± 0.18	1.0 ± 0.08
		MPP	28.3 ± 2.42[a]	416.9 ± 92.62[a]	4.1 ± 1.04[a]	0.9 ± 0.18
	17:00	Control	22.8 ± 3.43	58.3 ± 5.04	5.1 ± 0.56	65.0 ± 5.74
		MPP	27.2 ± 2.86	239.5 ± 27.46[a]	5.9 ± 0.98	5.9 ± 0.98[a]

The results are expressed as mean ± SEM

[a] $p < 0.01$ versus control sacrificed at same hour (ANOVA followed Tukey test)

$p < 0.0003$; proestrus day, left side: 6/6, right side: 6/6 vs. 5/24, $p < 0.001$).

Discussion

Our research group has previously shown an asymmetric expression of the mRNA for the two ER isoforms in the POA-AHA region. ERα mRNA expression was high in the right side of the estrus day and in the left side on proestrus day; in contrast, ERβ mRNA expression was only detected in the diestrus-2 day without asymmetry (Arteaga-Lopez et al. 2003). In the present study, the differential mRNA expression of ER isoforms did not correlate with the corresponding protein immunoreactivity suggesting post-translational regulation in the synthesis of ER isoforms in the POA-AHA region neurons.

According to Shughrue et al. (1997, 1998), numerous cells in the periventricular preoptic area, the medial preoptic nucleus and the preoptic area, express both the ERα and ERβ mRNAs, suggesting that ERβ is not a simply backup receptor for ERα. These authors proposed that the presence of both ERs may allow the cell to respond differentially to the actions of estrogen (Shughrue et al. 1998). In the current study, we observed that at diestrus-2, the blockade of ERα produced an increase in the serum levels of estradiol and a decrease in LH levels. The blockade of ERβ diminished LH levels without affecting E_2 serum levels. The blockade of ERα or ERβ performed at proestrus produced similar results on E_2, P_4, FSH, and LH levels, supporting the suggestion of Shughrue et al. (1998). Thus, we propose that the role of estradiol in the regulation of GnRH/LH secretion and ovulation depends on the hormonal environment that characterizes each day of the estrous cycle.

The percentage of neurons expressing ERα and ERβ in the rat POA-AHA at diestrus-2 and proestrus is similar to those reported by Skynner et al. (1999) in female mice. The changes in the proportion of ER-ir cells seem to be related to the fluctuation of serum E_2 levels. In fact, it has been reported that during the estrous cycle, E_2 regulates the expression of the ER gen in the anteroventral periventricular nucleus (Simerly et al. 1996).

It is of interest that the number of ERα-ir cells relative to that of ERβ cells is opposite on the days of diestrus-2 and proestrus, suggesting that each receptor would be regulated in the opposite way by circulating 17β-estradiol or by other factors released in the POA-AHA. That is, on the morning of diestrus-2, when E_2 serum levels are lower than at 17:00 h, the number of ERα-ir cells is low, while the number of ERβ-ir cells is high, which suggests that the ERα is up-regulated whereas ERβ is down-regulated by E_2. In this way, at 17:00 h, when the levels of E_2 are high, the number of ERα-ir cells are low and ERβ-ir cells increase. In proestrus, E_2 down-regulates ERα, when E_2 levels are the highest in the estrous cycle (the surge of estradiol). On the same day, by contrast, ERβ does not appear to be regulated by E_2.

Under normal conditions, in the morning of diestrus-2 day, ERα or ERβ activation in the right portion of POA-AHA induces the preovulatory secretion of LH and ovulation, since the blockade of ERα results in a decrease in ovulation rate and LH, E_2 serum levels were increased (Fig. 5a, right side). Thus, the control of this mechanism does not seem to be related to the number of positive cells for each receptor, because the number of cells is similar in either side of POA-AHA in the intact rat (Fig. 5a and b). Despite this, the participation of each ER seems to be different: ERα would regulate LH secretion through a mechanism dependent on E_2, as suggested by the observation that E_2 serum levels at 17:00 h are as high as those observed at 11:00 h of predicted proestrus day (Table 3, Fig. 5a and b), which leads to down-regulation of the ERα itself (Borras et al. 1994) consequently inhibiting the release of LH and ovulation. However, ERβ could be participating in a mechanism that is E_2 independent. These results indicate that on the morning of diestrus-2-day, ovulation depends on the activation of both ER located in the right side of POA-AHA region. This hypothesis is supported by the fact that the injection of synthetic LHRH restores ovulation.

In the proestrus day, the activation of ERα in either side of POA-AHA region or of ERβ in the left portion triggers ovulation. The asymmetric effect on ovulation of blocking ERβ could be related to the number of cells expressing the receptor but rather to the number of receptors per cell, since one side contains twice as many ERβ-ir cells (see right POA-AHA, Fig. 5d). This explains why ovulation was not blocked on the left side of POA-AHA. This effect could be conditioned by the fact that the ERα not being deactivated. The low serum levels of LH that results from blocking the ERβ in the proestrus day may not depend of a drop in E_2 serum levels, because the levels of this estrogen at 11:00 and 17:00 h are similar to that of their respective group of control rats (Table 4, Fig. 5d). In rodents, the stimulatory or inhibitory feedback effect of E_2 on the secretion of GnRH/LH occurs through ERα-ir interneurons (Couse et al. 2003; Dorling et al. 2003; Hewitt and Korach 2002; Lindzey et al. 2006), since most GnRH neurons do not express this receptor (Hrabovszky et al. 2000, 2001; Legan and Tsai 2003; Shivers et al. 1983). In this regard, secretory neurons of glutamate, noradrenaline, dopamine, serotonin, histamine, acetylcholine, kisspeptin, neurokinin B, and/or neuropeptide Y could be involved (Herbison 2015).

The blocking of the preovulatory secretion of LH by the deactivation of ERs could be related to a modification in the activity of GABA neurons (Liu et al. 2017). Some modifications in the presynaptic release of GABA/Glutamate would be more important than postsynaptic mechanisms

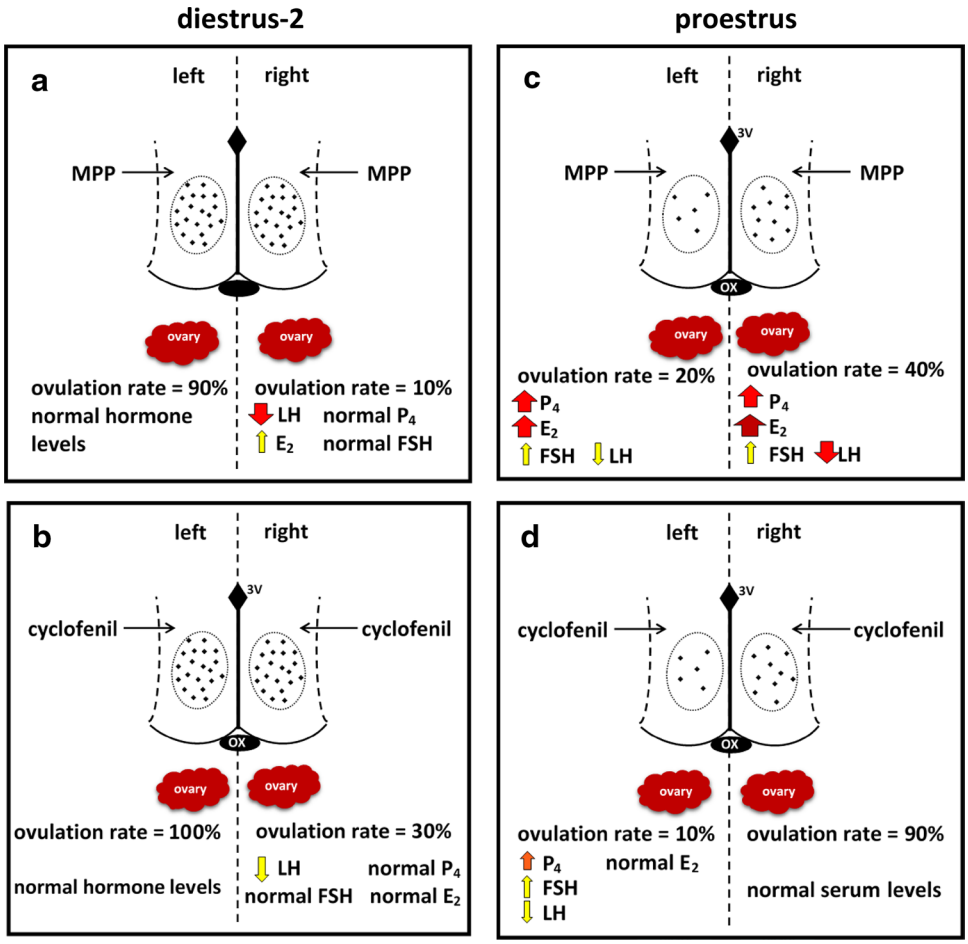

Fig. 5 Schematic summary of research findings. Schematic showing the relationship between the number of ERα-ir and ERβ-ir cells on each side of POA-AHA and the effects of the unilateral injection of MPP (**a**, **c**) or cyclofenil (**b**, **d**) on the left or right side of POA-AHA at 09:00 h of the day of diestrus-2 or proestrus. 3 V, third ventricle; OX, optic chiasm. decrease; increase

in the control of GnRH neurons firing across the ovarian cycle (Liu et al. 2017). In vivo, $GABA_A$ receptor signaling is determinant for GnRH neurons to exhibit normal firing patterns (Constantin et al. 2013); indeed, the GABAergic somas nearby GnRH neurons express ERα (Herbison et al. 1991; Jarry et al. 1988), and estrogens increase GABA release (Sullivan and Moenter 2003). Thus, the blockade of ERα activity would lead to a change in the release of GABA and their receptors. In vivo, $GABA_B$ receptors activation inhibits the electrical excitability of GnRH neurons (Liu and Herbison 2011; Zhang et al. 2009) and suppresses LH secretion in rats (Akema and Kimura 1991). Modifications in the excitability of GnRH neurons may explain changes observed in the FSH and LH levels in the present study. It has been shown that LHβ mRNA expression stimulates the pulse frequency of GnRH every 30 min, whereas FSHβ mRNA expression induces a slower GnRH pulse frequency, every 2 h (Kaiser et al. 1997).

The increase in P_4 and FSH observed 2 h after blocking ERs in either side of the POA-AHA region in the morning of proestrus day, suggests that E_2 levels could inhibit the secretion of both hormones. Cyclofenil treatment on ovariectomized rats depleted cytoplasmic ERs in the anterior and middle hypothalamus, while FSH and prolactin (PRL) serum levels were increased after 24 h of treatment (Bowman et al. 1982). Some brain regions that send direct and indirect projections to the paraventricular nucleus (PVN) such as the peri-PVN, bed nucleus of the *stria terminalis*, medial preoptic area, lateral septum, and hippocampus, express only ERβ. In the PVN, ERβ has been identified in PRL neurons (Handa and Weiser 2014; Suzuki and Handa 2005). Consequently, the increase in P_4 would be attributed to elevated levels PRL or FSH (Fortune and Vincent 1986). These high P_4 levels induce a strong inhibitory effect on the secretion and pulse frequency of GnRH/LH during the luteal phase, an action that occurs acutely, within around 50 min (Calogero et al. 1998). These facts may explain the increase in FSH secretion 2 h after of blocking either ER (Tables 2 and 3).

According to the World Health Organization, ovulatory disorders are the leading cause of infertility around the world (Mikhael et al. 2019). To our knowledge, there is currently no information about the modifications in estrogen receptors described in any of the circuits regulating ovulation in women. According to Dubois et al. (2015), in rodents ERα mediates most of the feedback effects by E_2. However, it is still unclear which cellular circuits and

which receptors are involved in this phenomenon. Because E_2 regulates the pulses of GnRH/LH by means of ERs (predominantly ERα) (Handa et al. 2014), any alteration in the feedback mechanisms of E_2 would invariably be associated with modifications, and even the absence, of ovulation and with infertility.

Conclusion

Altogether, our results showed that the activation of the ERα and ERβ asymmetrically regulates ovulation and suggest that activation of ERs on the morning of diestrus-2 and proestrus day appropriately regulate the secretion of FSH, P_4 y E_2 in the morning and afternoon of predicted proestrus day, thus ensuring that both the surge secretion of LH and ovulation occur at the right time. The present study supports that in POA-AHA region the neuroendocrine mechanisms that culminate with ovulation are asymmetric. This asymmetric activation not only depends of neurotransmitters such as acetylcholine and dopamine (Cruz et al. 1997, 1989; Moran and Dominguez 1995), also the stimulating feedback effect of estrogen linked to REα and/or REβ, regulates asymmetrically the GnRH/LH surge and ovulation in phases of diestrus-2 and proestrus day.

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Author Contributions MEC designed the experiments; MEC, IAC, RGJ, and RDC wrote the manuscript. IAC, MGL, and RLO performed the immunohistochemical analysis; RCh and CM quantified the concentrations of hormones; MEC, AF, IAC, RGJ, and RDC participated in the analysis and discussion of the results. All authors read and approved the final version of the manuscript.

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Compliance with Ethical Standards

Conflict of interest All authors declares that they have no conflict of interest.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All experiments were performed in strict accordance with the Mexican Law of Animal Treatment and Protection Guidelines and the specifications of the Mexican Official Standard NOM-062-ZOO-1999. The Institutional Committee of the Facultad de Estudios Superiores Zaragoza, Universidad Nacional Autónoma de México approved the experimental protocols (FES/DEPUCI/236/14). All efforts were made to minimize the number of animals used and their suffering.

Research Involving Human and Animal Participants This article does not contain any studies with human participants performed by any of the authors.

Abbreviations

POA-AHA: Preoptic and hypothalamic area; LH: Luteinizing hormone; FSH: Follicle stimulating hormone; GnRH: Gonadotropin-releasing hormone; P_4: Progesterone; E_2: E2; ERα: Estrogen receptors alpha; ERβ: Beta; -ir: Immunoreactive cells; MPP: Methyl-piperidino-pyrazole; SERM: Selective estrogen receptor modulator; ELISA: Enzyme-linked immunoassay; RIA: Radioimmunoassay; LHRH-Gly-OH: Synthetic LH-Releasing Hormone; GABA: Gamma aminobutyric acid; PRL: Prolactin; PVN: Paraventricular nucleus; MPA: Medial preoptic area; MPOL: Lateral part of medial preoptic nucleus; VMPO: Ventromedial preoptic nucleus; PE: Periventricular nucleus; 3V: Third ventricle; AVPe: Anteroventral periventricular nucleus; ox: Optic chiasm

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