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Differential mRNA Expression of Alpha and Beta Estrogen Receptor Isoforms and GnRH in the Left and Right Side of the Preoptic and Anterior Hypothalamic Area During the Estrous Cycle of the Rat

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Asymmetric mRNA expression was found in preoptic anterior and hypothalamic anterior areas of the two estrogen receptor isoforms and the gonadotropin-releasing hormone. On the right side of these areas, estrogen receptor alpha mRNA expression reached its peak on estrus day, while on the left side the peak was reached on proestrus day. Estrogen receptor beta mRNA expression peaked on both sides on the same day, diestrous–2 day, but at different hours, showing a sustained expression for the next measured hour on the left side, while peaking and dropping abruptly on the right side. Gonadotropin-releasing hormone also peaked on both sides on diestrous–2 day, being the left side peak expression significantly lower than the peak expression at the right side. The side expression differences suggest that different sides of the before mentioned areas may play different roles of endocrine reproductive functions, while differences of expression at different times may suggest interaction between sides for the same functions.

Key Words: GnRH; ER-α; ER-β; hypothalamic lateralization; estrous cycle; mRNA expression.

Introduction

Estrogen (E_2) is involved in the regulation of several functions of the central nervous system. In the hypothalamus one of the most important roles of E_2 is the regulation of reproductive functions, through the gonadotropin-releasing hormone (GnRH) and gonadotropin secretion *(1,2)*. The regulation of these peptides is mediated by two ER isoforms: the estrogen receptor alpha (ER-α) and the estrogen receptor beta (ER-β) *(3)*. Both ER-α and ER-β are expressed in the hypothalamus of the rat *(4)*. However, only ER-β is present in the hypothalamic GnRH neurons *(5,6)*.

The existence of an asymmetric function of different endocrine organs, including the gonads, the adrenals, and the thyroid gland, has been indicated in many studies *(7)*. Different studies have shown that manipulations of the right side of the hypothalamus (such as lesions, deafferentations, or pharmacological or hormonal implants), affect the response of the neuroendocrine reproductive axis asymmetrically *(8–12)*.

In female rats the development of sex-specific reproductive functions can be differentially disturbed, depending on which side in the hypothalamus an early implantation of estradiol is performed. Implantation of the hormone in the right side of the hypothalamus leads to masculinization (e.g., preference for other females), while implantation in the left side induces defeminization (e.g., loss of the estrous cycle) *(13)*. Unilateral implants of estradiol in the right side of the hypothalamus of adult rats induces better behavioral responses (lordosis) than in animals with implants in the left side *(14)*.

Furthermore, asymmetric activity in the hypothalamic and limbic structures, as well as in other brain structures related to the neuroendocrine control systems, has been reported *(7)*. Accordingly, in adult female rats the GnRH peptide content is higher in the right side of the median eminence than in the left.

The preceding observations indicate that an asymmetric expression of ER-α, ER-β, as well as GnRH may exist in different areas of the hypothalamus of female rats. Asymmetric mRNA expression of ER-α, ER-β, and GnRH, between the left and right side of the preoptic anterior and hypothalamic anterior areas (POA–AHA), during the estrous cycle of the rat, was found. The implication of asymmetric reproductive functions is then discussed.

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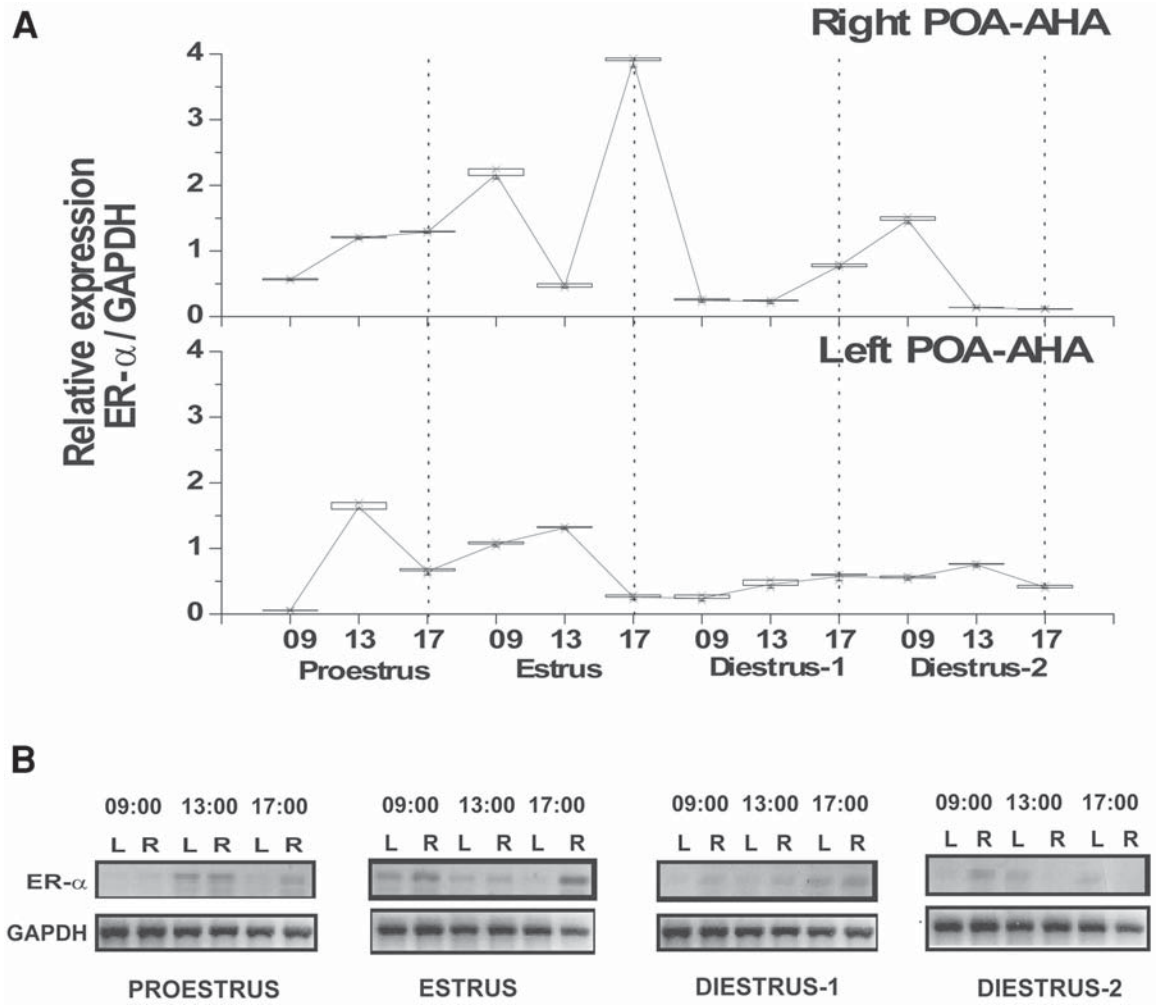

Fig. 1. Densitometric analysis of ER-α mRNA expression in the left and right side of POA–AHA of cyclic adult female rat at 09:00, 13:00, and 17:00 of estrous, diestrous–1, diestrous–2 and proestrous days. (**A**) Densitometric analysis of RT-PCR products. Each point in the graph is a box plot representing the whole distribution of the sample for each side, day, and hour. Note that each experimental group is represented by a normal distribution with no outlier points. Multivariate analysis of variance was performed (F-ratio = 2.0027; $p = 0.021$) showing statistical significance between sides, followed by pairwise Tukey tests, showing statistical significant differences ($p < 0.05$) between experimental groups, especially between left and right sides of POA–AHA at 17:00 of the estrous day. (**B**) Simultaneous RT-PCR representative assays for ER-α and GAPDH mRNA expression in the left (L) and right (R) side of POA–AHA. RT-PCR was performed using total RNA isolated from both sides of POA–AHA of adult female rats during the estrous cycle.

Results

When the results were analyzed, all together (by the day of the estrous cycle, the side of POA–AHA, and the hour of the day when the animals were sacrificed), we found that in the right side of POA–AHA, the ER-α mRNA expression reached its maximal level during the estrous day, with a peak at 17:00 (Fig. 1A, Table 1). Two other small peaks were observed at 09:00 of the day of diestrous–2 and at 13:00 on proestrus. However, only the 17:00 estrous day peak was statistically significant when compared to the values in the left side for the same day and hour (Table 1). ER-α mRNA expression on the left side of the POA–AHA show less variations along the different hours of the day of the estrous cycle and the small peaks at 13:00 of the proestrus and diestrous–2 days are not significatively different from the corresponding values on the right side. A larger expression activity of ER-α is seen on the right side especially at 17:00 of the estrous day.

ER-β mRNA expression peaked on both sides on diestrous–2 day, peaking at 9:00 on the right side and at 13:00 on the left side (Fig. 2A, Table 2). No expression was shown on any other day of the cycle indicating precise timing for the use of this receptor. The left side showed a little more expression than the right side and sustained its expression for a longer time, although peaking at 13:00 it showed the same drastic increase at 9:00 that the right side. It seems that ER-β, although slightly more expressed in the left than in the right side, is expressed for a precise timed action.

Table 1
ER-α Tukey Test Pairwise Comparison of mRNA Expression Between POA–AHA Sides

			Left side.										
			Proestrus			Estrus			Diestrus-1			Diestrus-2		
			9:00	13:00	17:00	9:00	13:00	17:0	9:00	13:00	17:00	9:00	13:00	17:00
Right side.	Proestrus	9:00	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
		13:00	0.99	1.0	1.0	1.0	1.0	0.99	0.99	1.0	1.0	1.0	1.0	1.0
		17:00	0.99	1.0	1.0	1.0	1.0	0.99	0.99	1.0	1.0	1.0	1.0	0.99
	Estrus	9:00	0.67	1.0	0.97	0.99	0.99	0.82	0.81	0.92	0.95	0.95	0.98	0.90
		13:00	1.0	0.99	1.0	1.0	0.99	1.0	1.0	1.0	1.0	1.0	1.0	1.0
		17:00	* **0.007**	0.56	* **0.05**	0.18	0.31	* **0.015**	* **0.014**	* **0.029**	* **0.042**	* **0.039**	0.073	* **0.025**
	Diestrus-1	9:00	1.0	0.99	1.0	1.0	0.99	1.0	1.0	1.0	1.0	1.0	1.0	1.0
		13:00	1.0	0.98	1.0	1.0	0.99	1.0	1.0	1.0	1.0	1.0	1.0	1.0
		17:00	1.0	0.99	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
	Diestrus-2	9:00	0.99	1.0	1.0	0.99	1.0	0.99	0.99	0.99	0.99	0.99	1.0	1.0
		13:00	1.0	0.97	1.0	0.99	0.99	1.0	1.0	1.0	1.0	1.0	1.0	1.0
		17:00	1.0	0.98	1.0	1.0	0.99	1.0	1.0	1.0	1.0	1.0	1.0	1.0

*Probabilities in bold and in shadowed cells indicate statistically significant differences between pairs ($p < 0.05$). Although Tukey test was performed for pairs within sides, the data are not shown because only the peak is statistically significant, as should be expected. Note that only the peak in the right side at 17:00 of estrous day is statistically significant between sides.

Just as ER-β, the expression of GnRH mRNA showed a peak at 13:00 of diestrous–2 day for both sides, however, showing a larger peak on the right side (Fig. 3A, Table 3). A tiny peak at 9:00 of proestrous day is also seen in both sides. Again, the absence of expression on the other days of the cycle suggests a precise timing for the expression of GnRH. The synchronicity of ER-β and GnRH expression while expressing in different sides, ER-β on the left side and GnRH on the right, suggests a complementary action between them but controlled by some synchronizing factor.

Single bands, corresponding to ER-α (Fig. 1B), ER-β (Fig. 2B), and GnRH (Fig. 3B) were detected in all gene amplifications.

Discussion

All the results show that the difference in mRNA expression of each gene studied depends on the side of POA–AHA analyzed, the hour of day, and the estrous cycle day.

The ER-α isoform is asymmetrically expressed in the right and left sides of POA–AHA, being highly expressed

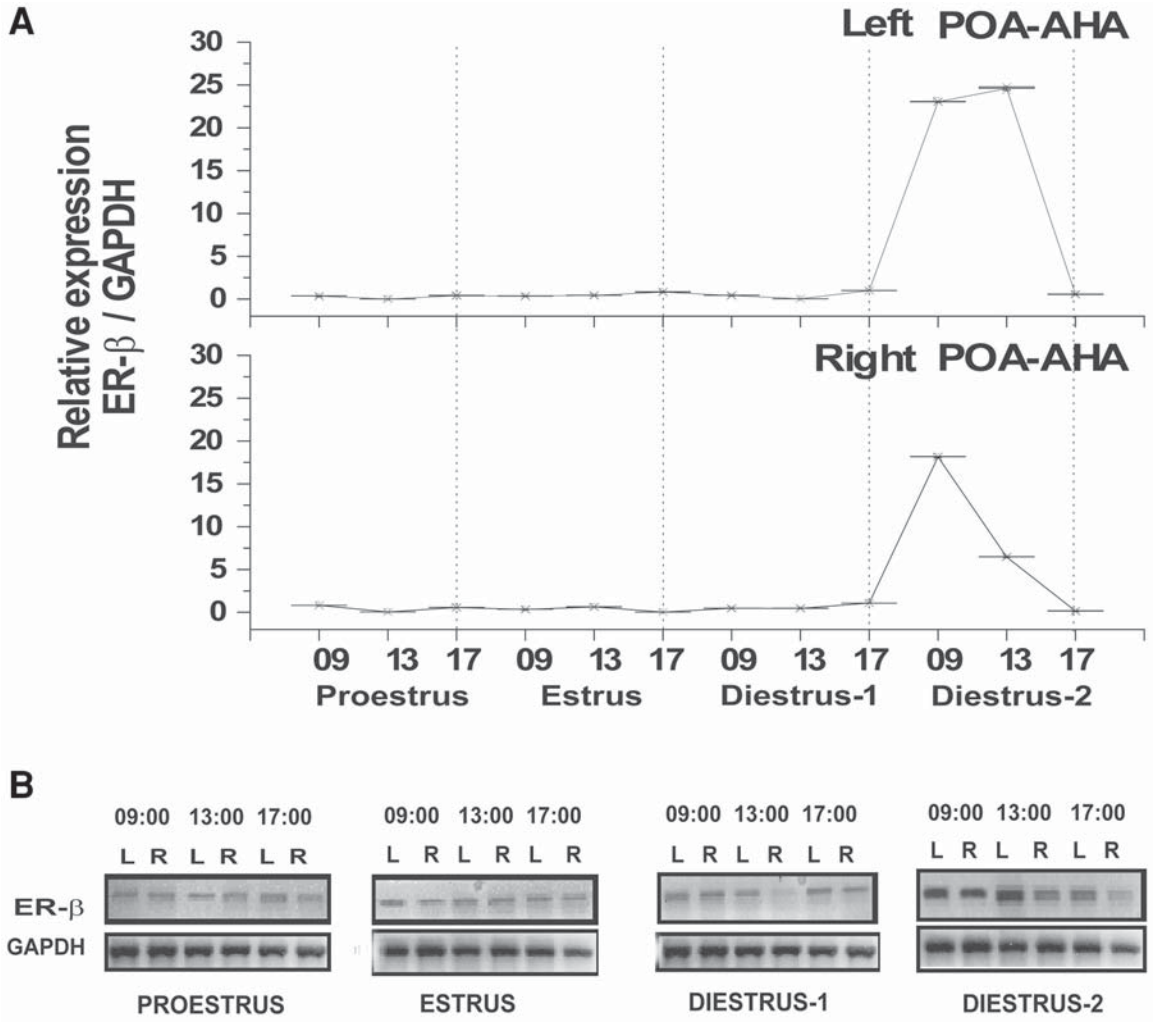

Fig. 2. Densitometric analysis of ER-β mRNA expression in the left and right side of POA–AHA of cyclic adult female rat at 09:00, 13:00, and 17:00 of estrous, diestrous–1, diestrous–2 and proestrous days. (**A**) Densitometric analysis of RT-PCR products. Each point in the graph is a box plot representing the whole distribution of the sample for each side, day, and hour. Note that each experimental group is represented by a normal distribution with no outlier points. Multivariate analysis of variance was performed (F-ratio = 9.59; p = 1e–5) showing statistical significance between sides, followed by pairwise Tukey tests, showing statistical significant differences ($p < 0.05$) between experimental groups, especially between left and right sides of POA–AHA at 13:00 of the diestrous–2 day. (**B**) Simultaneous RT-PCR representative assays for ER-β and GAPDH mRNA expression in the left (L) and right (R) side of POA–AHA. RT-PCR was performed using total RNA isolated from both sides of POA–AHA of adult female rats during the estrous cycle.

in the right side, especially on estrous day, on which the receptor is only expressed in the right side. This suggests a specialization of the receptor on the right side at precise timing on estrous day, allowing the other side to take care of another totally different function at the same time. Besides, the fact that female and male ERαKO are both infertile *(15–19)* suggests that this specialization of ER-α has to do with some neuroendocrine reproductive function.

The ER-β isoform is also asymmetrically expressed on both sides of POA–AHA, with a somewhat larger expression on the left side. In contrast with the ER-α receptor, the ER-β receptor is expressed at a very precise time on diestrous–2 day with no expression on any other day of the estrous cycle. However, the fact that the ERβKO female mice are just subfertile and that the difference between sides is not large indicates that ER-β plays some role on a neuroendocrine reproductive function but it possibly has another nonreproductive function that is related to both sides.

GnRH also shows asymmetry between sides, but like ER-β its expression is precisely timed on diestrous–2 day with no expression on any other day. In contrast with ER-β the difference between sides is noticeable with a larger expression on the right side. These results are in agreement with previous observations *(20,21)*, which show that on female rats, the GnRH peptide content is higher on the right half of the medial basal hypothalamus *(7)*. GnRH marked

Table 2
ER-β Tukey Test Pairwise Comparison of mRNA Expression Between POA–AHA Sides

			Left side.											
			Proestrus			Estrus			Diestrus-1			Diestrus-2		
			9:00	13:00	17:00	9:00	13:00	17:00	9:00	13:00	17:00	9:00	13:00	17:0
Right side.	Proestrus	9:00	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	* $1e^{-4}$	* $1e^{-4}$	1.0
		13:00	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	* $1e^{-4}$	* $1e^{-4}$	1.0
		17:00	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	* $1e^{-4}$	* $1e^{-4}$	1.0
	Estrus	9:00	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	* $1e^{-4}$	* $1e^{-4}$	1.0
		13:00	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	* $1e^{-4}$	* $1e^{-4}$	1.0
		17:00	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	* $1e^{-4}$	* $1e^{-4}$	1.0
	Diestrus-1	9:00	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	* $1e^{-4}$	* $1e^{-4}$	1.0
		13:00	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	* $1e^{-4}$	* $1e^{-4}$	1.0
		17:00	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	* $1e^{-4}$	* $1e^{-4}$	1.0
	Diestrus-2	9:00	* $5.8e^{-4}$	* $4.2e^{-4}$	* $6.2e^{-4}$	* $5.8e^{-4}$	* $6.3e^{-4}$	* $9.3e^{-4}$	* $6.3e^{-4}$	* $6.3e^{-4}$	* $4.3e^{-4}$	0.99	0.95	* $.1e^{-4}$
		13:00	0.99	0.95	0.97	0.97	0.97	0.99	0.97	0.97	0.95	* 0.002	* $4.3e^{-4}$	0.97
		17:00	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	* $1e^{-4}$	* $1e^{-4}$	1.0

*Probabilities in bold and in shadowed cells indicate statistically significant differences between pairs ($p < 0.05$). Although Tukey test was performed for pairs within sides, the data are not shown because only the peak is statistically significant, as should be expected. Note that only the peak at diestrous–2 day is statistically significant between sides.

asymmetrical mRNA expression in POA–AHA during the diestrous–2 day could be related to the differences in the number of GnRH neurons between the two sides of POA–AHA, since there is evidence that male mice have more GnRH immunoreactive neurons in the right side of the brain than in the left side *(22)*. The fact that the GnRH peak starts to disappear in diestrous–2 afternoon is consistent with observations made in male rats, where higher GnRH content in the right side of the hypothalamus was observed only in samples taken in the morning, but not in samples taken in the evening *(23)*.

Although not shown by the results of this study, the high levels of ER-α in the right side of POA–AHA together with the higher content of GnRH mRNA in the right side of POA–AHA, suggests that the stimulation of the ER-α receptor may be linked to the GnRH synthesis. Since the GnRH neurons do not have ER-α *(5,6)*, this stimulatory effect may be accomplished throughout an intermediary neuron,

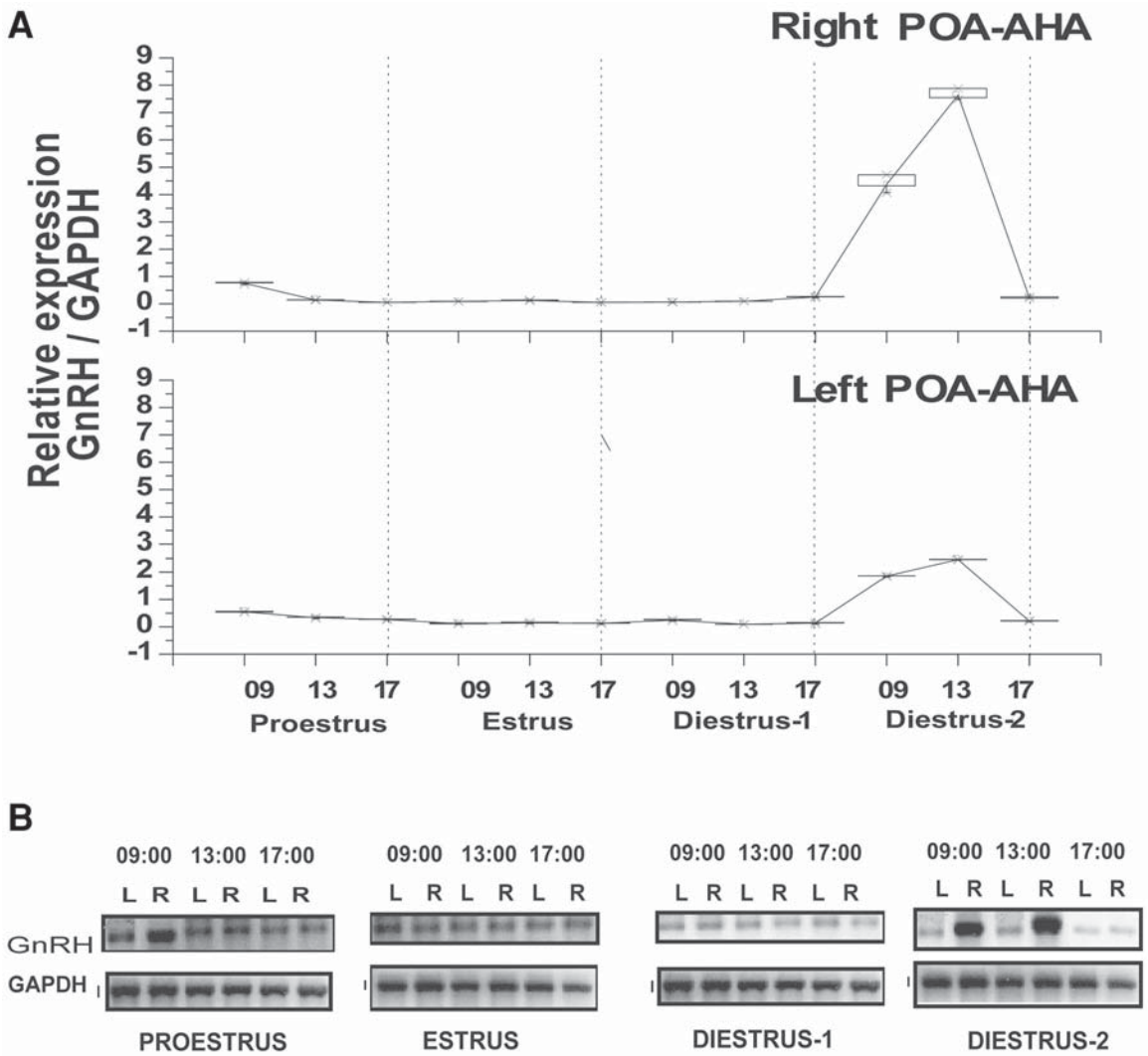

Fig. 3. Densitometric analysis of GnRH mRNA expression in the left and right side of POA–AHA of cyclic adult female rat at 09:00, 13:00, and 17:00 of estrous, diestrous–1, diestrous–2 and proestrous days. (**A**) Densitometric analysis of RT-PCR products. Each point in the graph is a box plot representing the whole distribution of the sample for each side, day, and hour. Note that each experimental group is represented by a normal distribution with no outlier points. Multivariate analysis of variance was performed (F-ratio = 1368; p = 1e–5) showing statistical significance between sides, followed by pairwise Tukey tests, showing statistical significant differences ($p < 0.05$) between experimental groups, especially between left and right sides of POA–AHA at 13:00 of the diestrous–2 day. (**B**) Simultaneous RT-PCR representative assays for GnRH and GAPDH mRNA expression in the left (L) and right (R) side of POA–AHA. RT-PCR was performed using total RNA isolated from the both sides of POA–AHA of adult female rats during the estrous cycle.

possibly an interneuron. Such an idea is supported by the fact that the ER-α is present in cholinergic neurons and possibly in GABAergic and catecholaminergic neurons *(2,24)*.

The GnRH peaks have also been observed previously on a RNase protected assay *(25,26)*, but, because there was no sampling between 11:00 and 18:00 on diestrous–2 day, this peak was missed. In that study both sides of POA–AHA were taken for their assay, while, as it is shown here, the large diestrous–2 day peak it is better observed when POA–AHA sides are taken separately. In that study an important unsolved issue was the origin for the mRNA expression peak on diestrous–2 day. Taking in consideration the synchrony of ER-β and GnRH, but acting on different sides, it is possible that the neurotransmitter which is responsible for synchronicity could also be responsible for a post-transcriptional mechanism that triggers the diestrous–2 day GnRH peak.

Hypothetically, the high levels of ER-β mRNA in the left side of POA–AHA on diestrous–2 day, one day before preovulatory release of GnRH, may be linked with the GnRH preovulatory triggering. This triggering effect is suggested by the presence of the ER-β in GnRH neurons *(5,6)*. At precisely the same time, GnRH mRNA is peaking, but on the right side, suggesting a complementary action between ER-β and GnRH on diestrous–2 day. If true, this necessarily means close communication between the neurons on both

Table 3
GnRH Tukey Test Pairwise Comparison of mRNA Expression Between POA–AHA Sides

			Left side.											
			Proestrus			Estrus			Diestrus-1			Diestrus-2		
			9:00	13:00	17:00	9:00	13:00	17:0	9:00	13:00	17:00	9:00	13:00	17:00
Right side.	Proestrus	9:00	0.27	* 3e^{-4}	* 1e^{-4}	* 1e^{-4}	* 1e^{-4}	* 1e^{-4}	* 1e^{-4}	* 1e^{-4}	* 1e^{-4}	* 1e^{-4}	* 1e^{-4}	* 1e^{-4}
		13:00	* 1e^{-4}	0.37	0.96	1.0	1.0	0.99	0.99	1.0	1.0	* 1e^{-4}	* 1e^{-4}	0.99
		17:00	* 6e^{-4}	* 0.019	0.25	1.0	1.0	0.99	0.45	1.0	0.99	* 1e^{-4}	* 1e^{-4}	0.75
	Estrus	9:00	* 1e^{-4}	0.09	0.61	1.0	1.0	1.0	0.83	1.0	1.0	* 1e^{-4}	* 1e^{-4}	0.97
		13:00	* 3e^{-4}	0.24	0.88	1.0	1.0	0.99	0.97	1.0	1.0	* 1e^{-4}	* 1e^{-4}	0.99
		17:00	* 1e^{-4}	* 0.02	0.25	1.0	1.0	0.99	0.45	1.0	0.99	* 1e^{-4}	* 1e^{-4}	0.76
	Diestrus-1	9:00	* 1e^{-4}	* 0.029	0.33	1.0	0.99	1.0	0.56	1.0	0.99	* 1e^{-4}	* 1e^{-4}	0.84
		13:00	* 1e^{-4}	0.099	0.64	1.0	1.0	1.0	0.85	1.0	1.0	* 1e^{-4}	* 1e^{-4}	0.98
		17:00	* 0.012	0.99	1.0	0.92	0.99	0.97	1.0	0.74	0.97	* 1e^{-4}	* 1e^{-4}	1.0
	Diestrus-2	9:00	* 1e^{-4}	* 1e^{-4}	* 1e^{-4}	* 1e^{-4}	* 1e^{-4}	* 1e^{-4}	* 1e^{-4}	* 1e^{-4}	* 1e^{-4}	* 1e^{-4}	* 1e^{-4}	* 1e^{-4}
		13:00	* 3e^{-4}	0.99	1.0	0.99	0.99	0.99	1.0	0.95	0.99	1.0	*1e^{-4}	1.0
		17:00	* 1e^{-4}	* 1e^{-4}	* 1e^{-4}	* 1e^{-4}	* 1e^{-4}	* 1e^{-4}	* 1e^{-4}	* 1e^{-4}	* 1e^{-4}	* 1e^{-4}	* 1e^{-4}	* 1e^{-4}

*Probabilities in bold and in shadowed cells indicate statistically significant differences between pairs ($p < 0.05$). Although Tukey test was performed for pairs within sides, the data are not shown because only the peaks are statistically significant, as should be expected. Note that only the peak at diestrous–2 and proestrous days are statistically significant between sides.

sides. However, the fact that ERβKO female mice are subfertile while the ERαKO are infertile *(15–19)* suggests that ER-β may not be completely involved in GnRH synthesis.

In summary, the results clearly indicate the existence of asymmetry in ER isoforms and GnRH mRNA expression in POA–AHA. This asymmetry seems to have distinct functional reproductive tasks and in the ER-β case possibly non-reproductive tasks. On one hand, the appearance of ER-α on the right side of POA–AHA could indicate the synthesis of GnRH; on the other hand, the appearance of ER-β on the left side of POA–AHA could indicate a triggering role on GnRH release. Complementation between ER-β and GnRH seem possible, indicating communication between sides. It is clear that the participation of estrogens in the regulation of reproductive functions depends on which side of the hypothalamus, as well as on the type of receptor, they act. It seems that the right and left side of neuronal structures in POA–AHA play different roles in the control of endocrine repro-

ductive functions, while keeping close communication for synchronization and time-dependent processes.

Materials and Methods

Animals

Virgin adult female rats CIIZ-V strain from our own stock, 195–225 g body weight, were used. Animals were kept under controlled lighting conditions (lights on from 0500 to 1900), with free access to food (Purina S.A., Mexico) and tap water. Estrous cycles were monitored by daily vaginal smears. Only those rats showing at least two consecutive 4-d cycles were used in the experiment.

At 09:00, 13:00, and 17:00 of diestrous-1, diestrous-2, proestrous, and estrous days, groups of six rats were sacrificed by decapitation. Each experimental group consisted of three repetitions. After decapitation, the brain was quickly removed and placed on a dry-ice-cooled plate. A slice (1.4 mm) was cut and the POA–AHA region was punched out with a needle (0.4 mm inner diameter), following the parameters (A-7020 to A-5660) of the Könnig and Klippel *(27)* rat's stereotaxic atlas. The left and right side of POA–AHA were separated.

Total RNA Isolation and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

The conventional methods for mRNA quantitation such as Northern blot or ribonuclease protection assay sometimes lack enough sensitivity to study low-abundance mRNA or to work with limited amounts of biological samples. PCR is a very powerful tool for evaluation of nucleic acids owing to its high efficiency, convenience, and better sensitivity, being chosen frequently for low-abundance mRNA and/or limited amounts of biological samples. Quantification using Northern blot and RT-PCR with ethidium bromide has been reported to show similar results within the standard error *(28,29)*.

Total RNA from each side of POA–AHA was extracted separately by a single-step method based on guanidine isothyocianate–phenol–chloroform extraction using TRIzol reagent (Gibco-BRL). RNA concentration was determined by absorbance at 260 nm, and its integrity was verified by electrophoresis on 1.2 % denaturing agarose gels in the presence of 2.2 *M* formaldehyde. A 2-µg amount of total RNA was reverse transcribed to synthesize single-stranded cDNA. Subsequently, 10 µL of the reverse transcriptase reaction was subjected to PCR in order to co-amplify a fragment of GnRH, ER-α and ER-β, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) genes.

The PCR reaction was done in an Eppendorf gradient thermocycler. The 50 µL PCR reaction included: 10 µL of previously synthesized cDNA, 20 m*M* Tris-HCl (pH 8.3), 50 m*M* KCl, 1 m*M* $MgCl^2$, 0.2 m*M* of each the four dNTPs, 0.5 µ*M* of each primer, and 2.5 U of Taq DNA polymerase. Negative controls without RNA and with nontranscribed RNA were included in all experiments.

Initial standard conditions were established for GnRH, ER-α, ER-β, and GAPDH amplification. The cycle number was performed within the exponential phase of the amplification process. All PCR products were always studied and analyzed together throughout the experiments. In all samples a single product of 250 bp corresponding to GnRH fragment, one of 337 bp corresponding to ER-α, and another of 301 bp corresponding to ER-β were obtained. Primers selected for the amplification of GnRH, ER-α, and ER-β had the following sequences: GnRH sense: CAA CCC ATA GGA CCA GTG CTG G, GnRH antisense: CAC TAT GGT CAC CAG CGG GG, ER-α sense: TTC ACA CCA AAG CCT CGG G, ER-α antisense: TGC AGC AGC ATC AGC GGA, ER-β sense: TCC CGG CAG CAC CAG TAA C, ER-β antisense: CCC AGA TGC ATA ATC GCT GC. For semiquantitative analysis of mRNA from GnRH, ER-α, and ER-β, RT-PCR amplification of mRNA corresponding to GAPDH was carried out routinely in parallel as an internal control of messenger quality and quantity.

After the PCR, amplified products (25 µL) were resolved on 2% agarose gel stained with ethidium bromide, and photographed under an UV transilluminator. The image was captured with the Kodak EDAS 206 system. To determine changes in the expression of GnRH, ER-α, and ER-β mRNA, the density and area of each band of the GnRH, ER-α, and ER-β PCR products were analyzed with the Scion Image program and values were normalized to densitometric values of corresponding GAPDH PCR products.

Reagents

Chemical reagents were purchased from Sigma Chemical Corp. (St. Louis, MO) and Gibco-BRL, Inc. (Gaithersburg, MD). Taq DNA polymerase was purchased from Perkin-Elmer (Branchburg, New Jersey).

Statistic Analysis

Normalized densitometric GnRH, ER-α, and ER-β mRNA expression values of left and right sides of POA–AHA at 09:00, 13:00, and 17:00 of each day of the estrous cycle were analyzed. Each experimental group ($n = 3$) was analyzed separately using box plots to find outlier distribution values. In all the groups, the values fell within normal distribution limits, thus there was no need for more repetitions within groups. Complete box plots were used instead of the mean ± SEM in Figs. 1A, 2A, and 3A. As an example Fig. 4 shows the box plot of two experimental groups with the individual values of each group. From the broken relative expression axis it can be seen that the distributions seem independent of each other and that the values in each distribution should be significantly different from the values of the other distribution. To determine if there were statistical significative differences between experimental groups, especially between experimental groups of different sides, multivariate analysis of variance (MANOVA) was used, taking as variables the side, day, and hour, independently,

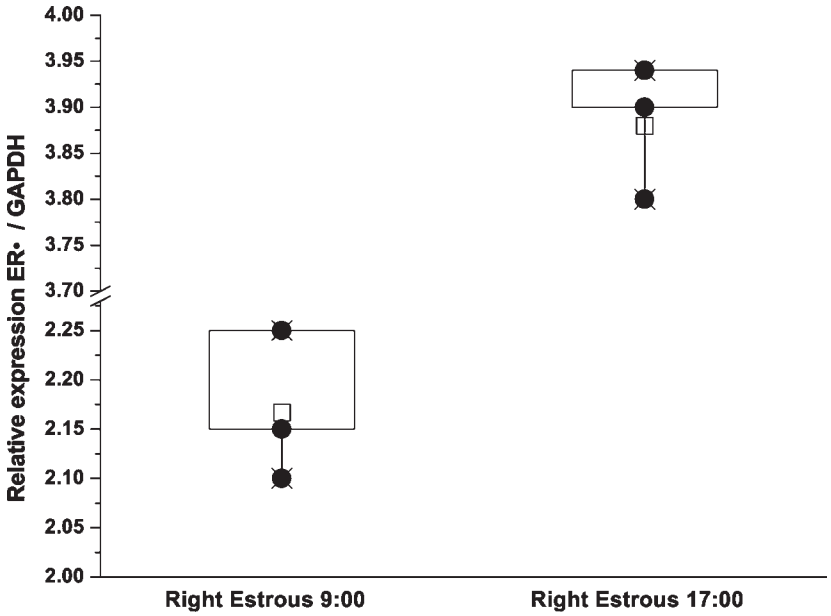

Fig. 4. Example of box plot distribution for two different experimental groups. Box plots for two different experimental groups are shown together with the sampled values. The box represents the range between the 25–75 percentiles, the square represents the mean, the X's represent 1% and 99%, and the underscores represent the minimum and maximum of the distribution. Filled circles represent the values of the sampled data. The relative expression axis has being broken to accommodate both distributions in the same graph. Note that, although the distributions are not symmetrical, the data stays within the distributions.

and in nested combinations of the three variables. Significant results were only obtained when side was used as the variable and when day and hour were nested under the side. The *F*-ratio was calculated and a corresponding probability of $p < 0.05$ was considered as statistically significant between at least two experimental groups. To find which groups had the statistical differences, pairwise Tukey test was performed between pairs of experimental groups where $p < 0.05$ was accepted as significant. Tables 1, 2, and 3 show those groups that had significative differences between them for ER-α, ER-β, GnRH, respectively. The tables do not show pairwise statistics within sides because only the peaks were statistically significant, as should be expected. Tables are included for the reader to check which pairs are significant and which not because is difficult to see it from the figures directly, especially for nonpeak values. All statistics were performed using SYSTAT 7.0 for Windows (SYSTAT, Inc., Evanston, IL).

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