Changes in α-estradiol receptor and progesterone receptor expression in the locus coeruleus and preoptic area throughout the rat estrous cycle

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Abstract

We have previously shown that the locus coeruleus (LC) is essential for triggering surges of LH. Since LC neurons are responsive to estradiol, which induces progesterone receptor (PR) expression, this study aimed to investigate whether LC neurons express the α-estradiol receptor (αER) and PR as well as comparing such responses to that observed in the preoptic area (POA). Female rats were perfused at 10, 14 and 16 h on each day of the estrous cycle, and a blood sample was collected for estradiol, progesterone and LH measurements. αER- and PR immunoreactive (ir) neurons were detected in POA and LC by immunocytochemistry (ICC). Higher plasma estradiol levels were observed on the day of proestrus, when a smaller number of αER-ir POA neurons were detected. An increase in the number of αER-ir neurons were observed at 16 h of proestrus and estrus. The number of PR-ir neurons increased in POA only at 16 h of proestrus, and remained unchanged during all other days and times. The profile of αER-ir and PR-ir neurons in LC changed over the estrous cycle, with a lower expression on metestrus morning and reaching a peak on diestrus afternoon before declining on the day of proestrus. However, on estrus afternoon, αER-ir neurons increased, while PR-ir neurons decreased which may be related to the prolactin surge of estrus. These data show that LC neurons express αER and PR and seem to be more sensitive to variations in estradiol than POA. Also, the fluctuation in αER and PR observed for LC neurons seems to accompany the hormonal events that occur during the estrous cycle. This profile of αER and PR expression might be related to the ability of estradiol and progesterone in regulating the activity of LC neurons, which could be associated to the control mechanisms of LH and prolactin release.

Journal of Endocrinology (2006) **188,** 155–165

Introduction

Luteinizing hormone (LH) secretion is under control of the gonadotropin releasing hormone (GnRH), which in rodents, is mainly produced by the preoptic area (POA) neurons and is released at terminals of the median eminence (Levine & Ramirez 1982, Park & Ramirez 1989). Although it is well known that ovarian steroids control GnRH and LH secretion, the precise sites where estradiol and progesterone exert such control are not clear.

Two isoforms of the estradiol receptor (ER) have been described: αER and βER (Kuiper *et al.* 1996). Since αER knockout mice are completely infertile and βER knockout mice show only a decreased fecundation rate (Lubahn *et al.* 1993), it seems that the activation of αERs is more strictly related to the regulation of reproductive events. However, although the presence of αER has been reported in 20% of GnRH neurons in ovariectomized rats treated with colchicine (Butler *et al.* 1999), most authors have demonstrated that GnRH neurons do not express αER (Herbison & Theodosis 1992, Herbison *et al.* 1995, Laflamme *et al.* 1998, Herbison & Pape 2001). These data suggest that estradiol may control GnRH indirectly via αER-sensitive neurons which project to GnRH neurons located inside or outside POA (Herbison 1998).

Furthermore, one of the main effects of estradiol in the central nervous system (CNS) is the induction of progesterone receptor (PR) expression (MacLusky & McEwen 1978).

DOI: 10.1677/joe.1.06268
0022–0795/06/0188–155

PR activation seems to be a critical step in the full-length generation of the preovulatory LH surge, since progesterone administration to estradiol-primed ovariectomized rats results in the amplification and anticipation of GnRH (Levine & Ramirez 1980) and LH (Everett 1948, Krey *et al.* 1973) surges, and administration of PR antagonist RU486 to proestrus rats blocks LH surge (Bauer-Dantoin *et al.* 1993). Also, PR knockout mice are anovulatory and fail to show an LH surge when exposed to male odors (Chappell *et al.* 1997). Since most GnRH neurons do not contain PRs (Fox *et al.* 1990), progesterone, like estradiol, may indirectly influence the activity of GnRH neurons.

Noradrenaline is known to be one of the excitatory neurotransmitters for LH release (Tima & Flerko 1974, Kalra 1985, Osterburg *et al.* 1987). Locus coeruleus (LC) is the major noradrenergic nucleus and sends projections to the entire CNS, including areas involved in GnRH synthesis and secretion (Swanson & Hartman 1975, Foote *et al.* 1983, Wright & Jennes 1993). Data from our laboratory have demonstrated that electrolytic lesion of LC decreases noradrenaline content in medial POA and medial basal hypothalamus and blocks the preovulatory gonadotropin surges observed during proestrus as well as LH pulses (Anselmo-Franci *et al.* 1999) and the steroid-induced surge in ovariectomized rats by decreasing GnRH release (Franci & Antunes-Rodrigues 1985, Anselmo-Franci *et al.* 1997, 1999, Helena *et al.* 2002, Martins-Afferri *et al.* 2003) . Also, an increased number of FOS-immunoreactive (FOS-ir) neurons was observed in LC simultaneously with the preovulatory gonadotropin surges (Martins-Afferri *et al.* 2003).

Since LC neurons concentrate estradiol (Heritage *et al.* 1980), express mRNA for ER (Shughrue *et al.* 1997) and PR (Curran-Rauhut & Petersen 2002), and are responsive to estradiol treatment, increasing the expression of noradrenaline synthetic enzymes (Serova *et al.* 2002), it is likely that LC neurons are a target for estradiol action, as described for noradrenergic neurons A1 and A2 (Haywood *et al.* 1999). If so, LC neurons should express ER and, consequently, PR. Therefore, this study aimed at investigating whether LC neurons express αER and PR and whether changes in these expressions occur during the estrous cycle as well as at comparing such pattern to that observed in the POA.

Materials and Methods

Animals

Adult female Wistar rats weighing 250–300 g were housed in collective cages (5 per cage) under controlled temperature (24 ± 0·5 °C) and light conditions (lights on from 6:00 to 18:00 h). Food and water were supplied *ad libtum*. Only rats showing at least three consecutive regular 4-day estrous cycles were included in this study. In addition, the proestrus group studied at 16:00 h only included rats that exhibited LH levels higher than 3 ng/ml, which indicates the occurrence of a preovulatory surge, since basal LH levels generally do not exceed 1·0 ng/ml.

Tissue preparation

The animals were anesthetized with 2,2,2-tribromoethanol (250 mg/kg body weight; i.p.; Aldrich Chemical Co. Inc., Milwaukee, WI, USA) and perfused at 10:00, 14:00 and 16:00 h of each day of the estrous cycle ($n=6$–8 per group). Transcardial perfusion was performed with 50 ml 0·01 M phosphate buffered saline (PBS), pH 7·4, containing 5 IU/ml heparin, immediately followed by 300 ml ice-cold 4% paraformaldehyde in 0·1 M phosphate buffer (4% PFA). The brains were quickly removed, postfixed in 4% PFA for 2 h, and cryoprotected in 30% sucrose in 0·1 M phosphate buffer at 4 °C, where they were kept until sinking (approximately 48 h). The fixed brains were frozen by immersion in iso-pentane (Riedel-de Haën, Seelze, Germany) at − 40 °C for one minute and frontal serial 30 μm sections were cut with a cryostat throughout the rostrocaudal extension of LC and POA between 0·26–1·30 mm post Bregma for POA limits and between 9·16–10·52 mm post Bregma for LC neuron limits according to the Paxinos atlas (Paxinos & Watson 1998). They were stored in a cryoprotectant solution (Watson *et al.* 1986) at − 20 °C until processed.

Antibody specificity

The antiserum recognizing αER (AS 409; Okamura *et al.* 1992) was raised in a rabbit against the conjugated protein of beta galactosidese-rat αER, which was produced in *E. coli* cells containing rat αER cDNA (for a detailed description, see (Okamura *et al.* 1992)). The antibody recognizes bound and unbound αER (Okamura *et al.* 1992) and preadsorption with αER protein results in no immunolabeling (Papka *et al.* 1997, Weiland *et al.* 1997). For PR ICC, we used a polyclonal antibody directed against the DNA-binding domain (B region) of human PR (Host: rabbit, A0098, DAKO Corp., Carpinteria, CA, USA). This antibody shows similar reactivity to that of the monoclonal antibody, clone PR AT 4·14, and recognizes a specific site in the unactivated and activated PR and distinguishes between intact and proteolyzed receptors (Traish & Wotiz 1990). The specificity of the PR antibody was determined by incubating control sections from animals perfused in the afternoon of metestrus and diestrus with an anti-PR that had been previously preabsorbed overnight at 4 °C with 200 μg/ml of the antigen peptide (amino acids 533–547; Alpha Diagnostic International Inc, TX, USA), which eliminated nuclear labeling throughout the preoptic area and locus coeruleus. For tyrosine hydroxylase (TH) staining, we used a monoclonal TH antibody (Host: mouse, anti TH-2;

Sigma Chemical Co.). The antibody recognizes an epitope present in the N-terminal region (approximately amino acids 9–16) of both rodent and human TH. Clone TH-2 reacts with the intact TH subunits. No nuclear labeling was observed when αER or PR primary antibodies were replaced with PBS containing 0·3% TX-100 and 1% bovine serum albumin (BSA), indicating the specificity of the antibodies for these proteins (data not shown). No cytoplasm immunoreactivity was detected for the TH antibody by using the same procedure.

Double-labelling immunocytochemistry

Every first and second section from sets of four sections was used for immunocytochemistry (ICC) of αER and PR, respectively. All ICC steps were performed at 22 °C, except for incubation with the primary antibodies, which was performed at 4 °C. Free-floating sections were placed on culture dishes and rinsed five times in PBS to wash out the cryoprotectant. Immediately thereafter, the sections were rinsed in 0·1 M glycine in PBS, incubated with 0·3% Triton X-100 (TX-100) in PBS for 30 min, followed by incubation with 1% H_2O_2 in PBS for 1 h and, finally, with 3% BSA in PBS for 1 h. The first series of sections was then incubated with anti-αER (AS 409) at a dilution of 1:10 000 in PBS containing 0·3% TX-100 and 1% BSA for 40 h (all primary and secondary antibodies were diluted in the same buffer). The second series of sections was incubated with anti-PR antibody at 1:250 dilution for 70 h. After washing with PBS, all sections were incubated with biotinylated anti-rabbit IgG (Host: goat, Elite kit, Vector Laboratories, Burlingame, CA, USA) at 1:400 dilution for αER and PR, for 2 h, and with the avidin DH-biotinylated horseradish peroxidase complex (ABC at 1:100 in PBS for each A and B Elite kit reagents, Vector Laboratories) for 1 h. The final reaction was carried out using a solution containing 3,3′-diaminobenzidine-HCl (0·2 mg/ml DAB; Sigma) and H_2O_2 (1 μL/ml from a 30% solution) with nickel chloride (25 mg/ml) in PBS. After washing with PBS and 1% H_2O_2 in PBS for 15 min, the LC-containing sections were incubated with the TH antibody at 1:30 000 for 40 h. After washing with PBS, the sections were incubated for 1 h with a biotinylated horse anti-mouse secondary antibody (1:1000). After washing in PBS, the tissues were incubated for 30 min with ABC, washed again with PBS and developed using DAB (0·1 mg/ml) plus 1 μL/ml H_2O_2 from a 30% solution in 0·05 M Tris–HCl buffer, pH 7·6 (Shu *et al.* 1988). Sections were mounted on gelatin-coated glass slides, air-dried, rinsed in ethanol, cleared in xylene and coverslipped with Entellan (Entellan, Merck).

Analysis

The sections were blindly examined under a light microscope (Axioskop 2 plus, Zeiss, Hallbergmoos, Germany) using an image analysis system (Axiovision 3·1, Zeiss). The number of αER-ir and PR-ir neurons was counted unilaterally (right side) in a total of 6 POA sections and 8 LC sections per animal, at a magnification of ×200. The LC sections were grouped in accordance to the morphology previously described (Grzanna & Molliver 1980). αER-ir and PR-ir neurons of LC were counted in 2 sections from the rostral portion and 6 from the LC proper using TH immunostaining to identify the precise boundaries of the LC region. The number of αER-ir and PR-ir neurons in the POA was quantified in 6 sections beginning in the most rostral region of POA (AVPV) until the medial POA, in an area measuring 400 × 400 μm using the third ventricle as the medial limit. The results are expressed as the average of the total number of αER-ir or PR-ir neurons in all counted sections for each rat.

Blood samples

One minute before the beginning of perfusion, 1 ml blood samples were collected from the right ventricle of anesthetized rats into heparinized syringes, centrifuged at 1200 ***g*** for 15 min at 4 °C, and plasma was separated and stored at −20 °C until the time for RIA. Plasma LH concentrations were determined using specific kits provided by the Institute of Diabetes and Digestive and Kidney Diseases (NIDDK, Baltimore, MD, USA). The antiserum for LH was LH-S10 and the reference preparation was RP3. The lower limit of detection was 0·2 ng/ml and the intra-assay coefficient of variation was 4%. Plasma estradiol and progesterone concentrations were determined using the Estradiol and Progesterone Maia kits (Biochem Immunosystems, Serotec, Italy), respectively. The lower detection limit and the intra-assay coefficient of variation were respectively 7·5 pg/ml and 2·5% for estradiol and 4·1 ng/ml and 3·7% for progesterone. All samples were measured in duplicate and at different dilutions, if necessary. In order to prevent interassay variation, all samples were assayed in the same RIA.

Statistical analysis

The influence of the estrous cycle phases on the number of αER-ir and PR-ir neurons in LC and POA was assessed by two-way ANOVA. When the F values indicated significant differences, *post hoc* comparisons were made between groups using one-way ANOVA followed by the Newman–Keuls test for multiple comparisons. Values were considered to be significant when $P<0·05$.

Results

Figure 1 shows a schematic drawing depicting the region of LC (A) and POA (B) where αER-ir and PR-ir neurons

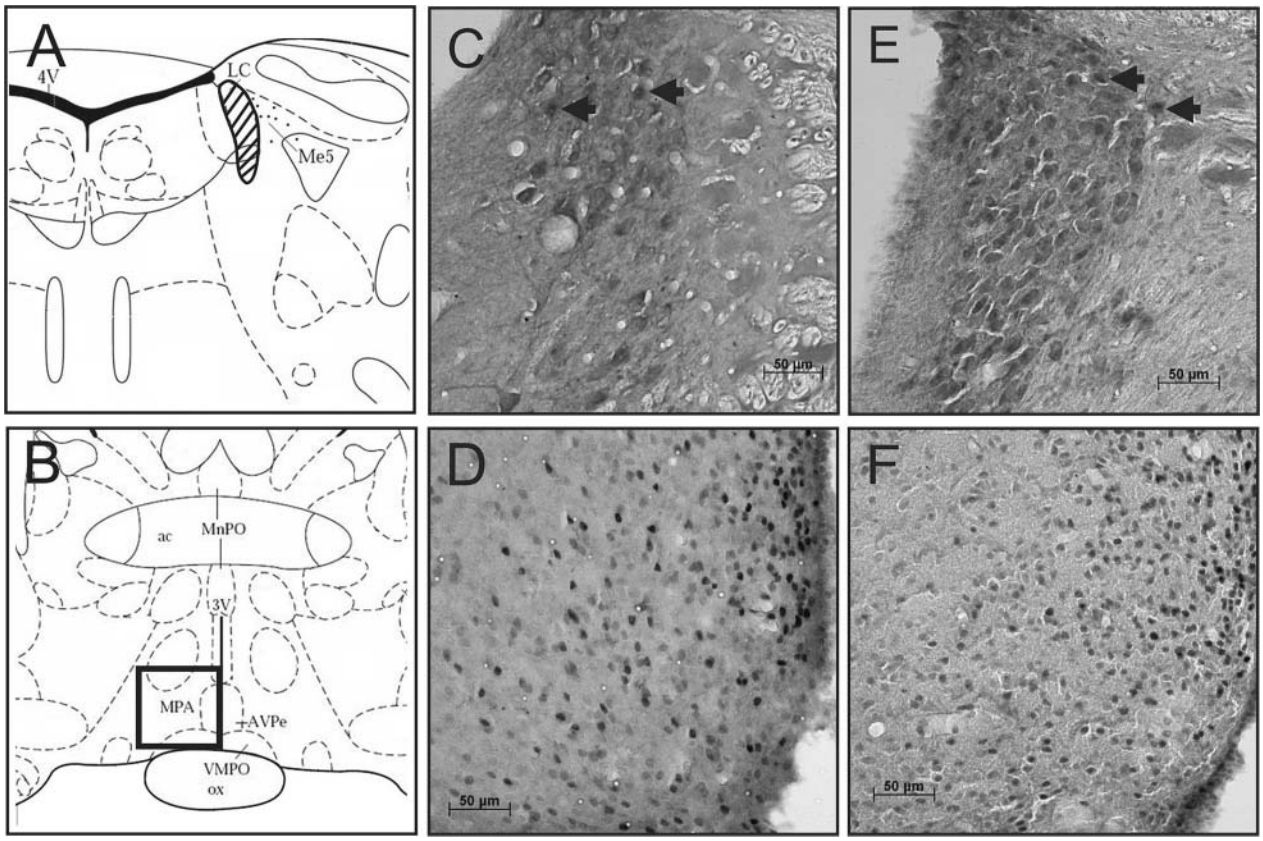

Figure 1 Schematic view of the locus coeruleus (LC; hatched area in A) and preoptic area (POA; box in B), and photomicrographs of αER (C, D)- and PR (E, F)-immunoreactive neurons in LC (C, E) and POA (D, F) of a female rat perfused at 16:00 h of diestrus. In LC, all αER- and PR-immunoreactive neurons were colocalized with tyrosine hydroxylase (arrows). The box in B shows the selected area used for quantification.

were evaluated as well as αER-ir and PR-ir neurons in LC (C and E) and POA (D and F) as examples. Since most LC neurons express TH (Pickel *et al.* 1975), immunostaining (only seen in the neuronal cytoplasm) was efficient in delimiting the LC boundaries, as shown in panels C and E. In LC, αER-ir and PR-ir neurons were always colocalized with TH, but not all TH-ir neurons were positive for both receptors. Moreover, the number of PR-ir neurons was much larger than that of αER-ir neurons, not only in the LC but also in the POA region. No PR labeling was found in the TH-expressing cells of the LC and in POA region when anti-PR was preabsorbed with the antigen peptide, indicating specificity of this antibody to detect PR in these regions, as it is shown in Fig. 2.

Regarding the days of the estrous cycle, the profile of αER-ir and PR-ir cells in LC changed in a similar way over the estrous cycle, as we can observe by the curves superimposed to the bars in Figures 3A and 3C. The expression of both receptors increased gradually from a lower expression on metestrus morning and reached its maximum level on diestrus afternoon ($P<0{\cdot}01$ and $P<0{\cdot}001$, respectively), before declining on proestrus day. However, on estrus afternoon, the number of αER-ir neurons increased ($P<0{\cdot}001$), while that of PR-ir neurons decreased ($P<0{\cdot}05$). As expected, the highest plasma estradiol levels during the estrous cycle were observed during all times of proestrus day ($P<0{\cdot}001$) while they were lower and constant on all other days and times studied (Fig. 3B). Progesterone levels were higher on metestrus ($P<0{\cdot}05$), decreased on diestrus and tended to increase on proestrus afternoon (Fig. 3D). These variations occurred in an opposite direction from that of PR expression in LC (Fig. 3C). Basal LH concentrations were observed during all times, except at 16:00 h of proestrus (time of the preovulatory surge, $P<0{\cdot}01$), as shown in Fig. 3E.

In contrast to LC, a constant number of αER-ir cells was found in the POA (Fig. 4A) over the days of the estrous cycle, except on proestrus day when a decrease in this number was observed ($P<0{\cdot}001$). Concerning the times studied, the number of αER-ir cells did not differ among them on metestrus and diestrus days, whose values were similar to those observed at 10:00 and 14:00 h

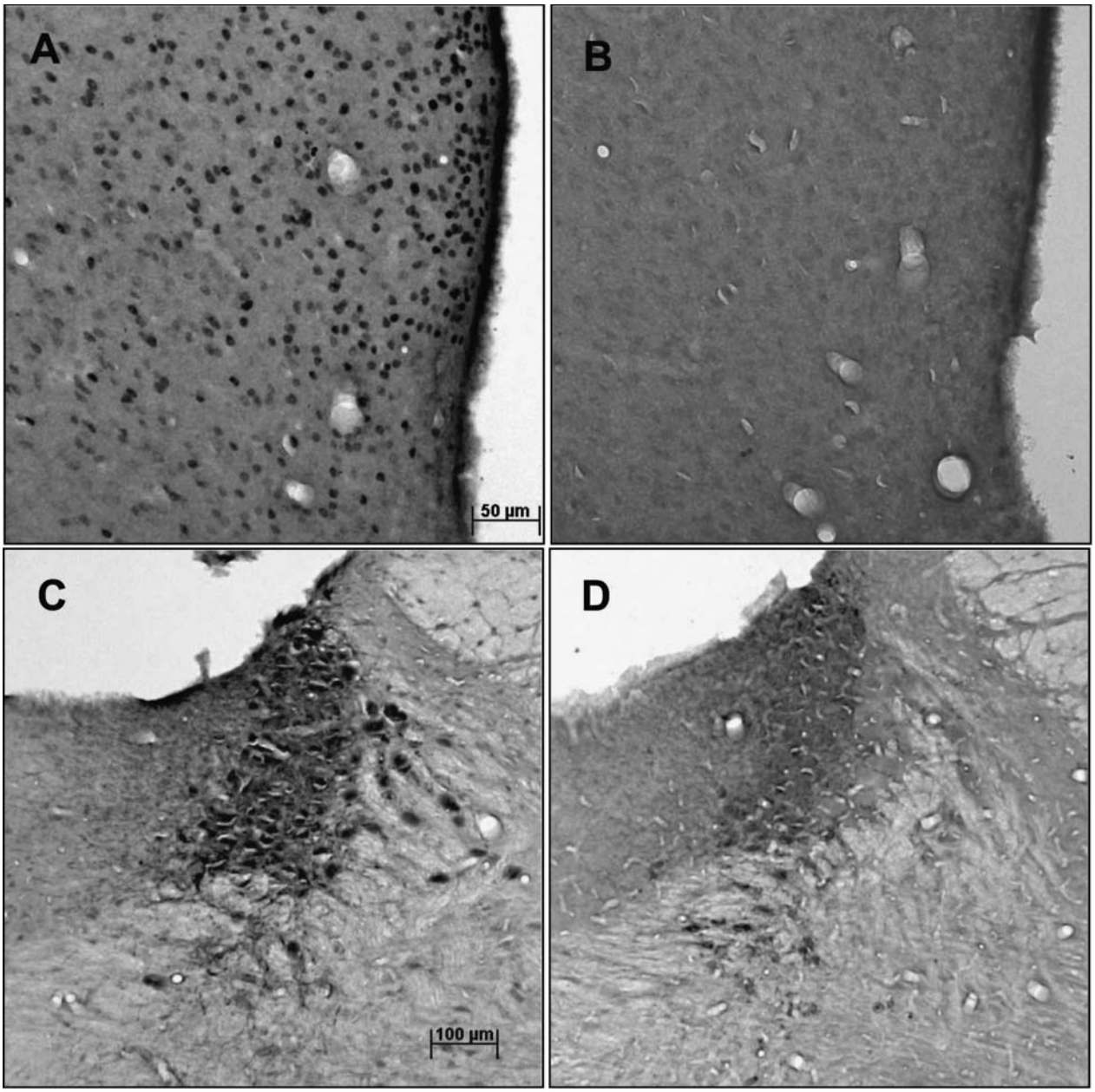

Figure 2 Photomicrographs of PR-immunoreactive neurons in POA (A and B) and LC (C and D) illustrating the preabsorption test. Sections from rats perfused in the afternoon of diestrus were incubated with the anti-PR alone (A and C) or with an anti-PR that has been pre-absorbed overnight with the antigen peptide (B and D). The pre-absorption completely abolished nuclear staining of PR in both areas, while TH immunoreactivity persisted in the cytoplasm of the LC neurons.

on estrus day. However, there was an increase in the number of αER-ir cells at 16:00 h on proestrus and estrus day ($P<0{\cdot}05$ and $P<0{\cdot}01$) as compared with the other times studied. The number of PR-ir cells did not vary in POA (Fig. 4B), except at 16:00 h of proestrus, when a significant increase was observed ($P<0{\cdot}01$).

Discussion

In the present study, we described the presence of αER and PR in LC neurons of female rats as well as their pattern of expression during the estrous cycle by comparing it to that observed in POA. Expression of αER in LC

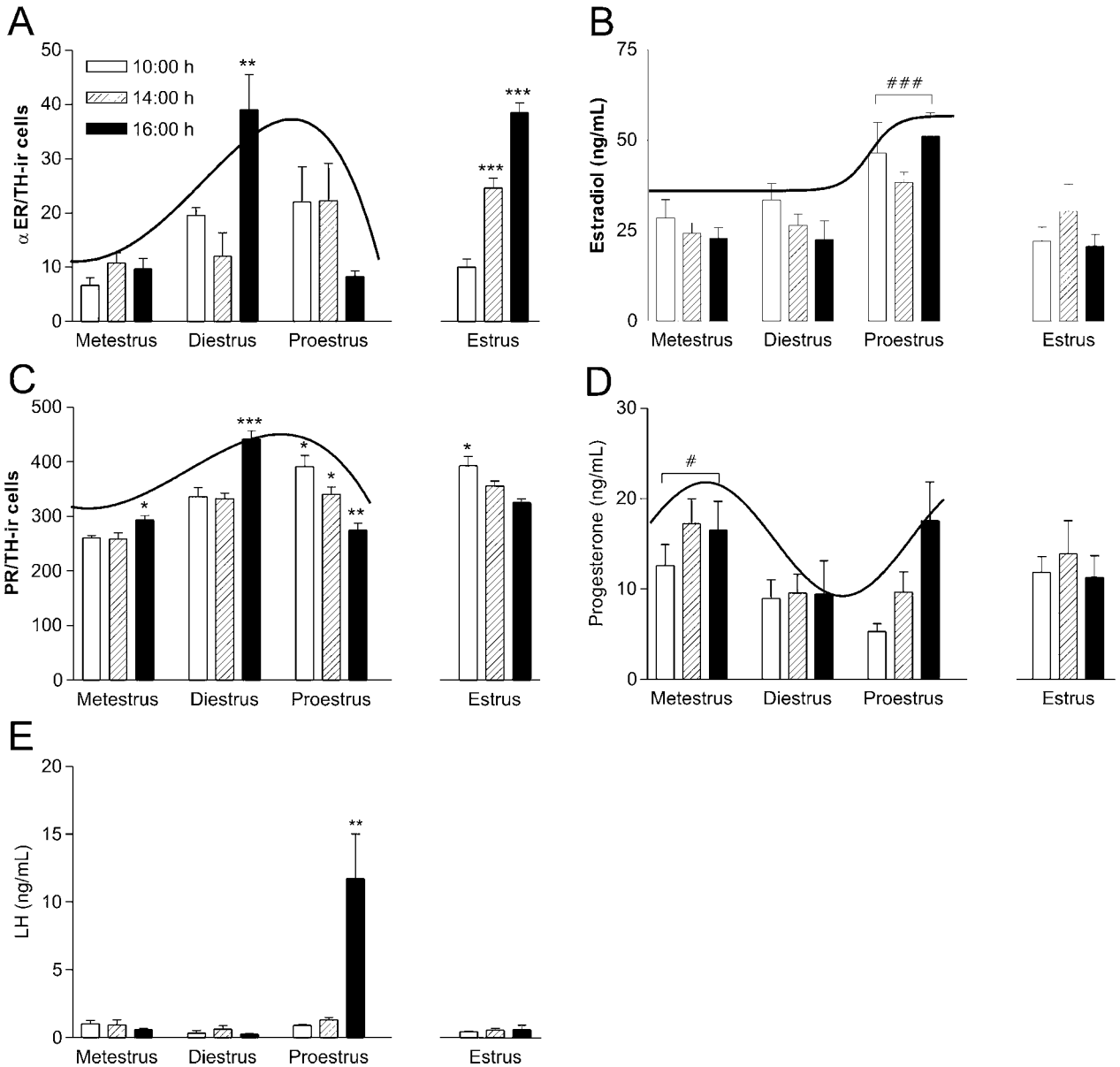

Figure 3 Number of αER and PR-immunoreactive (ir) neurons in LC (A and C, respectively), plasma estradiol (B), progesterone (D) and LH (E) concentrations of cycling female rats on each day of the estrous cycle. #P<0·05 and ###P<0·001 compared with other days of the estrous cycle; *P<0·05, **P<0·01 and ***P<0·001 compared with the other times in the same group (n=6–8).

has previously been described for male mice (Mitra *et al.* 2003), while no data regarding the expression of PR in this nucleus are available thus far. Although we did not colocalize αER and PR, it is possible that PR was expressed in αER-positive neurons since some studies conducted on guinea pigs support the hypothesis that estradiol-inducible PRs occur only in cells expressing ERs (Blaustein & Turcotte 1989, Warembourg *et al.* 1989). Interestingly, we found that the number of PR-ir cells was much larger than that of αER-ir cells in LC and POA in all groups studied. Thus, if PR-positive neurons should also express ER, it is possible that PR synthesis was induced by an ER other than αER. In fact, disruption of the αER gene suppressed, but did not completely inhibit, the induction of PR in several brain regions, including POA, of αER-knockout mice (Moffatt *et al.* 1998). This suggests that the induction of PR in αER-knockout mice may also be mediated by βER. In this regard, it has been

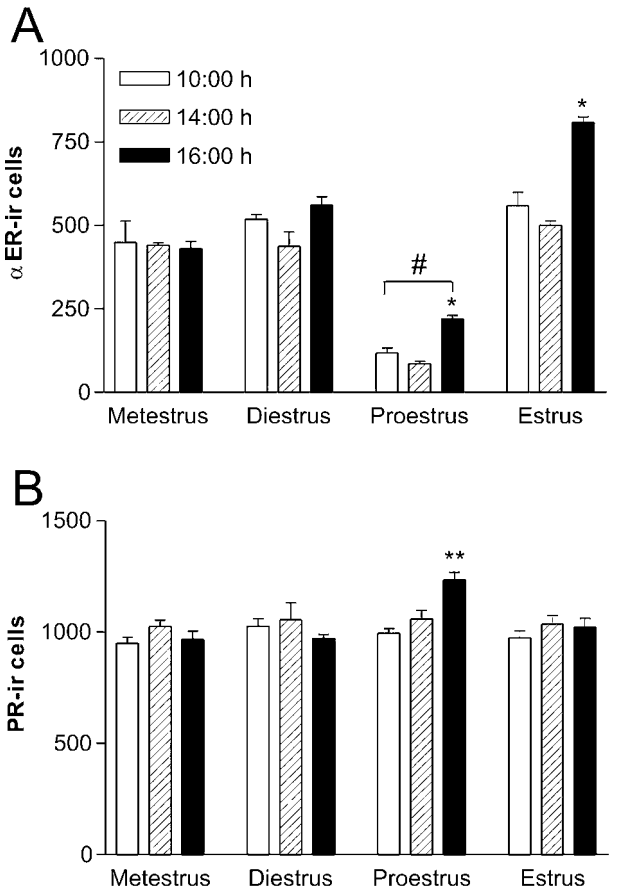

Figure 4 Number of αER and PR-immunoreactive (ir) neurons in POA (A and B, respectively) in cycling female rats on each day of the estrous cycle. #$P<0{\cdot}05$ compared with other days of the estrous cycle; *$P<0{\cdot}05$ and **$P<0{\cdot}01$ compared with the other times in the same group ($n=6$–8).

demonstrated that POA and LC neurons express βER mRNA (Shughrue *et al.* 1997*b*) and that the two receptors (αER and βER) bind to estradiol with equal affinity (Kuiper *et al.* 1997). In addition, βER has been shown to be expressed in αER- and PR- containing cells in the female rat forebrain (Greco *et al.* 2001). Thus, although it is not known whether βER induces PR synthesis, this could be a possibility to explain the larger number of PR-ir cells (when compared with αER-ir cells) found in LC and POA neurons.

Locus Coeruleus

From metestrus to proestrus The profile of expression of αER-ir and PR-ir in the LC neurons changed significantly during the estrous cycle. Although basal estradiol levels were observed on metestrus and diestrus days, the expression of both receptors was lower on metestrus and increased gradually until reaching its maximum level at 16 h of diestrus afternoon. Increased levels of estradiol are required to activate the LH surge on proestrus afternoon since administration of estradiol inhibitors (Shirley *et al.* 1968) and estradiol antiserum (Neill *et al.* 1971) on diestrus blocks the proestrus LH surge. In this regard, this increase in the number of αER-ir cells in LC may be a way to prepare this nucleus to respond to the increase of estradiol levels which starts in the late evening of diestrus (Smith *et al.* 1975) in order to activate the LH surge on proestrus afternoon, which was observed at 16 h in our study.

On proestrus day, when the highest levels of estradiol were observed, there was a decreased number of αER-ir cells in LC at all times studied, when compared with 16 h of diestrus, which may suggest that αER synthesis was down-regulated by estradiol. In fact, although estradiol has been demonstrated to induce an increase in its own receptors in peripheral tissues (Sarff & Gorski 1971), this control seems to occur in an inverse manner in CNS (Zhou *et al.* 1995).

As expected, higher progesterone levels were observed in metestrus (as a consequence of luteal secretion). In proestrus, although no significant difference in progesterone levels was observed among the times studied, the clear tendency toward an increase in the afternoon probably indicates the beginning of follicular secretion which coincides with the LH surge and reaches its maximum after the LH surge in late proestrus (Smith *et al.* 1975). These plasma progesterone levels may regulate PR expression in LC since the lowest and the highest plasma progesterone levels observed in diestrus and metestrus respectively were accompanied by the highest and lowest expression of PR in LC neurons, correspondingly. Similarly, the clear tendency toward a gradual increase in plasma progesterone levels during proestrus was accompanied by a gradual decrease in PR expression. Thus, one may suggest that PR synthesis is down- or up-regulated by its ligand. In addition, PR expression also seems to be regulated by αER since the profile of PR expression in LC neurons from metestrus to proestrus followed that of αER.

It should be noted that estradiol increases noradrenergic turnover before and during the proestrus LH surge in several brain areas, including the POA and median eminence (Rance *et al.* 1981, Mohankumar *et al.* 1994), and that an increased noradrenergic input to POA is essential for the synthesis as well as for the release of GnRH (Herbison 1997). In LC neurons, estradiol stimulates gene expression of TH and dopamine beta hydroxylase (Serova *et al.* 2002), probably by acting through αER. Besides, it is well established that estradiol induces PRs synthesis in CNS, and that their activation is critical for the LH surge occurrence, since it is blocked by RU-486 administration (Tebar *et al.* 1998).

Consequently, since we have previously shown that noradrenaline of LC neurons plays an important role in

GnRH release and gonadotropin surges (Anselmo-Franci *et al.* 1997, Helena *et al.* 2002), we may hypothesize that estradiol would act in LC αER-ir cells in order to increase the synthesis of noradrenaline and PRs during the late follicular phase. Subsequently the rising progesterone of proestrus would induce the noradrenaline release, which would induce GnRH, and consequently gonadotropin release. In fact we have shown an increase of FOS expression in LC during proestrus afternoon (Martins-Afferri *et al.* 2003), which may represent an increase in noradrenaline release in POA, which is required for the release of these hormones.

Estrus Interestingly, on estrus day, the number of αER-ir cells of LC increased, while the number of PR-ir cells decreased, both gradually. We assume that this variation is not related to the variation in gonadotropins or estradiol secretion, since the concentrations of these hormones are constant during this period. However, this result seems to be quite interesting if correlated with prolactin secretion. We have demonstrated that an acute and robust increase in prolactin secretion occurs in the afternoon of estrus (between 15:00 and 17:00 h) in female rats (Szawka & Anselmo-Franci 2004). Since estradiol is the main hormone inducing prolactin secretion (Freeman *et al.* 2000) and estradiol concentrations during estrus are constant, an increased number of LC αER-ir neurons by the time of the prolactin surge on estrus day may represent a mechanism to render this nucleus more sensitive to the positive action of estradiol on prolactin secretion. On the other hand, the precise role of progesterone in the secretion of prolactin is not clear. Progesterone is able to advance and amplify the prolactin surge in a time- and dose-dependent manner (Caligaris *et al.* 1974, Yen & Pan 1998). In addition, progesterone has been reported to be responsible for the plateau aspect of the proestrus prolactin surge (Arbogast & Ben-Jonathan 1990). Since progesterone concentrations are high in the afternoon of proestrus and low during estrus, these low plasma progesterone levels, together with the decreased number of LC PR-ir neurons observed in this study, may be responsible for the absence of a plateau phase in the estrus prolactin surge. Thus, even in the absence of alterations in plasma estradiol and progesterone levels, the increase in the number of αER-ir cells and the decrease in the number of PR-ir cells in LC observed here during estrus suggest that LC neurons become more sensitive to the action of estradiol while being less sensitive to progesterone, thus determining not only the occurrence of the secondary prolactin surge, but also its acute shape. Indeed, recent data from our laboratory have demonstrated that LC neurons are essential for the occurrence of this surge, since it is blocked by LC lesion (Poletini *et al.* 2004).

Preoptic area

Differently from LC, from metestrus to diestrus the number of αER and PR-ir cells of POA was constant. On proestrus day, although a small increase was observed at 16 h, the number of αER-ir cells was the lowest in the whole estrous cycle, suggesting that these neurons could be under the control of the same down-regulation mechanism as described for LC neurons. The number of PR-ir cells on proestrus day was similar to that of metestrus and diestrus, except for an increase observed at 16 h. In fact a higher content of PR in POA on the day of proestrus has been demonstrated (McGinnis *et al.* 1981). Interestingly, the reduced number of αER-ir cells found in POA on proestrus day did not cause a decrease in the number of PR-ir cells. Thus, one may hypothesize that the higher estradiol levels observed on this day may compensate for the lower αER synthesis, thus maintaining PR expression constant. The fact that both receptors presented an increased expression at 16 h of proestrus afternoon, even without significant changes in estradiol levels, suggests that this pattern may be driven by mechanisms, other than hormonal plasma levels, that are triggered at this critical time of the occurrence of gonadotropin surges.

In the afternoon of estrus, as observed in LC, an increase in the number of αER-ir cells was observed in POA. This increased number of αER-ir cells supports data reported by Shughrue and colleagues (Shughrue *et al.* 1992), who demonstrated that the content of ER mRNA in POA is high on the afternoon of estrus. As discussed for LC, this finding may be related to the occurrence of the secondary prolactin peak. Indeed, POA neurons have been implicated in the control of prolactin secretion since lesion of this area blocks the prolactin peak induced by estradiol in ovariectomized rats (Pan & Gala 1985), and electric stimulation of POA increases prolactin secretion in male rats (Colombo 1984).

Thus, although expressive variations on αER and PR expression were observed in LC, these expressions were practically constant through the whole estrous cycle in POA, with minor variations in a few periods. One possibility to explain these different results could be that while LC is almost exclusively constituted by noradrenergic neurons, POA presents several neuronal phenotypes, including neurons that could exert excitatory or inhibitory roles on GnRH release. Consequently, once we did not access the phenotype of the POA neurons expressing αER and PR, it is possible that during the estrous cycle a decreased expression of these receptors in inhibitory neurons occurs, which was accompanied by an increased expression in excitatory neurons or vice-versa. Thus, these possible opposite variations would mask real changes in the receptors expression.

In summary, the present data demonstrate that LC neurons do express αER and PR, and these expressions fluctuate throughout the estrous cycle in a more variable

way than observed for POA neurons. In addition, the results suggest that LC may be a primary temporal structure signaling the genesis of hormonal surges, playing an important role in the occurrence of the secondary prolactin peak. Thus, the expression of αER and PR in LC and POA neurons seems to be regulated by complex and distinct factors that act in a coordinated manner to guarantee adequate endocrine and/or behavioral responses for the success of reproduction.

Acknowledgements

The authors would like to thank Ms Rute A Freitas Marcon, Sonia A Zanon and Ariana Mathias Fernandes for technical support.

Funding

This study was supported by FAPESP, CNPq, CAPES and CREST Project of JPST. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received 31 October 2005
Accepted 8 November 2005
Made available online as an Accepted Preprint
22 November 2005