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REGULAR ARTICLE

The content of gonadotropin-releasing hormone (GnRH), kisspeptin, and estrogen receptors (ERα/ERβ) in the anteromedial hypothalamus displays daily variations throughout the rat estrous cycle

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Abstract

The content of gonadotropin-releasing hormone (GnRH), its mRNA, and estrogen receptor alpha (ERα) and beta (ERβ) in the hypothalamus varies throughout the estrous cycle. Furthermore, the abundance of these molecules displays asymmetry between the right and left side. In the present study, we investigated the changes in the content of ERα, ERβ, kisspeptin, and GnRH by western blot in the left and right anteromedial hypothalamus, at four different times during each stage of the rat estrous cycle. The serum levels of the follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were also measured. ERα and ERβ levels changed depending on the stage of the estrous cycle, meanwhile that of kisspeptin was modified according to both the hour of the day and the stage of the cycle. Except in estrus day, ERβ was higher in the right hypothalamus, while ERα was similar in both sides. During both proestrus and estrus, the content of kisspeptin and GnRH was higher in the right hypothalamus. The highest levels of FSH and LH occurred at 17:00 h of proestrus. But at estrus, the highest FSH levels were observed at 08:00 h and the lowest at 17:00 h. Thus, the current results show that the content of ERα, ERβ, kisspeptin, and GnRH in the anteromedial hypothalamus are regulated as a function of the stage of the estrous cycle and the hour of the day. Furthermore, the content of these proteins is regularly higher in the right anteromedial hypothalamus, regardless of the stage of the cycle or time of the day.

Keywords GnRH · Estrogen receptor · Kisspeptin · Estrous cycle · Hypothalamic asymmetry

Introduction

Gonadotropin-releasing hormone (GnRH) is the main stimulating factor that regulates the secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), both of which then regulate the development of ovarian follicles and ovulation (Herbison 2015). In the rodent brain, GnRH neurons are located in rostral areas of the encephalon as part of the

María-Esther Cruz is deceased. This paper is dedicated to her memory.

This article is dedicated to the memory of our beloved colleague, who recently passed away.

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diagonal band of Broca, septum, medial preoptic area (mPOA), and anterior hypothalamic area (AHA) (Melmed et al. 2016). POA-AHA GnRH-neurons project their axons to the median eminence where the nerve terminals are in appositions with the vessels of the hypothalamic-pituitary portal system. The secretion of GnRH is regulated by 17β-estradiol (E_2) acting at the alpha- and beta-isoform of the estrogen receptor (ERα and ERβ) (Herbison 2015). GnRH neurons express ERβ but not ERα (Shughrue et al. 1998). The latter is expressed in astrocytes (Garcia-Ovejero et al. 2002) and in neurons innervating GnRH cells (Kalló et al. 2001) which in turn synthesize GABA (Herbison 1997), glutamate (Eyigor et al. 2004), noradrenaline (Simonian et al. 1999), serotonin (Leranth et al. 1999), neurotensin (Herbison and Theodosis 1992), galanin (Bloch et al. 1992), substance P (Okamura et al. 1994), calcitonin gene-related peptide (Yuri and Kawata 1994), acetylcholine (Turi et al. 2008), and kisspeptin (Adachi et al. 2007).

Thompson et al. (2004) have shown the pivotal role played by kisspeptin in the neuroendocrine regulation of GnRH secretion. Kisspeptin is a 145-amino acid peptide from the RF-amide family, encoded by the Kiss1 gene (Putteeraj et al. 2016; Smith et al. 2006; Kotani et al. 2001; Dedes 2012). Kisspeptin was first isolated from human placental tissue and named metastin due to its activity as a metastasis suppressor (Bilban et al. 2004; Hussain et al. 2015) There are two dense population of kisspeptin neurons in the central nervous system, one in the anteroventral periventricular area (AVPV) and the other in the arcuate nucleus (ARC) (Pineda et al. 2010; Putteeraj et al. 2016; Muir et al. 2001; Ishii et al. 2013; Dedes 2012). In addition, a small population of kisspeptin neurons has been described in the periventricular nucleus (Smith et al. 2006). AVPV-kisspeptin neurons innervate the soma of GnRH-neurons located in the POA (Polston and Simerly 2006; Yip et al. 2015). Meanwhile, nervous terminals from the ARC-kisspeptin neurons can be found in close apposition with axons of GnRH-neurons in the median eminence and with the soma of AVPV-kisspeptin neurons (Yip et al. 2015). It has been shown that both populations of kisspeptin neurons express ERα and that estradiol exerts opposite responses on their activity and on the expression of kisspeptin mRNA. High circulating levels of estradiol stimulate Kiss1 expression and the activity of AVPV-kisspeptin neurons but inhibit the ARC-kisspeptin neurons. An opposite response occurs in each population in the presence of low levels of estradiol (Smith et al. 2005). Based on these findings, it has been proposed that the kisspeptinergic population at the AVPV participates in the positive feedback mechanisms by which estrogens trigger the preovulatory secretion of gonadotropins (Pinilla et al. 2012; Yip et al. 2015; Dubois et al. 2015). In contrast, ARC-kisspeptin neurons would be responsible for the estrogen negative feedback that occurs during most of the estrous cycle (Dubois et al. 2015). According to this model, the negative feedback by estrogens is the result of the inhibition of kisspeptin release by AVPV neurons, thus leading to the lack of the phasic discharge of GnRH. During the estrogen negative feedback, the stimulatory activity of the ARC-kisspeptin neurons in the median eminence regulates the tonic secretion of GnRH. Positive feedback, on the other hand, results from the stimulation of kisspeptin release from terminals of AVPV-kisspeptin neurons (Dubois et al. 2015). In summary, the activity and discharge of GnRH-neuron increases in the presence of kisspeptin whose secretion depends on the concentration of estradiol (Radovick et al. 2012).

In addition to the regulatory mechanisms described above, it has been shown that the hypothalamus regulates in an asymmetric way the secretion of gonadotropins and the functions of the ovary (Nance and Moger 1982; Fukuda et al. 1984; Nance et al. 1984; Cruz et al. 1989; Cruz et al. 1990a, b; Inase and Machida 1992; Morán et al. 1994; Sánchez et al. 1994; Arteaga-López et al. 2003). This functional asymmetry is exemplified by a number of observations. For example, in female rats, the content of GnRH in the right medio-basal hypothalamus is higher than in the left side (Gerendai et al. 1978; Bakalkin et al. 1984). Similarly, in male mice, the number of GnRH cell bodies is also higher in the right hypothalamus (Inase and Machida 1992). These findings are in agreement with the results showing an increase in the amount of the GnRH mRNA in the right preoptic area, compared with the left, at 13:00 h of diestrous-2 stage (Arteaga-López et al. 2003). The transcription of estradiol receptor mRNA is also asymmetric in this area as indicated by the observation that at 17:00 h of estrus, the content of ERα mRNA is higher in the right side. In contrast, ERβ mRNA is not asymmetric at this stage, but at 13:00 h of diestrus-2, it is higher in the left side (Arteaga-López et al. 2003). Because estradiol modulates GnRH secretion by acting on its receptors in afferent neurons, including AVPV- and ARC-kisspeptin neurons, the goal of this study was to examine the changes in the content of ERα, ERβ, kisspeptin, and GnRH in the anteromedial hypothalamus throughout the rat estrous cycle. Special attention was paid to highlighting the asymmetries between the left and right sides of the hypothalamus. The secretion profile of 17β-estradiol, LH, and FSH was also investigated.

Methods and materials

Animals

All experiments were approved by the Ethics Committee of Facultad de Estudios Superiores Zaragoza, UNAM (license number: FES/DEPUCI/236/14) and were conducted in strict adherence with the Mexican regulations for laboratory animals handling described in the Official Norm NOM-062-ZOO-1999. These regulations conform to international

guidelines. We used the 96-day-old CIIZ-V (hooded) rats weighing 230–260 g for each day of estrous cycle. The animals were housed under a 14:10 light cycle (lights on from 05:00 to 19:00 h) with a temperature of 22 ± 2 °C and with free access to tap water and pelleted food (Teklad, 2018S, 18% protein rodent diet, Envigo, RMS, Inc., USA). Vaginal smears were taken daily to determine the stage of their estrous cycles (diestrus-1, diestrus-2, proestrus, and estrus). Only animals displaying at least two 4-day cycles were included on the study.

Acute effects of pentobarbital anesthesia on FSH and LH serum levels

To determine whether the dose of pentobarbital, 40 mg/kg b.w. has any effect on the secretion of gonadotropins and ovulation, animals ($n = 8$ per group) were anesthetized with pentobarbital intraperitoneally at 11:00 or 17:00 h of proestrus day and sacrificed 15 min after by decapitation. In both groups, blood was collected, and the serum was retrieved and stored at − 20 °C until determination of LH and FSH levels.

Brain tissue dissection and processing

Because pentobarbital anesthesia modified the LH serum levels, the animals used for this study were euthanized by decapitation without anesthesia. Female intact rats were sacrificed at 08:00, 11:00, 14:00, and 17:00 h of each stage of the estrous cycle, and the left or right portion of the anteromedial hypothalamus region from eight rats were pooled per time point ($n = 3$). After decapitation, the brains were immediately removed from the skull and placed in cold 0.9% saline solution. A tissue block spanning the entire anteromedial hypothalamus region, this region includes from the anterior border of the preoptic area (bregma − 0.30 mm, anterior hypothalamus) to the posterior end of the medio-basal hypothalamus (bregma −2.80 mm, posterior hypothalamus), was obtained using a stainless-steel brain matrix, according to rat brain atlas (Paxinos and Watson 2005; Fig. 1). An antero-posterior section was across the middle of the third ventricle in order to separate the left and right portion of the anteromedial hypothalamus region, and then two portions were stored separately in liquid nitrogen until further processing.

SDS-PAGE electrophoresis and western blotting

Each half of pooled anteromedial hypothalamus region per time point was homogenized in RIPA lysis buffer with cold protease inhibitor cocktail (MilliporeSigma, St. Louis, MO). The supernatant obtained from homogenized sample was stored at − 70 °C. Protein concentration was determined by the bicinchoninic acid method. One hundred fifty microgram of total protein per sample in Laemmli buffer was loaded for electrophoresis into 4 to 20% polyacrylamide gels (Criterion™ TGX™ BIO-RAD, Hercules, CA). The proteins were electrophoretically transferred onto nitrocellulose membranes (Amersham™ Protran™, Chicago, IL). The membranes were incubated with a solution containing the corresponding primary antibodies: ERα (Dil 1:1000), ERβ (Dil 1:1000), GnRH (Dil 1:100) (rabbit polyclonal, Santa Cruz Biotechnology, Dallas, TX); kisspeptin (Dil 1:500) (rabbit polyclonal, Abcam, Cambridge, UK); and β-tubulin (Dil 1:10000) (mouse monoclonal, Cell Signaling Technology, Danvers, MA). Finally, the membranes were incubated with secondary antibodies coupled with fluorochromes and analyzed with an Odyssey CLx imaging system (LI-COR Biosciences, USA). β-tubulin was used for normalization purposes.

Determination of 17β-estradiol, FSH, and LH serum levels

Concentrations of 17β-estradiol (E_2) in serum ($n = 10$) were measured using radioimmunoassay, with kits purchased from Diagnostic Products (Los Angeles, CA). Results are expressed in pg/ml. The intra- and inter-assay percent variation coefficients for E_2 were 6.9 and 10.8, respectively. The detection limits of E_2 were 0.2680 to 900.00 pg/ml; correlation coefficient was 0.9960.

The concentration of FSH and LH in serum ($n = 10$) was measured by liquid-phase double-antibody radioimmunoassay. The reagents and methods were kindly provided by the National Hormone and Pituitary Program (NIDDK, Baltimore, USA). For LH, the antibody (NIDDK-anti-rLH-S-11) was diluted 1:252000, while for FSH, the dilution of the antibody (NIDDK-anti-rFSH-S-11) was 1:62500. Secondary antibodies (sheep anti-rabbit gamma globulin serum) were diluted 1:10 in PBS + 8% polyethilenglycol. Next, 12,000 counts per minute of rLH-I^{125} (NIDDK-rLH-I-10) or rFSH-I^{125} (NIDDK-rFSH-I-9) were added to each tube in a volume of 100 μL. Samples were incubated for 2 h at room temperature and counted in a gamma emission counter model 5005 (Packard, Canberra Company, Austria). The coefficients of inter- and intra-assay variation were 12.1% and 14.6%, respectively. Sensitivity of the assay was 0.1 ng/ml.

Statistics

For specific protein content and the concentration of gonadotropins, the data were analyzed with the Shapiro-Wilk and the D'Agostino-Pearson normality tests to check if they conformed to a Gaussian distribution. The Bartlett's test was used to check for homogeneity of variance. Protein content in the anteromedial hypothalamus was analyzed by a two-way

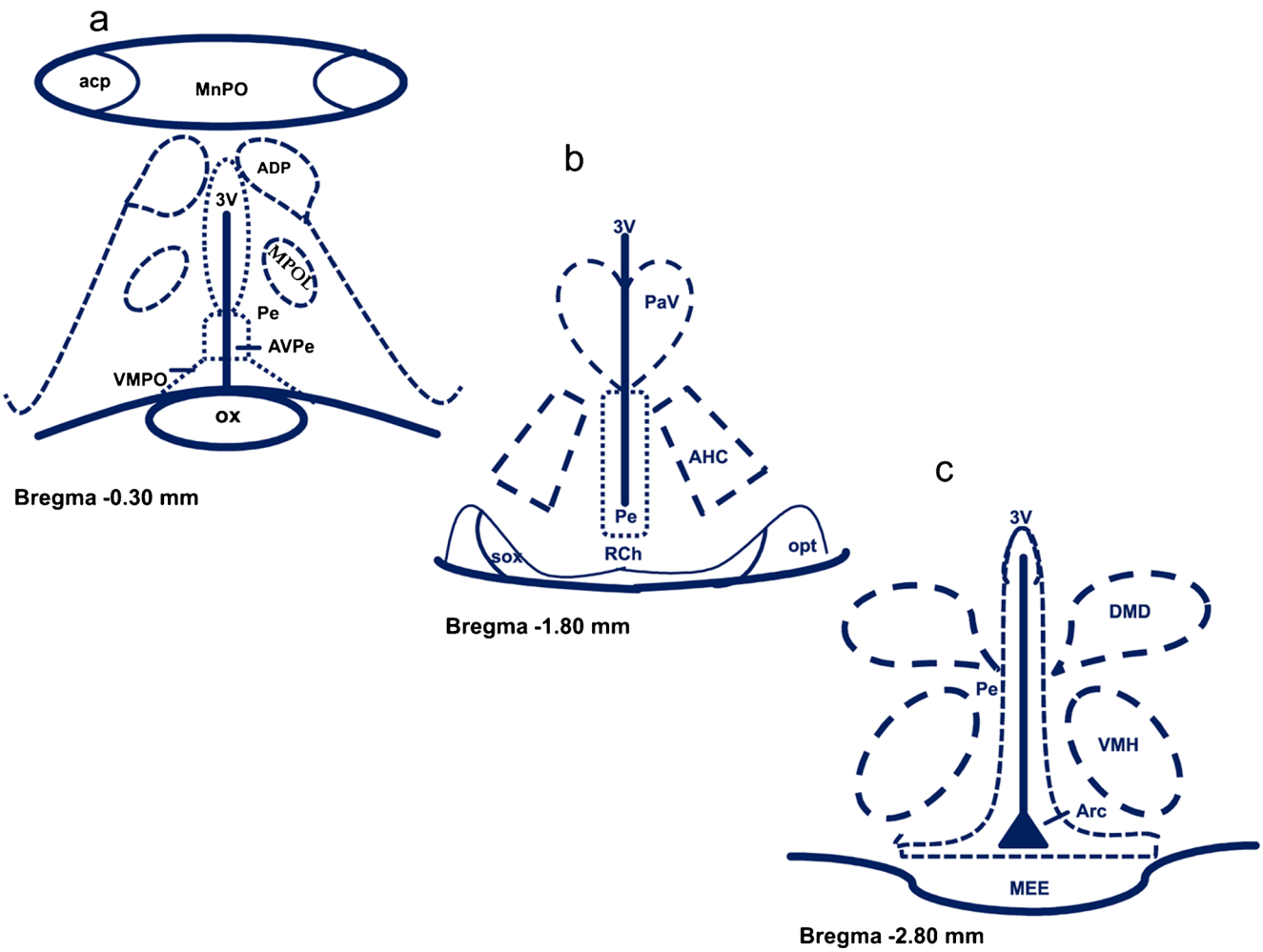

Fig. 1 Representative diagram of the entire anteromedial hypothalamus region. The diagram is based in the Paxinos and Watson's stereotaxic atlas for the rat brain. The brain tissue wedges used in the study spanned from preoptic area (**a**), passing through anterior hypothalamic area (**b**) to medio-basal hypothalamus region (**c**). See materials and methods section for details. 3 V, third ventricle; acp, anterior commissure, posterior part; ADP, anterodorsal preoptic nucleus; AHC, anterior hypothalamic area, central part; Arc, arcuate nucleus; AVPe, anteroventral periventricular nucleus; DMD, dorsomedial hypothalamic nucleus, dorsal part; MEE, medial eminence, external layer; MnPO, median preoptic nucleus; MPOL, lateral part of medial preoptic nucleus; opt, optic tract; ox, optic chiasm; PaV, paraventricular hypothalamic nucleus, ventral part; Pe, periventricular nucleus; RCh, retrochiasmatic area; sox, supraoptic decussation; VMH, ventromedial hypothalamic nucleus; VMPO, ventromedial preoptic nucleus

ANOVA considering the stage of the estrous cycle and the time of the day as the independent variables. The Pearson correlation coefficient between each pair of proteins was also calculated for this sum considering or not the stage of the cycle. For each stage of the cycle, the differences between the side of the anteromedial hypothalamus and the hour of the day by a two-way ANOVA considering the time of the day and the side of the anteromedial hypothalamus as the independent variables were performed. A one-way ANOVA followed by the Tukey's test was used to analyze the differences between sampling points on each stage of the cycle. Statistical comparisons were made using GraphPad Prism 7.0 for Windows (San Diego, CA). To further correct of multiple comparisons in ANOVA tests, differences were considered as significant when $p \leq 0.01$.

Results

Effects of pentobarbital anesthesia on FSH and LH serum levels

Pentobarbital anesthesia did not modify proestrus FSH serum levels compared with not anesthetized rats (11:00 h, 2.78 ± 0.2 vs. 3.90 ± 0.8 ng/mL; 17:00 h, 11.2 ± 3.6 vs. 14.9 ± 5.2 ng/mL), while LH levels were lower than not anesthetized rats sacrificed at 17:00 h (71.8 ± 10.1 vs.104.9 ± 10.3 ng/mL, $p < 0.05$).

Hormone serum levels across the estrous cycle

In diestrus-1, the E_2 serum levels remain constant from 08:00 to 14:00 h and fall at 17:00 h. In diestrus-2, the hormone levels

remain low until 17:00, when the concentration increases. During the proestrus, the levels of E_2 increase progressively from 08:00 h, reach their maximum values at 14:00 h, and decrease at 17:00 h to levels like those in the afternoon of diestrus-1 and the morning of diestrus-2. In estrus, E_2 serum levels are low throughout the day (Fig. 2).

In rats sacrificed at diestrus-1, no differences in FSH levels were observed. In diestrus-2, the highest value was observed at 11:00 h, and it was significantly different from the lowest values occurring at 14:00 and 17:00 h ($p \leq 0.01$, Tukey's test). During proestrus, the highest value occurred at 17:00 h was different when compared against the rest of the time points ($p \leq 0.01$, Tukey's test). During estrus, the highest value occurred at 08:00 h and diminished to the lowest value at 17:00 h, but no significant differences were found. The serum levels of LH were no different during estrus, diestrus-1, and diestrus-2, while in proestrus, the highest value was observed at 17:00 h (Fig. 2).

The content of ERα, ERβ, kisspeptin, and GnRH in the entire anteromedial hypothalamus region

We analyzed whether there was a correlation between the content of the studied proteins across the each stage of the estrous cycle. ERα showed a moderate positive correlation with ERβ ($r = 0.6306$, $p = 0.0001$, $R^2 = 0.3977$); this was dependent on the stage of the cycle, since the strength of the correlation increased gradually from diestrus-1 to proestrus (diestrus-1, $r = 0.7936$, $p = 0.0021$, $R^2 = 0.6297$; diestrus-2, $r = 0.8401$, $p = 0.0006$, $R^2 = 0.7057$; proestrus, $r = 0.9562$, $p = 0.0001$, $R^2 = 0.9144$) and then decreased abruptly on the day of estrus ($r = 0.4963$, $p = 0.1007$, $R^2 = 0.2464$). In contrast, the content of both estradiol receptors did not show any correlation with the content of kisspeptin when the data of all the stages was analyzed (ERα, $r = 0.2068$, $p = 0.1585$, $R^2 = 0.0427$; ERβ, $r = 0.1061$, $p = 0.4727$, $R^2 = 0.01127$). The same was observed when the data of each stage of the cycle was analyzed separately: proestrus (ERα, $r = 0.101$, $p = 0.0754$, $R^2 = 0.0102$; ERβ, $r = 0.1973$, $p = 0.5388$, $R^2 = 0.03892$), estrus (ERα, $r = 0.1112$, $p = 0.7548$, $R^2 = 0.0.0123$; ERβ, $r = 0.1559$, $p = 0.6284$, $R^2 = 0.0243$), diestrus-1 (ERα, $r = 0.0929$, $p = 0.7739$, $R^2 = 0.0086$; ERβ, $r = 0.1682$, $p = 0.6014$, $R^2 = 0.0282$), or diestrus-2 (ERα, $r = 0.4743$, $p = 0.1193$, $R^2 = 0.2250$; ERβ, $r = 0.4317$, $p = 0.1611$, $R^2 = 0.1864$).

Asymmetries of content of ERα, ERβ, kisspeptin, and GnRH in the left or right portion of the anteromedial hypothalamus region

We did not find any differences in the content of ERα between the right and left side of the brain in any of the stages of the estrous cycle; moreover, in diestrus-1 and estrus, no differences regarding the time of the day were observed. In diestrus-2, an interaction between the estrous cycle day and hour of the day ($F_{3,\ 16} = 3976$, $p = 0.0271$) and also an effect of the time of the day was detected in the right portion ($F_{3,\ 16} = 11.31$, $p = 0.0003$), since the content is lower at 11:00 and 14:00 h when compared with 08:00 h. A similar effect was found in proestrus in the left portion (interaction, $F_{3,\ 16} = 3.876$, $p = 0.0294$; time of the day, $F_{3,\ 16} = 3.978$, $p = 0.0269$), where the lowest content occurs at 11:00 and 14:00 h and the highest at 17:00 h (Fig. 3a).

ERβ was higher in the right portion of the hypothalamus during diestrus-1, diestrus-2, and proestrus, while the opposite occurred during estrus day. In diestrus-1, we found that the content was lower at 08:00 h ($F_{3,\ 16} = 6.0$, $p = 0.0061$). In diestrus-2, we found an interaction between factors ($F_{3,\ 16} = 6.432$, $p = 0.0046$) and an asymmetry between both sides of the hypothalamus ($F_{1,\ 16} = 86.38$, $p = 0.0001$). A difference between both sides was also found in proestrus ($F_{1,\ 16} = 51.17$, $p = 0.0001$) and estrus ($F_{1,\ 16} = 24.02$, $p = 0.0002$) (Fig. 3b).

In the case of kisspeptin, no interaction between factors was found in any of the stages of the cycle. We did not find differences in both days of diestrus that depends on the side of the hypothalamus, while the content was higher in the right hypothalamus at 08:00 h of proestrus day ($F_{1,\ 16} = 6.154$, $p = 0.0246$) and did not reach statistical significance. During this stage, the content decreases from 11:00 to 17:00 in the right side, while it remains constant on the left one. In estrus, the content is lower in the left side ($F_{1,\ 16} = 68.59$, $p = 0.0001$) (Fig. 3c).

GnRH was not detected at any time point studied during both days of diestrus and at 08:00 and 17:00 h of proestrus. At this stage of the cycle, GnRH was higher in the right side of the brain at 14:00 h. In estrus, we did not find interaction between factors, but a weak effect of the time of the day ($F_{3,\ 16} = 4.947$, $p = 0.0128$) and the side of the brain ($F_{1,\ 16} = 6.154$, $p = 0.0246$) was apparent; in this regard, a higher amount of GnRH on the right side was observed at 08:00 h. GnRH gradually decreased from 11:00 to 14:00 h on the right hypothalamus remaining constant in the left one (Fig. 3d).

Discussion

The current study shows that in the female rat, the content of ERα, ERβ, kisspeptin, and GnRH in the anteromedial hypothalamus changes as a function of the stage of the estrous cycle and the hour of the day. Overall, the content of the various proteins studied is higher in the right than in the left portion of this region of the brain. Gore and Roberts (1997) showed that in the preoptic area-anterior hypothalamus of rats maintained under 12/12 light/dark conditions, the GnRH gene expression, measuring both the primary transcript in the nucleus and the mRNA levels in the cytoplasm, reaches

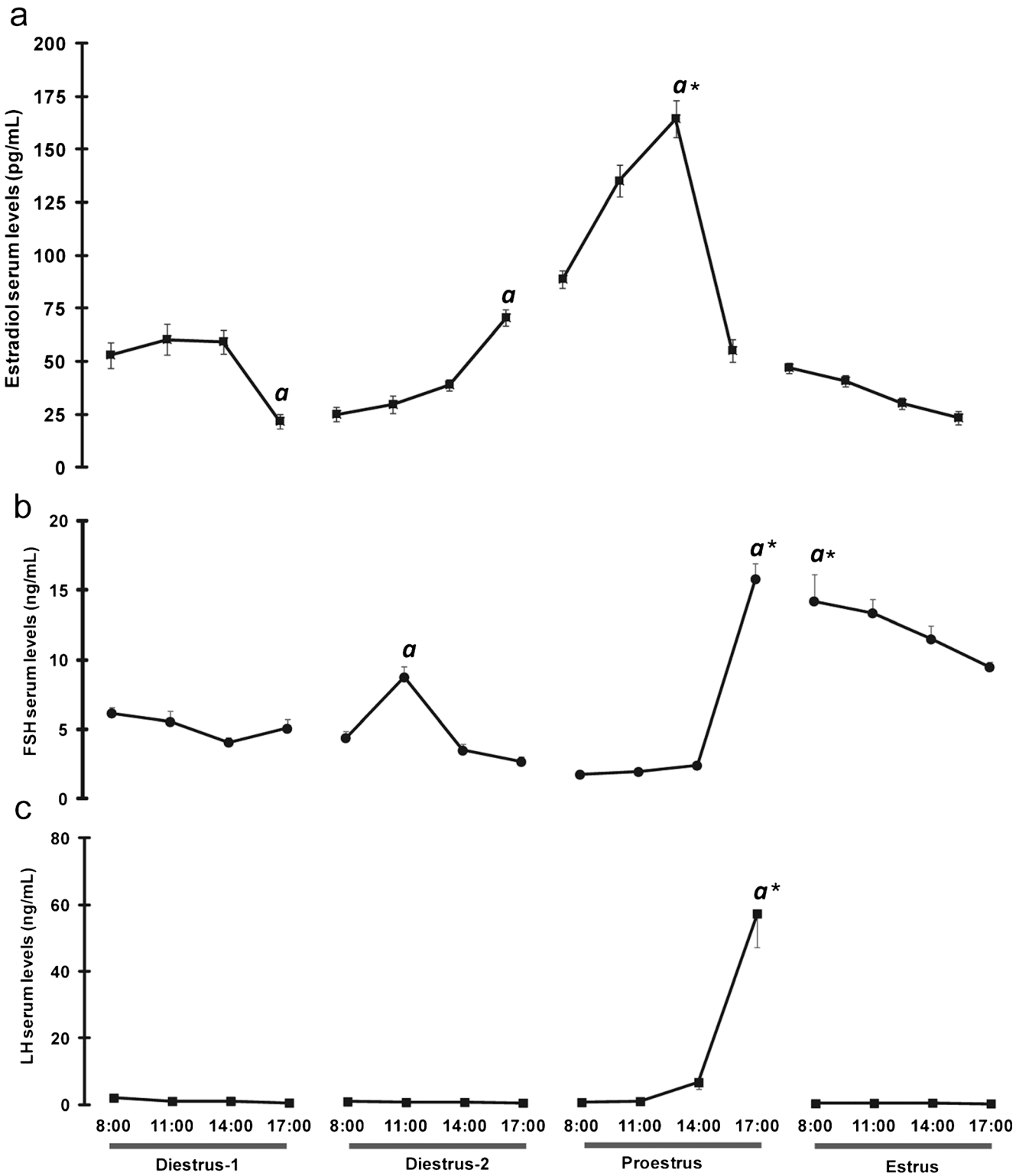

Fig. 2 Serum Levels of E_2 (**a**), FSH (**b**), and LH (**c**) in CIIZ-V rats (n = 10) at 08:00, 11:00, 14:00, and 17:00 h of each stage of the estrous cycle. Data are expressed as Mean ± SEM; a, $p \leq 0.001$ vs. the other hours of the same stage of the cycle; * represents the highest value of the estrous cycle (ANOVA followed by Tukey's test)

the highest values at 15:00 h of proestrus. In the present study, we used rats maintained under a 14/10 conditions and observed that the content of GnRH increases at 14:00 h of the same day. Taken together, the results by Gore and Roberts (1997) and those in the present study suggest that the primary transcript, the mRNA, and the GnRH protein increase

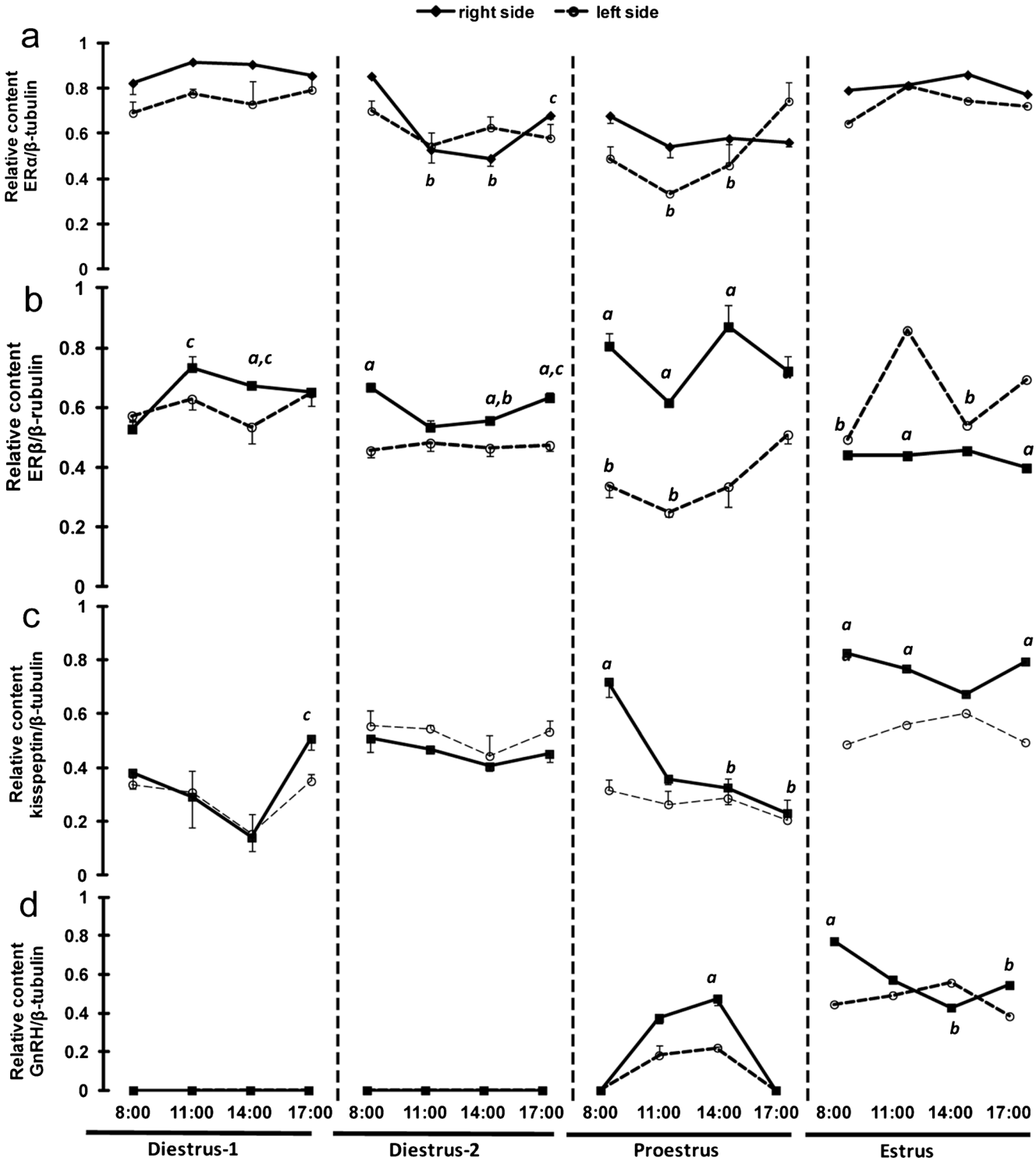

Fig. 3 Relative content of ERα (**a**), REβ (**b**), kisspeptin (**c**), and GnRH (**d**) in the left or right portion of the anteromedial hypothalamus region of adult female rats sampled at 8:00, 11:00, 14:00, and 17:00 h on each day of the estrous cycle. Data are expressed as Mean ± SEM; a, $p < 0.05$ vs. left side at the indicated time point; b, $p < 0.05$ vs. the highest value of the same side; c, $p < 0.05$ vs. the lowest value on the same side

simultaneously before the pre-ovulatory secretion of LH/FSH, i.e., when there is a major demand of the neurohormone. We have previously shown that both sides of the hypothalamus respond differentially to this demand since the expression of the GnRH mRNA (Arteaga-López et al. 2003) in POA-AHA and the content of the protein (present study) in the right anteromedial-hypothalamus are higher than in the left side. In the present study, the content of GnRH protein peaks at

08:00 h of the estrus day, suggesting that this mRNA is being translated. Since the increase in the content of GnRH peptide during the different hours of the estrus day correlates with the serum levels of FSH, we suggest that the frequency and amplitude of the pulses were low, which facilitates FSH but not LH secretion as previously proposed (Marshall et al. 1991).

It is well documented that kisspeptin triggers the pre-ovulatory secretion of LH acting directly at the GnRH-neuron. Papaoiconomou et al. (2011) and Navarro et al. (2005) suggest that, in short term, the effects of kisspeptin on such neurons do not involve the transcriptional activation of the gene that encodes GnRH, and hence it only stimulates the release of the decapeptide that is already synthesized. Our results support this idea, since the content of kisspeptin is high at 8:00 h of proestrus and drops 4 h later, exactly when the content of GnRH rises. It is worth noting that the hypothalamus regulates asymmetrically this process, since the content of kisspeptin in the right hypothalamus is higher than that on the left side. The same is true during most of the time points in estrus, which is similar to the pattern of the content of GnRH in this same stage.

In mice and rats, the positive feedback of estradiol that results in the triggering of the pre-ovulatory surge of GnRH/gonadotropins depends on the estradiol action on the kisspeptin-neurons of the AVPV throughout the ERα (Navarro et al. 2004; Roa et al. 2008a). In proestrus, the injection of a selective antagonist of the ERβ results in the attenuation in the kisspeptin-stimulated release of FSH while incrementing LH secretion (Roa et al. 2008b). Based on these results, the authors proposed that the ERβ plays an inhibitory role on the secretion of LH (Roa et al. 2008c) and a stimulant one on the secretion of FSH (Roa et al. 2008c). The increase in the ERβ protein content at 11:00 and 17:00 h of estrous day supports the findings by Roa et al., and also suggests that the stimulating role of estradiol acting at the ERβ on FSH secretion occurs mainly on the left anteromedial hypothalamus, since at estrus the increase in the content of this protein matches with a high serum level of the hormone during all the time points studied.

In a previous study, we showed that in diestrus-2, the specific blockade of both estradiol receptors in the right POA-AHA leads to the inhibition of ovulation in most of treated animals. Also, we proved that the number of ERα- and ERβ-immunoreactive cells was significantly higher in the right POA-AHA at 09:00 h of proestrus and that the specific blockade of ERα or ERβ in the left POA-AHA inhibited ovulation in 84% of the animals (Arrieta-Cruz et al. 2019). Taken together, the results suggest that the positive feedback action of estradiol on POA-AHA on the secretion of gonadotropins varies along the estrous cycle and in proestrus occurs mainly in the right side of the hypothalamus, which is in line with the present study.

In summary, our results indicate that the content of the ERα, ERβ, kisspeptin, and GnRH changes during the light phase of each stage of the estrous cycle. A correlation between both estradiol receptors was found, and it depends on the stage of the cycle, being stronger during the diestrus-2 and proestrus days, when the action of estrogens turns to a positive feedback. ERα is symmetrical in both sides of the brain during all the estrous cycle, while the content of the ERβ is higher in the right side during most of the estrous cycle. In proestrus, however, the right hypothalamus seems to be the target site for stimulating actions of estradiol on gonadotropin secretion and ovulation during this stage of the cycle.

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Authors' contribution EOJ and MEC designed the research. EOJ, AF, IAC, LMG, HMC, HELV, and MC performed the research. CCS, IAC, RD, RGJ, and MEC participated in the analysis and discussion of the results. CCS, IAC, RGJ, and MEC wrote the manuscript. All authors made critical revision of the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The animal research protocol was approved by the local Ethics Committee of Facultad de Estudios Superiores Zaragoza, UNAM (license number: FES/DEPUCI/236/14).

Statement on the welfare of animals All animal experiments were performed in compliance with the Mexican laws for animal handling, Official Norm NOM-062-ZOO-1999, which instead conforms to international guidelines.

Abbreviations GnRH, gonadotropin-releasing hormone; FSH, follicle-stimulating hormone; LH, luteinizing hormone; E_2, 17β-estradiol; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; 3 V, third ventricle; acp, anterior commissure, posterior part; ADP, anterodorsal preoptic nucleus; AHC, anterior hypothalamic area, central part; Arc, arcuate nucleus; AVPe, anteroventral periventricular nucleus; DMD, dorsomedial hypothalamic nucleus, dorsal part; MEE, medial eminence, external layer; MnPO, median preoptic nucleus; MPOL, lateral part of medial preoptic nucleus; opt, optic tract; ox, optic chiasm; PaV, paraventricular hypothalamic nucleus, ventral part; Pe, periventricular nucleus; RCh, retrochiasmatic area; sox, supraoptic decussation; VMH, ventromedial hypothalamic nucleus; VMPO, ventromedial preoptic nucleus

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