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Progesterone blockade of a luteinizing hormone surge blocks luteinizing hormone-releasing hormone Fos activation and activation of its preoptic area afferents

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Abstract

Progesterone is capable of facilitating or blocking the luteinizing hormone (LH) surge, depending on the timing of its administration. However, the precise targets of progesterone's actions are unknown. Since recent studies described the presence of a periventricular preoptic area (pePOA) neuron population afferent to LH-releasing hormone (LHRH) neurons that is co-activated to express c-Fos with LHRH neurons at the time of the LH surge, the present study was designed to determine if the pePOA neurons contain progesterone receptors (PRs) and whether progesterone inhibition is manifested by a failure of LHRH and pePOA neurons to become activated at the time of an LH surge. For progesterone facilitation, a group of immature rats each received a silastic capsule (1.57 mm i.d., 3.18 mm o.d., 1.5 cm long) containing estradiol-17β (E_2) in peanut oil (150 μg/ml) at 09.00 h on postnatal day 28 followed 24 h later by a progesterone implant (crystalline, 1.57 mm i.d., 3.18 mm o.d., 1.5 cm long). For progesterone inhibition, a second group of rats received the estrogen capsule and a progesterone capsule (3.35 mm i.d., 4.65 mm o.d., 3.0 cm long) together at 09.00 h on day 28, and 24 h later received only a blank capsule. On the afternoon of postnatal day 29, all animals were anesthetized and perfused for localization of c-Fos and LHRH, PRs alone, or c-Fos and PRs. The present studies determined that following a progesterone-inhibition paradigm, along with blockade of the LH surge, both activation of LHRH and pePOA neurons was low or absent. Staining of PRs in progesterone-facilitated and progesterone-inhibited rats indicated that the pePOA neurons contained PRs in similar patterns. Double labeling of c-Fos and PRs in progesterone-facilitated rats indicated that nearly all the c-Fos-positive neurons of the pePOA ($80 \pm 4.2\%$) co-expressed PRs; in progesterone-inhibited rats, only $32 \pm 12\%$ of few c-Fos-positive neurons also contained PRs. In no instance were LHRH neurons found to contain PRs. Taken together, these data suggest that both progesterone facilitation and inhibition likely involve direct actions of progesterone on the pePOA neurons, and are consistent with a role for the pePOA neurons in transducing steroid effects on LHRH neurons. © 1997 Elsevier Science B.V.

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1. Introduction

Progesterone, when administered to rats after estrogen (E_2) priming augments and synchronizes the preovulatory luteinizing hormone (LH) surge. Actions of progesterone are exerted both in the brain and at the pituitary gland. For example, progesterone treatment of estrogen-primed rats is associated with a greater release of LH-releasing hormone (LHRH) from perfused hypothalamic/preoptic area explants [23] and greater activation of immediate early gene proteins in LHRH neurons [13,20] than similar tissue from ovariectomized rats treated with estrogen only. Although times during the estrous cycle when endogenous progesterone is highest, and similar times after progesterone

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treatment of estrogen-primed ovariectomized rats are both associated with enhanced responsiveness of the pituitary to exogenous LHRH in vivo [21] and in vitro [18,19], the mechanism underlying this effect has not been elucidated. The levels of LHRH receptors in the pituitary and expression of LHRH receptor mRNAs are either attenuated or unchanged by progesterone [2,4,22,28], suggesting a more complicated route for progesterone's effect.

When progesterone is presented before or concurrently with estrogen, progesterone blocks rather than enhances the LH surge [1]. The majority of evidence points to the brain as the site of action of progesterone in blocking the LH surge. Progesterone can prevent E_2-induced LHRH release [25] and decrease LHRH output in response to electrical stimulation of the preoptic area [26]. Placement of appropriately timed progesterone implants into the anterior preoptic area of pentobarbital-treated intact rats or estrogen-treated ovariectomized rats also prevents LH discharges [3]. Whereas some evidence is available that progesterone can reduce LHRH binding in the pituitary [28] and suppress gene expression of the LHRH receptor [4], those studies were not designed in a way that could rule out a primary CNS effect in evoking pituitary changes. Moreover, in sheep, where portal blood LHRH measurements are possible without the confounds of anesthesia during collection, progesterone abolishes the surge of LHRH [17], unequivocally indicating that a CNS effect of progesterone is the primary cause of the progesterone-block in LH secretion.

For a progesterone effect to take place within the central nervous system and inhibit LHRH neurons, the site and mechanism of progesterone's action must be considered. PRs are not present in LHRH neurons of the rat [11]. Yet, progesterone could affect the functions of LHRH neurons by impacting on afferents to the LHRH system. One potential locale for this interaction is the periventricular preoptic area (pePOA), a region that projects to LHRH neurons and is activated in synchrony with LHRH neurons at the time of an LH surge [6] The present study was thus designed to determine whether progesterone inhibition is manifested by a failure of both LHRH and pePOA neurons to become activated, and whether progesterone receptors (PRs) are present in those pePOA neurons that are activated at the time of an LH surge.

2. Materials and methods

2.1. Animals

Twenty-seven immature female Sprague-Dawley rats, 21 days old, were purchased from Zivic-Miller (Allison Park, PA). The rats were acclimated to the laboratory environment for 1 week prior to the onset of the experiment. All rats were maintained on a 14:10 h light/dark schedule (lights on 05.00–19.00 h) and were given free access to food and water. Protocols for the experiments were approved by the University Committee on Animal Research according to NIH Guidelines.

2.2. Preparation of implants

Silastic capsules (1.57 mm i.d., 3.18 mm o.d., 1.5 cm long) containing estradiol-17β (E_2) in peanut oil (150 μg/ml) and implants containing crystalline progesterone (3.35 mm i.d., 4.65 mm o.d., 3.0 cm long for progesterone inhibition; 1.57 mm i.d.; 3,18 mm o.d., 1.5 cm long for progesterone facilitation) were prepared as described previously [1,12]. Implants were soaked in phosphate-buffered saline (PBS)–0.1% gelatin overnight at room temperature before use to avoid release of a bolus of steroid upon implantation.

2.3. Hormone treatments

For demonstration of progesterone facilitation of the LH surge, a group of 28-day-old rats ($n = 15$) was implanted s.c. with Silastic capsules containing E_2 at 09.00 h to initiate the LH surge. Twenty-four hours after E_2 treatment, each rat received a 1.5 cm progesterone implant to enhance the LH surge.

For progesterone-inhibition of the LH surge, a group of 28-day-old rats ($n = 12$) received 3.0-cm Silastic progesterone capsules concomitantly with E_2 capsules at 09.00 h. Twenty-four hours later, the progesterone inhibition group of rats received a blank capsule. All implants were inserted under light methoxyfluorane anesthesia (Metofane, Pitman-Moore).

2.4. Perfusion of tissue for immunocytochemistry

Each animal was anesthetized with an overdose of pentobarbital (100 mg/kg, i.p.) between 14.00 and 18.00 h on day 29. A blood sample (0.1 ml) for determination of plasma LH was collected directly from the heart. The animals were then perfused transcardially first with saline containing 2% sodium nitrite (Fisher, Pittsburgh, PA) and then with 2.5% acrolein (EM grade; Polysciences) in phosphate-buffered 4% paraformaldehyde (pH 7.0) as previously described [14]. After fixation, the brains were sunk in 30% aqueous sucrose and sectioned at 25 μm on a freezing microtome. Serial sections were collected into 1-in-12 series and stored in cryoprotectant solution [27] at -20°C until staining was initiated.

2.5. Immunocytochemical staining and analyses

2.5.1. c-Fos and LHRH

The methods used for staining of c-Fos and LHRH have been published previously [14]. The anti c-Fos antibodies

used in this study came from two sources: one was obtained from Oncogene Sciences (antibody 2, 0.02 μg/ml, or 1:50 000, with reconstituted serum considered 1:1) and the second was a generous gift from Dr. Philip Larson (1:100 000). Both were generated from amino acids 4–17 of the c-Fos protein and provided comparable results in assays. These antisera do not recognize other immediate–early gene products or Fos related antigens [14,15]. Briefly, the sections for dual immunocytochemical staining of c-Fos and LHRH were moved from the cryoprotectant, rinsed, treated with a 1% $NaBH_4$ solution (Sigma, St. Louis, MO), rinsed, and then incubated with rabbit anti-c-Fos for 48 h at 4°C. After rinsing, the tissue was incubated in biotinylated goat anti-rabbit IgG 1:600 (heavy and light chains, Vector Laboratories, Burlingame, CA) for 1 h at room temperature, rinsed and incubated for 1 h in AB reagents ('elite' ABC kits, Vector Laboratories, 4.5 μl each per ml of PBS mixture). After first rinsing in PBS, then in 0.175 M sodium acetate (NaOAc), the c-Fos antibody–peroxide complexes were stained in 3,3-diaminobenzidine–HCl (DAB, 0.2 mg/ml) with nickel sulfate (25 mg/ml) and H_2O_2 (0.83 μl of a 3% solution/ml). After approximately 15–20 min, c-Fos immunoreactivity was visualized as blue–black in the nuclei of neurons. For colocalization of LHRH neurons, c-Fos-labeled sections were rinsed in PBS and incubated with rabbit-anti LHRH antiserum for 48 h at 4°C. Anti-LHRH (LR-1 from Drs. Benoit and Guillemin) was used at 1:150 000. After rinsing, sections were incubated in goat anti-rabbit IgG and AB complex reagent as described above and then stained by using a mixture of H_2O_2 and DAB–HCl in Tris buffer (Sigma, 0.05 M, pH 7.2). After staining for 7–10 min, LHRH immunoreactivity was visualized as brown reaction product in the cytoplasm. The tissue was transferred to Tris buffer to stop the reaction, rinsed in saline and mounted on gelatin/chrome alum-subbed slides. After drying overnight, the slides were dehydrated in graded alcohols, cleared in Histoclear (VWR Scientific) and coverslipped with Histomount (VWR scientific). This procedure resulted in permanently stained sections that were used to both determine the degree of LHRH activation and the extent of pePOA neuronal activation.

2.5.2. PRs alone or with c-Fos or LHRH

Two different PR antibodies were used in this study. One from Stress Gen Biotechnology Corp (Victoria, BC) was generated against the hinge region of the chicken PR; the other from Dako (#533–547, Carpenteria, CA) was generated against the DNA binding domain of human PR. For single labeling of PRs, the rabbit anti- human PR from Dako was used at a concentration of 1:20 000. Double labeling of PRs and c-Fos or LHRH was accomplished with the Stress Gen antiserum and required first treating the sections with microwaves. The procedure used [16] follows. After rinsing sections to remove cryoprotectant and treating them with $NaBH_4$, the rinsed sections were placed in 5 ml of a 10 mM citrate buffer, pH 6.0, that had been preheated 2 min in a microwave oven (Logik, 650 W) at full power. The sections were microwaved for 10 s and quickly washed in PBS (at room temperature).

Incubation of the tissue from this point on was the same whether the Stress Genm or Dako antibody was used. Briefly, the sections were incubated with anti-PR (1:20 000) at 4°C for 48 h. After rinsing, sections were incubated in biotinylated anti-mouse (when Stress Gen antiserum was used) or anti rabbit IgG (when Dako antibody was used) and the sections were carried through the ABC elite (Vector Laboratories) method as described for c-Fos above. After staining for 10–20 min, PR immunoreactivity was visualized as punctate blue–black product in the nuclei of neurons.

For detection of activation of c-Fos in progesterone-receptive pePOA neurons, immunoperoxidase and amplified biotin-immunofluorescent methods were combined. For PR staining, the procedures were the same as described above for single labeling of PR immunoreactivity using the Stress Gen antibody. For colocalization with c-Fos, the c-Fos was detected using amplified biotin-immunofluorescence [5]. For this procedure, sections were incubated with the Larson c-Fos antiserum (1:75 000 in PBS/Triton X-100 containing 1% BSA) for 48 h at 4°C. The tissue was rinsed in PBS and incubated in biotinylated goat anti rabbit IgG (1:5000 in PBS/Triton X-100) for 1 h at room temperature. After rinsing, the tissue was incubated for 30 min with 'elite' avidin–biotin complex reagents (1.125 μl each per ml of mixture in PBS with 0.4% Triton X-100), rinsed again for 30 min and incubated for 20 min in a biotinylated tyramine solution (5 μl/ml in PBS) to which H_2O_2 at final concentration of 0.005% was added. The tissue was rinsed for 30 min in PBS and incubated with streptavidin-CY3 (Sigma, 1:5000 in 0.4% Triton X-100) for 2 h at 37°C. After rinsing in PBS followed by normal saline, the tissue was mounted on gelatin/chrome alum-subbed glass slides, dehydrated through graded ethanol solutions, cleared in xylenes and mounted with DPX medium (British Drug Houses, BDH Laboratories Chemical Division, Poole, UK). The nuclei containing c-Fos fluoresced bright orange when viewed with a Cy3 excitation cube adapted for a Nikon Optiphot microscope (Fryer Company, Huntley, IL).

In a few cases, sections stained for PR were double-labeled for LHRH. In these instances, LHRH was localized as described above for c-Fos and LHRH.

2.6. Assessment of c-Fos expression in pePOA and LHRH neurons

A 1-in-6 series of sections (25 μm) was analyzed for each rat. We evaluated c-Fos immunoreactivity within LHRH neurons contained within the entire rostral caudal extent of the forebrain. c-Fos immunoreactivity in LHRH

neurons was expressed as the percentage of LHRH neurons expressing c-Fos. For pePOA neurons possessing c-Fos, we analyzed 5 levels of the pePOA from the initial opening of third ventricle, 150 μm caudal to the organum vasculosum of the lamina terminalis (OVLT), to 750 μm behind the OVLT, the region where the suprachiasmatic nucleus, SCN, reached its maximal width. The location of the OVLT was designated '0 μm'. Since earlier studies indicated that the principal zone of activation of the preoptic area at the time of the LH surge was restricted to a narrow strip of cells close to the third ventricle [7], we counted and plotted c-Fos-positive cells in the zone 80 μm from the ventricular surface at 100× using a 10×10 counting grid. We expressed the values as numbers of c-Fos-positive nuclei in the entire dorsal-to-ventral periventricular zone on both sides in each section. Analysis of PR and c-Fos was performed in a similar fashion.

2.7. LH radioimmunoassay

The radioimmunoassay we used for plasma LH has been published previously [1]. Plasma volumes of 20 μl were assayed for each animal. Standard curves were constructed using the rat LH RP-3 standard from the National Hormone and Pituitary Program of the NIDDK.

2.8. Statistical analysis

Analysis of variance (ANOVA) was used to evaluate whether the pePOA neurons were activated in synchrony with LHRH neurons following progesterone-facilitation and progesterone-inhibition of immature rats and for determination of the proportion of pePOA neurons that expressed both c-Fos and PRs. Differences between experimental groups were further evaluated using the Scheffe *F*-test. A confidence level of $P < 0.05$ was considered significant.

3. Results

3.1. Effects of progesterone on the LH surge and c-Fos activity in LHRH and pePOA neurons

As expected, animals administered progesterone concurrently with E_2 (progesterone-inhibition model) failed to

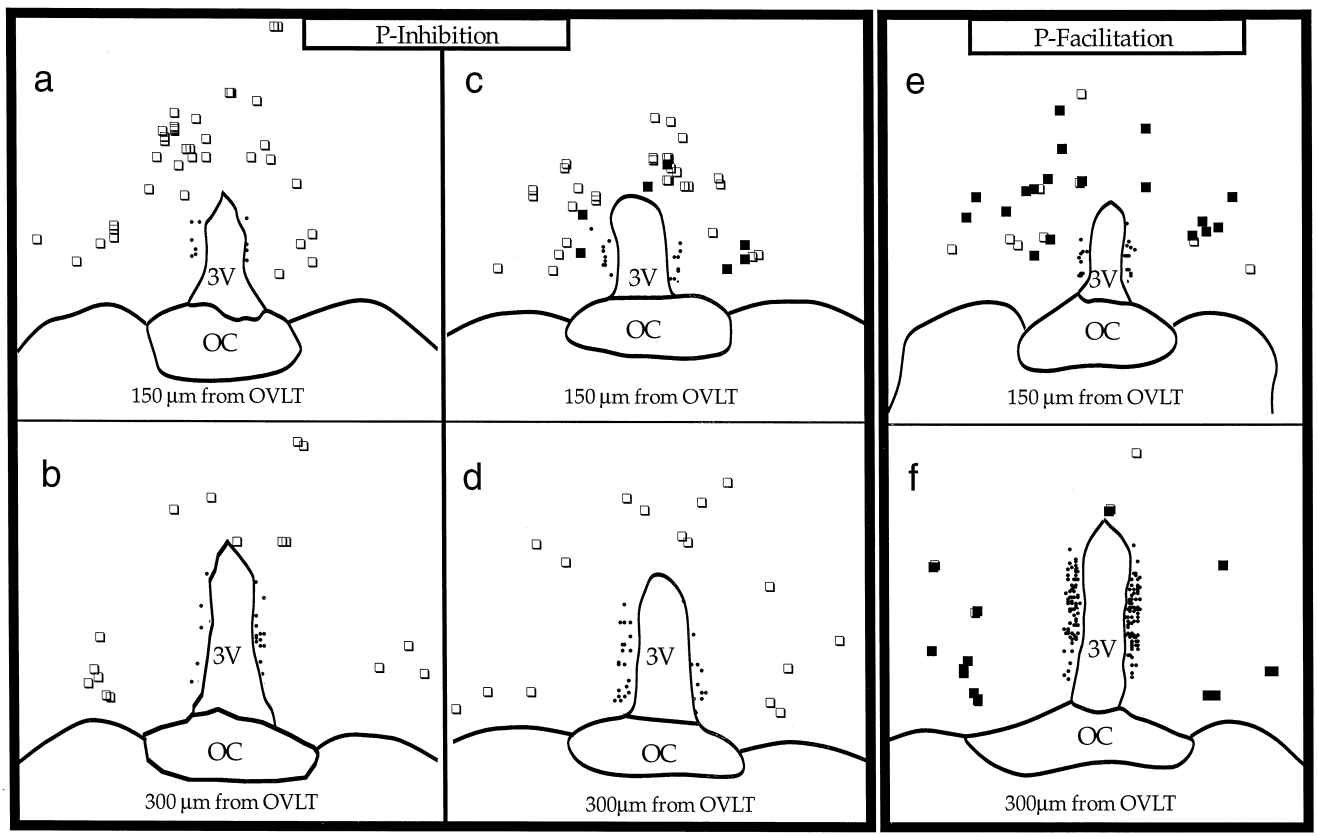

Fig. 1. Representative plots of LHRH and pePOA c-Fos-positive neurons in progesterone-inhibited rats (a–d) and progesterone-facilitated rats (e and f) at the levels 150 μm (a, c, and e) and 300 μm (b, d, and f) caudal to the OVLT where LHRH and pePOA neurons, respectively, reach their maximum numbers. Filled squares indicate LHRH neurons which expressed c-Fos; open squares indicate LHRH neurons devoid of c-Fos staining; small dots represent c-Fos-positive neurons of the pePOA (with unknown phenotypes). Note that c-Fos-positive nuclei other than those within LHRH neurons and those in the 80-μm wide pePOA area are not illustrated. AC, anterior commissure; 3V, third ventricle; OC, optic chiasm; VL, lateral ventricle.

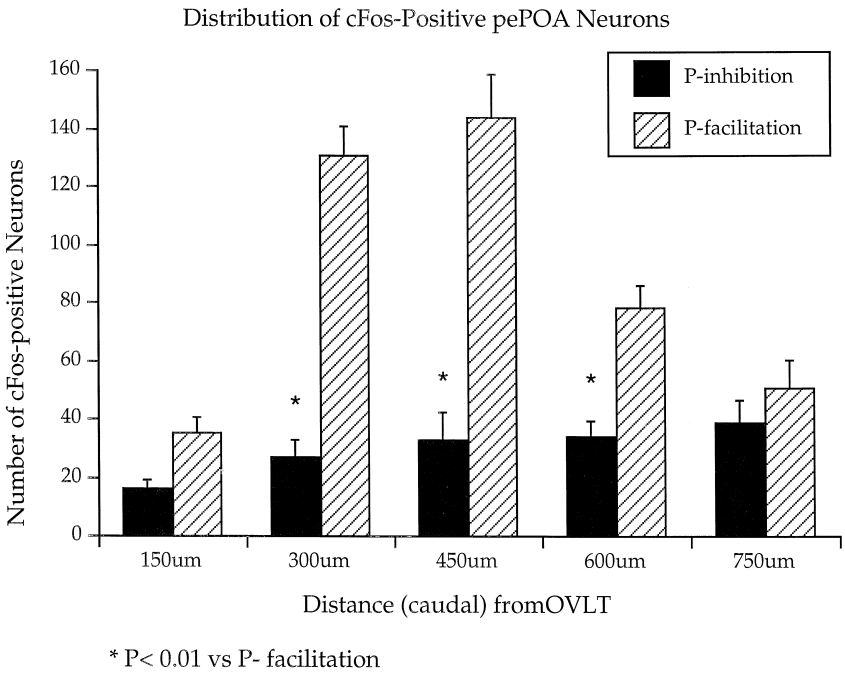

Fig. 2. Graph of the numbers of c-Fos-positive nuclei in pePOA neurons throughout the rostral–caudal extent of the preoptic area. Progesterone-inhibited rats (filled bars); progesterone-facilitated rats (striped bars). * $P < 0.01$.

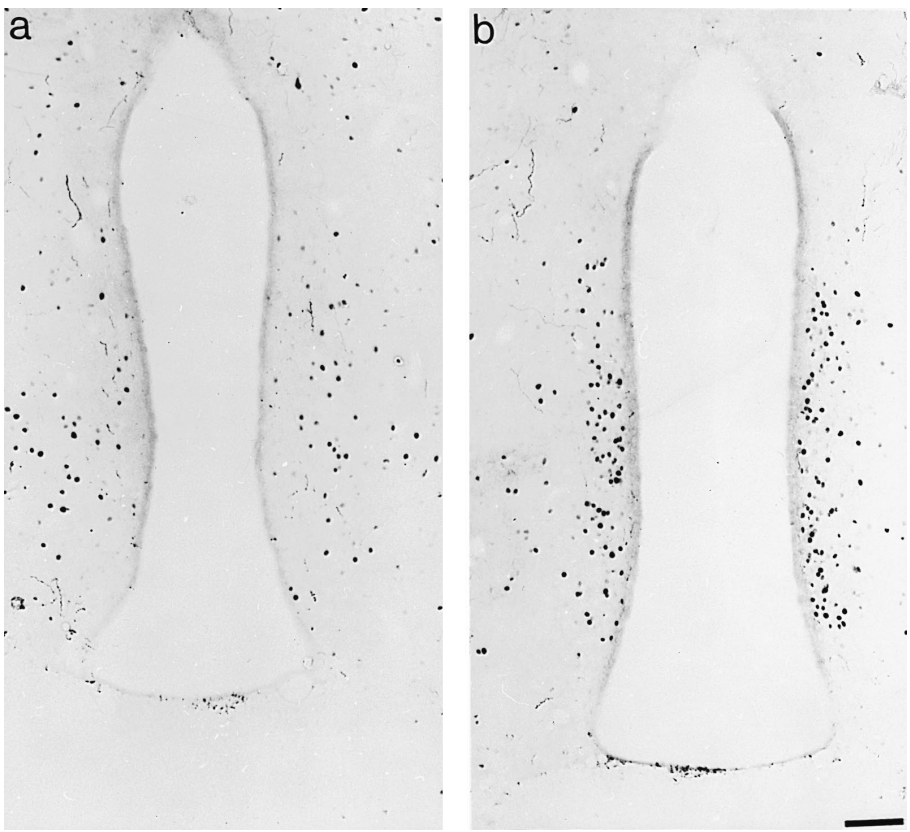

Fig. 3. Micrograph taken from the pePOA approximately 300 μm from the OVLT. a: progesterone-inhibition group; b: progesterone-facilitation group stained for c-Fos. Note the lack of significant c-Fos after progesterone inhibition. Bar = 100 μm.

exhibit an LH surge (plasma LH concentration 0.3 ± 0.1 ng/ml RP3), whereas progesterone administered after E_2 priming (progesterone-facilitation model) evoked a large surge in LH (10.39 ± 1.88 ng/ml; $P < 0.0001$), and these two groups of rats had very different patterns of c-Fos activation within LHRH and pePOA neurons. Fig. 1 illustrates the pattern of c-Fos activation in LHRH and pePOA neurons at two levels of the preoptic area (150 μm, and 300 μm caudal to the OVLT) in two animals treated with the progesterone-inhibition paradigm and in one rat treated for progesterone-facilitation. Activation of c-Fos within LHRH neurons was either completely absent (4 of 12 rats, Fig. 1a and b) or very low (under 8%, 8 of 12 rats, Fig. 1c and d) in the progesterone-inhibited rats (average, all animals, 2.6 ± 0.8%). In contrast, the progesterone facilitated rats showed marked LHRH activation (44.6 ± 2.4%, Fig. 1e and f). The pattern of pePOA activation, like that of LHRH neurons, was quite different in the two groups of animals. After progesterone inhibition, only a few neurons in the pePOA showed c-Fos staining at any rostral–caudal level. In the progesterone facilitation group of rats, a striking increase in the number of c-Fos-positive neurons was observed. With a more complete analysis of the rostral–caudal distribution of c-Fos-positive neurons in the pePOA, there were significantly greater numbers of neurons activated within the region 300–600 μm caudal to the OVLT (Fig. 2). Examples of the c-Fos staining in the pePOA of a progesterone-inhibited and progesterone-facilitated rats are shown in Fig. 3.

3.2. Expression of PR in pePOA neurons of the progesterone-facilitated and progesterone-inhibited rats

Examination of PR staining in progesterone-facilitated and progesterone-inhibited rats revealed the presence of pePOA neurons containing PR in both groups of rats. While quantitative measures were not made, the general patterns of staining of PR were quite similar (Fig. 4), although there was a slight tendency for PR staining to be weaker in the progesterone-inhibited rats.

3.3. Co-expression of c-Fos and PR in pePOA neurons (and lack of co-expression of PR in LHRH neurons)

Double labeling of pePOA neurons for both c-Fos and PR at the time of a progesterone-facilitated LH surge revealed 51 of 63 (80 ± 4.2%) c-Fos-positive pePOA neurons (per section) of the pePOA which also possessed PR (Fig. 5); in progesterone-inhibited rats, 5 of 13 (32 ± 12%) c-Fos-positive cells were PR-positive. The differences between these two conditions in terms of the percent of c-Fos-positive cells that were double-labeled was signifi-

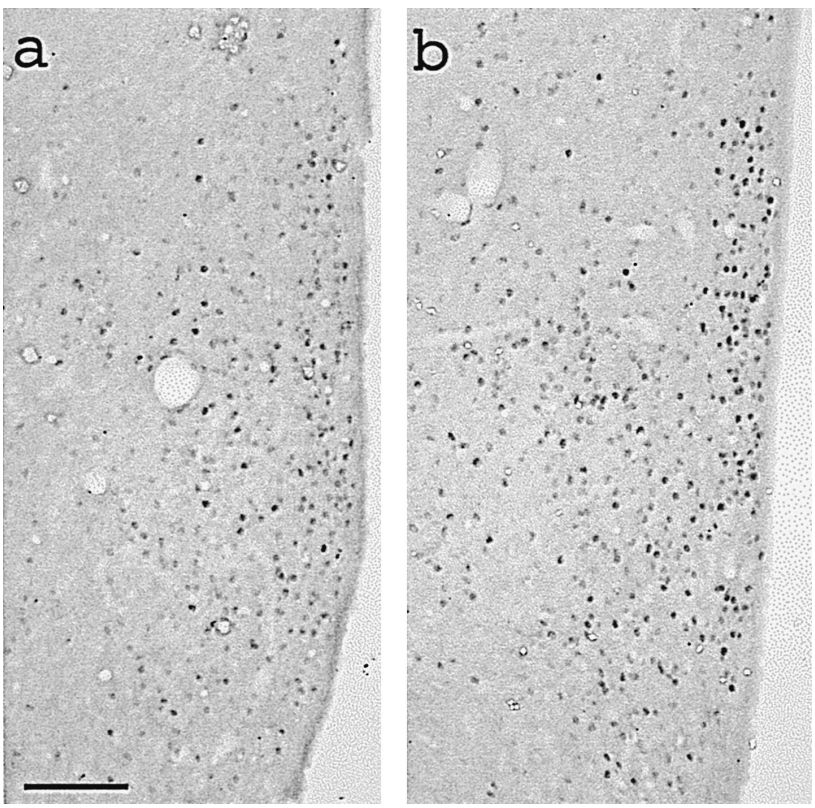

Fig. 4. Micrographs of sections of the pePOA stained for PR taken from a: a progesterone-inhibited rat, and b: a progesterone-facilitated rat. Note that after both treatments, progesterone receptors are abundant in the pePOA. In the progesterone-inhibited rat, however, the intensity of PR staining is slightly reduced. Bar = 100 μm.

cant ($P < 0.01$). Double labeling of PR and LHRH failed to reveal any double-labeled cells (data not shown).

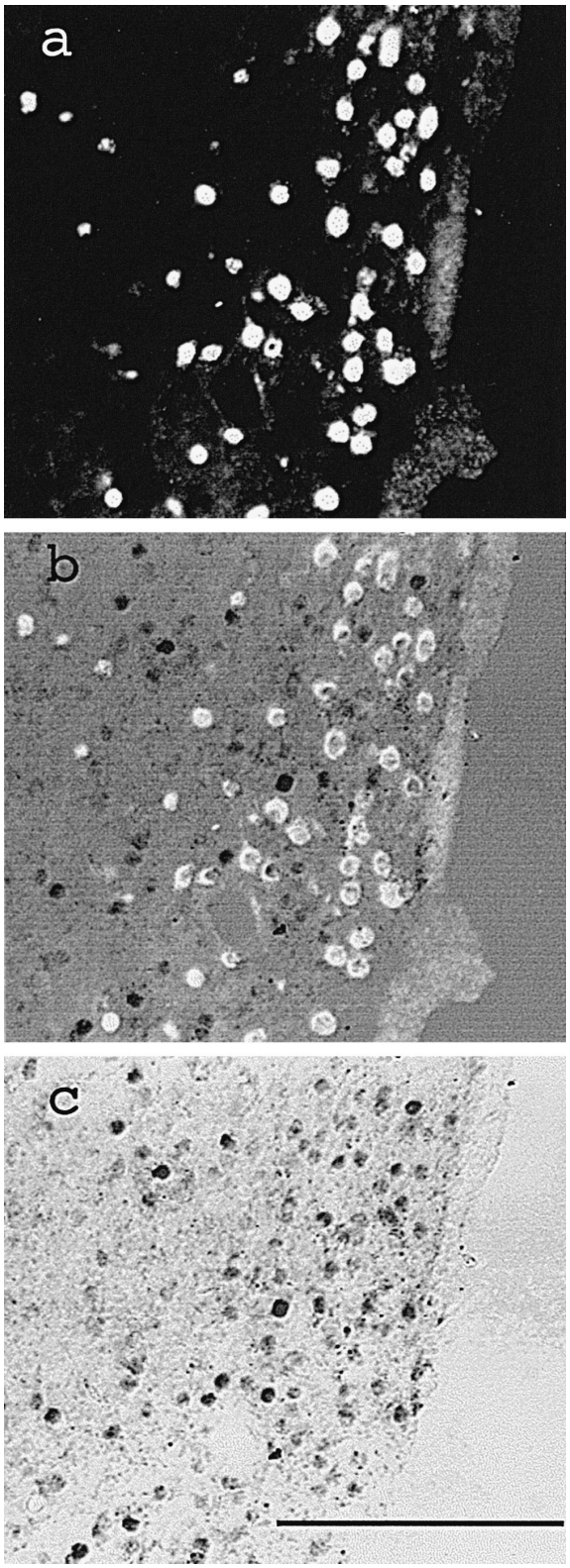

Fig. 5. Micrographs of a pePOA section from a progesterone-facilitated rat double-labeled for a: PR (black nuclei), and c: c-Fos (white nuclei). b: the superimposition of both images to emphasize cells that are double-labeled (white with gray centers). Bar = 100 μm.

4. Discussion

The effects of progesterone administered concurrently with E_2 ('progesterone inhibition') or following E_2 ('progesterone facilitation') on plasma LH values verified earlier results from our laboratory [1], demonstrating that the timing of progesterone administration with respect to E_2 treatment could block or increase the LH surge, respectively. Our subsequent studies in both immature and adult rats showed that one role of progesterone was to increase the strength of LHRH cell activation at the time of the LH surge [13,20].

The actions of progesterone in facilitating LH secretion were accompanied by not only activation of LHRH neurons, but also by clear activation of neurons of the pePOA. The pattern of activation of the pePOA in the immature rat closely resembles that seen in spontaneous cycling adult rats at the time of the preovulatory LH surge [6], with the greatest extent of activated neurons positioned in a narrow strip of cells within 80 μm of the ventricular surface 300–600 μm caudal to the OVLT. The only difference between the activation pattern in adults and immature animals is that the area of greatest activation in the immature animal is slightly more compressed, as might be anticipated from the smaller size of the brain in the immature rat. Studies in the adult using both anterograde and retrograde labeling reveal a direct projection from the pePOA to LHRH neurons [6]. Thus, pePOA activation may be at least partially responsible for stimulation of the LHRH neurons. Following the progesterone-inhibition paradigm, the pePOA neurons and LHRH neurons showed little activation suggesting that the pePOA was unable to activate LHRH neurons when progesterone was administered along with E_2.

Progesterone's actions on LHRH neurons could theoretically arise not only from afferent input but also from direct actions of the steroid. Earlier studies in the rat have ruled out direct genomic effects of progesterone on LHRH neurons, since the LHRH cells lack nuclear PRs [24]. Membrane effects are possible [10] but are not likely since progesterone levels need not remain elevated at the time the LH surge is evoked [9]. With no obvious mechanism for PR to affect directly LHRH neurons, an afferent to the LHRH system remains the more feasible target of progesterone's effects. The pePOA neurons, on the other hand, are likely targets for a genomic action of progesterone. Animals treated both for progesterone inhibition as well as progesterone facilitation contain a large numbers of progesterone receptor-containing neurons in the pePOA. The progesterone receptive neurons are the same cells that will express c-Fos at the time of a progesterone facilitated LH surge. Moreover, since the pePOA neurons provide affer-

ent input to the LHRH neurons [6], these observations suggest that the pePOA neurons transduce progesterone signals to the LHRH system, whether stimulatory or inhibitory.

It is noteworthy that preliminary examination of pePOA neurons activated at the time of an LH surge also reveals the presence of E_2 receptors in the activated neurons (Le, unpublished data). Since E_2 receptors are important regulators of the level of progesterone receptors, the co-expression of both E_2 and progesterone receptors in the pePOA neurons could explain the increased levels of progesterone receptor protein noted in the progesterone-facilitated rats as compared with the progesterone-inhibited rats.

Our data describing co-expression of PR and c-Fos indicated that PRs were present in some, but not all c-Fos-activated pePOA neurons in the progesterone-facilitated rats. It is quite possible that we underestimated the total degree of co-expression. High progesterone levels are associated with a decrease in staining intensity and gene expression of PR [8]. Thus we may have missed detecting some of the PR. In addition, we used nickel-DAB to reveal PR. This strategy, while producing clear staining of PR with nickel DAB in the pePOA, could quench the c-Fos fluorescence when PR staining is very intense, limiting detection to those PR cells that are in the low-to-middle intensity range. Thus, from our study we can definitively demonstrate that many of the activated pePOA neurons were PR-expressing, but we cannot rule out the possibility that all the c-Fos-positive neurons expressed PR.

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