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Progesterone treatment increases Fos-immunoreactivity within some progestin receptor-containing neurons in localized regions of female rat forebrain

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Abstract

In female rats, the sequential release of estradiol and progesterone from the ovaries is required for the expression of sexual behavior during the estrous cycle. Many of the neuronal effects of estradiol and progesterone involve estrogen and progestin receptors. Treatment with a behaviorally-effective dose of estradiol increases Fos expression, suggestive of neuronal response, and subsequent treatment with a behaviorally-effective dose of progesterone further increases Fos expression within a few hours in female rat brain. In order to determine if neurons that respond to progesterone with increased Fos expression also contain progestin receptors, we used a double-label immunofluorescent technique to label both progestin receptors and Fos protein following progesterone or vehicle treatment of estradiol-primed female rats. As shown previously, progesterone treatment increased Fos expression in progestin receptor-containing regions, such as the ventromedial nucleus of the hypothalamus and the medial preoptic area. In addition, progesterone treatment induced a statistically-significant increase in Fos-immunoreactivity within progestin receptor-containing cells in the medial preoptic area and the ventromedial nucleus of the hypothalamus, but not in the arcuate nucleus. Therefore, many but not all of the neurons that respond to progesterone with increased Fos expression also contain progestin receptor-immunoreactivity. The progesterone-induced Fos expression within progestin receptor-containing neurons may or may not be associated with the effects of progesterone on sexual or other reproductive behaviors, as it remains to be tested. However, the Fos expression provides a useful marker to aid in identification of neurons that respond to a behaviorally-relevant dose of progesterone.

Keywords: Progesterone; c-*fos*; Immediate early gene; Progestin receptor; Hypothalamus; Preoptic area

1. Introduction

During the estrous cycle of rats, the sequential release of estradiol and progesterone from the ovaries predicts the timing and occurrence of sexual behavior [12,37,42]. These behaviors can be abolished by ovariectomy and reinstated by sequential treatment with estradiol followed one or more days later by progesterone. Although estradiol treatment alone can induce sexual receptivity, sequential treatment with estradiol and progesterone allows lower doses to be used [50], makes the onset and termination of reproductive behavior more predictable, and increases solicitation behavior [17].

Many of the neuronal actions of steroid hormones on sexual behavior are believed to be mediated through intracellular steroid receptors [10], although some effects may be mediated by membrane receptors [21,34]. One mechanism by which intracellular steroid hormone receptors are activated is by a cognate ligand binding to the receptor. Binding of a ligand to steroid receptors results in a conformational change that allows the steroid–receptor complex to bind to a hormone response element located on DNA [28,46]. Once the steroid–receptor complex binds to DNA, it may regulate gene transcription, therefore protein synthesis, and ultimately neuronal function [13,46].

Some cellular outcomes of estradiol and/or progesterone treatment are changes in second messenger systems [19], neurotransmitter/peptide receptor levels [29,30,40], and neurotransmitter release [20]. Another outcome of estradiol treatment is the induction of progestin receptors

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[32] within estrogen receptor-containing cells [11]. Progestin receptors are found throughout the guinea pig and rat brain [7,32]. However, the highest concentrations of estradiol-induced progestin receptors are found within the preoptic area, arcuate nucleus, and the ventromedial hypothalamus [8,35].

The evidence suggests that neuronal progesterone receptors are involved in mediating the effects of progesterone on sexual behavior, as prevention of ligand binding to progestin receptors with a progesterone antagonist blocks the actions of progesterone on sexual behavior [10]. In addition, blocking the synthesis of progestin receptors with antisense oligonucleotides directed at progestin receptor mRNA inhibits progesterone facilitation of sex behavior [33].

Using Fos-immunocytochemistry, it is possible to identify cells that respond genomically to a stimulus. Fos, as other immediate early gene products (e.g. jun), is a transcription factor that may be expressed rapidly in response to cellular stimulation and may then regulate the expression of other proteins [16,41]. Fos-immunocytochemistry has been used to identify neurons that respond to physiologically-relevant stimuli, such as vaginal-cervical [43] and photic stimulation [15]. Recently, we [1] and others [14,26,27] have reported that treatment with estradiol increases Fos expression in the female rat brain. Treatment with a low or high dose of estradiol results in increased Fos expression in female rat brain within 3 to 96 h later, depending on anatomical regions [1,26,27]. In addition, subsequent treatment with a behaviorally-relevant dose of progesterone further increases Fos expression in estradiol-primed female rat brain within 1 to 5 h [1,23]. As progesterone increases Fos expression in the female rat brain [1,23], it is possible to determine the phenotype of progesterone-responsive neurons using a double-label immunocytochemical technique. Many of the progesterone-dependent effects on behavior require progestin receptors [10]; therefore, it is likely that some, but not all, progesterone-responsive neurons contain progestin receptors. In order to determine if neurons that respond to a behaviorally-effective dose of progesterone with increased Fos expression also contain progestin receptors, we used a double-label fluorescent technique to label both progestin receptors and Fos protein.

2. Materials and methods

2.1. Animals

Female Sprague–Dawley rats (200–250 g) obtained from Charles River Breeding Laboratories, Inc., (Wilmington, MA, USA) were group-housed for one week in a 14:10 h light:dark cycle. All rats were then ovariectomized under methohexital sodium anesthesia (52 mg/kg b.wt., Brevital, Eli Lilly and Co., Indianapolis, IN) prior to experiment.

2.2. Hormone treatment

One week following surgery, all rats were injected subcutaneously with 5 μg of estradiol benzoate (dissolved in 0.1 ml sesame oil) followed by either 500 μg of progesterone (dissolved in 0.1 ml sesame oil containing 5% benzyl alcohol and 15% benzyl benzoate, $n = 5$) or vehicle (0.1 ml sesame oil, $n = 5$) approximately 48 h later. Injections were given about 2 h after the lights went out. Each rat was perfused exactly 1 h after receiving the second injection, as this paradigm reliably induces Fos expression [1].

2.3. Perfusion

Animals were anesthetized with sodium pentobarbital (89 mg/kg) and chloral hydrate (425 mg/kg). The heart was then exposed, and the left ventricle was injected with 5000 U of sodium heparin dissolved in 1 ml of saline (0.15 M). Following an incision of the right atrium, a cannula was inserted through an incision of the left ventricle, so that its tip lay in the aorta. Seventy-five ml of saline preceded the flow of 250 ml of fixative (2% acrolein in 0.1 M sodium phosphate buffer; pH 7.2) through the cannula. Perfusion pressure was maintained at 100 mmHg with a flow rate of 25 ml/min for 10 min. After the brains were removed and blocked, they were placed into 0.1 M sodium phosphate buffer (pH 7.2) containing 20% sucrose overnight at 4°C. Forty μm sections from the preoptic area to the midbrain region were cut on a freezing microtome and placed into cryoprotectant [47] at -20°C until processed for immunocytochemical detection of both Fos protein and progestin receptors.

2.4. Immunofluorescence

For each animal, a set of one in four sections was removed from cryoprotectant and rinsed three times for 5 min each in Tris-buffered saline (TBS; pH 7.6). Sections were then pretreated in 1% sodium borohydride for ten min to remove residual aldehydes. Following pretreatment and three additional rinses in TBS for 5 min each, sections were placed into 1% H_2O_2, 20% normal goat serum, and 1% bovine serum albumin for 20 min to reduce nonspecific staining and endogenous peroxidase activity. Sections were then incubated in a cocktail of: (1) rabbit polyclonal c-*fos* antiserum directed against the N-terminal of the Fos protein (1 μg/ml; SC 52, Santa Cruz Biotechnology, Inc., Lot# H024, Santa Cruz, CA, USA) and (2) mouse monoclonal progestin receptor antibody directed against the hinge region of both A and B progestin receptor isoforms (3 μg/ml; H928, StressGen, Biotechnologies Corp., Lot# 1.0, Victoria, BC, Canada) diluted in modified TBS (pH

7.6, 4°C) containing 0.1% gelatin, 0.02% sodium azide, 0.5% Triton X-100 and 1% normal goat serum for 3 days at 4°C. Following three rinses with the above modified TBS buffer (pH 7.6) for 5 min each, the tissue sections

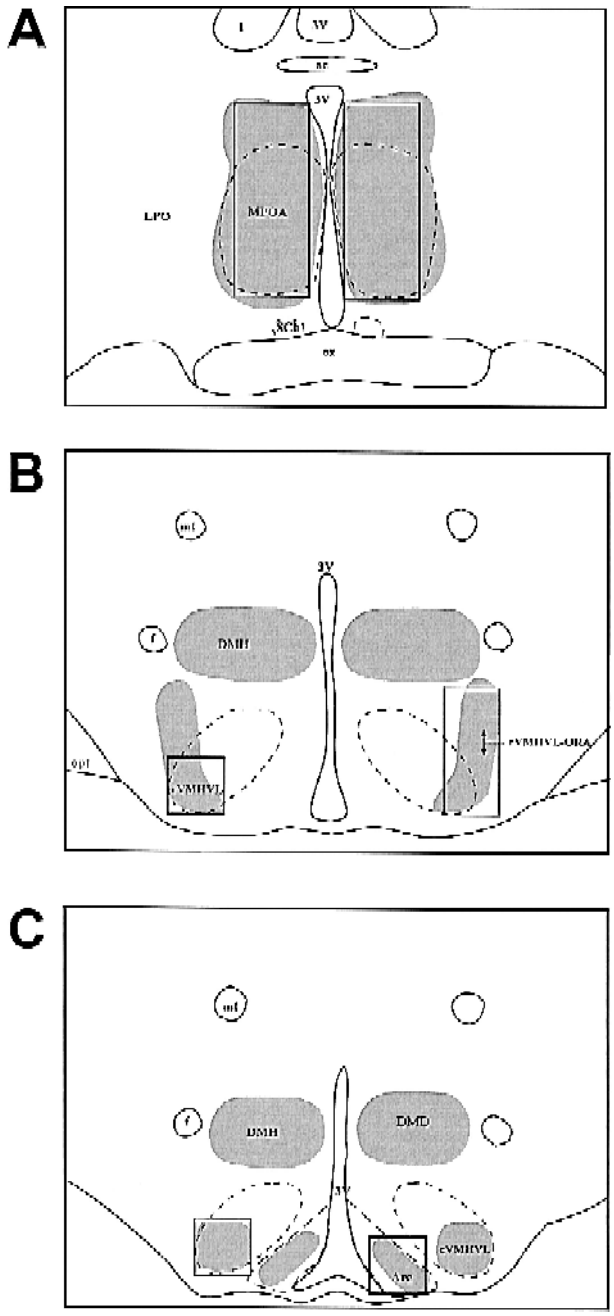

Fig. 1. Schematic drawings of areas in which Fos-IR cells were counted. Shaded regions represent distribution of Fos-IR and boxes indicate areas that were examined for Fos-IR and PR-IR colocalization. A: LPO, lateral preoptic area; MPOA, medial preoptic area; Sch, suprachiasmatic nucleus. B: DMH, dorsomedial hypothalamic nucleus; rVMHVL, rostral ventrolateral ventromedial nucleus of the hypothalamus; rVMHVL-ORA, ovarian steroid receptor-containing area associated with the rostral ventrolateral ventromedial nucleus of the hypothalamus. C: cVMHVL, caudal ventrolateral ventromedial nucleus of the hypothalamus; Arc, arcuate nucleus. f, fornix; ac, anterior commissure; ox, optic chiasm; mt, mammillothalamic tract; opt, optic tract; 3 V, third ventricle.

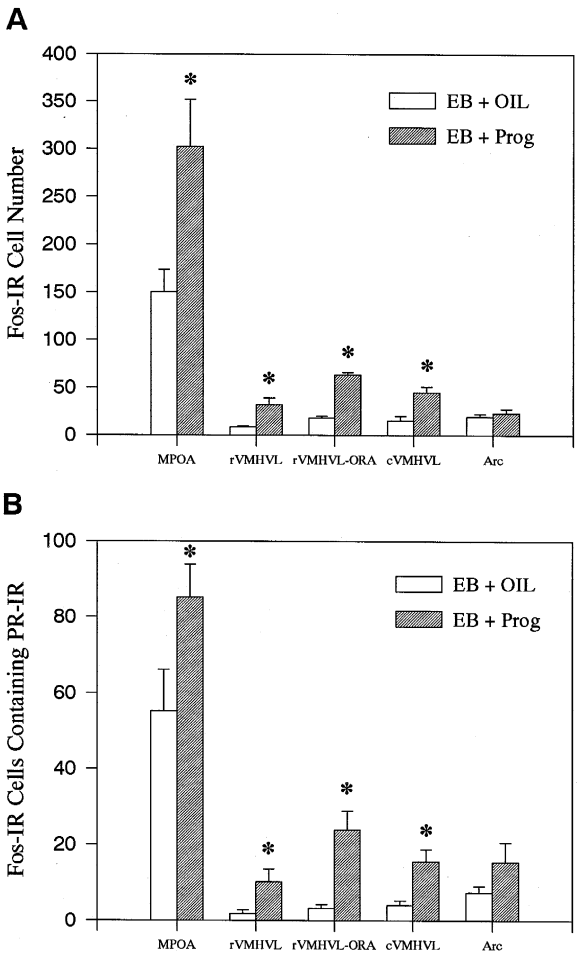

Fig. 2. A: mean number (±S.E.M.) of Fos-IR cells following treatment with estradiol benzoate and progesterone (EB+Prog) or estradiol benzoate and oil (EB+Oil). B: mean number (±S.E.M.) of Fos-IR cells containing PR-IR following EB+Prog or EB+Oil. Mean numbers were obtained bilaterally from one section per area from each rat ($n = 5$ per group).

were then incubated in a cocktail of fluorescently-labeled secondary antiserum: (1) Texas Red goat anti-rabbit serum (5 μg/ml; Jackson ImmunoResearch, Inc., West Grove, PA, USA) for detection of Fos-IR and 2) fluorescein isothiocyanate (FITC) goat anti-mouse serum (5 μg/ml; Jackson ImmunoResearch, Inc.) for detection of PR-IR diluted in the modified TBS (pH 7.6) with 1% normal goat serum for 90 min at room temperature. Following three additional rinses, sections were then placed in modified TBS (pH 7.6) containing (1) rabbit (5 μg/ml, Sigma Chemical, St. Louis, MO, USA) and (2) mouse (5 μg/ml, Sigma Chemical) immunoglobulins with 1% normal goat serum for 90 min at room temperature. Sections were then rinsed three times for 5 min each in modified TBS (pH 7.6) and placed back into the fluorescently-labeled secondary antiserum for 60 min. After bridging of the fluorescently-labeled secondary antiserum, sections were rinsed three times for 5 min each in TBS (pH 7.6) and mounted onto glass slides with 5% *n*-propyl gallate in glycerol to

reduce fading of the immunofluorescence [22]. Immunocytochemical controls consisted of omission of either the Fos or H928 antibody in the immunocytochemical procedure.

2.5. Data analysis

Sections were analyzed using a Nikon Optiphot-2 fluorescent microscope fitted with a G-1B filter module (narrow band green range, 541–551) to visualize Texas Red labeled Fos-IR and a B-2 E filter (broad band blue range, 450–490) to visualize FITC labeled PR-IR. The sections were mounted onto glass slides immediately following immunocytochemistry and counted manually at 200× power. One section per area from each rat was counted with the experimenter blind to the treatment group, and the total number of cells obtained bilaterally was analyzed using SigmaStat Statistical Analysis System 1.01 (Jandel Scientific, Corta Madera, CA, USA). Two-tailed *t*-tests were used to analyze data in all areas except the MPOA in which a one-tailed *t*-test was used. As previous research from our laboratory [1] and another [23] show progesterone treatment increases Fos expression in estradiol-primed female rat brain, we predicted direction of Fos-immunoreactive cell number following progesterone treatment. Data were considered statistically significant at a probability level of less than 0.05.

2.6. Neuroanatomical areas analyzed

Representative sections of areas containing PR-IR such as the medial preoptic area (MPOA), arcuate nucleus (Arc), and a rostral and caudal of the ventromedial hypothalamus (VMH), were matched using the rat brain atlas of Paxinos and Watson (Ref. [36]; Fig. 1A–C). In the rostral section, the VMH/PR-IR rich area was analyzed on the bases of the nissl defined cell cluster, the ventrolateral ventromedial nucleus of the hypothalamus (rVMHVL), as well as the ovarian progestin receptor-containing area associated with the ventrolateral ventromedial nucleus of the hypothalamus that extends dorsally (rVMHVL-ORA; Fig. 1B). In the caudal section, only the ventrolateral ventromedial nucleus of the hypothalamus (cVMHVL) was analyzed, as the PR-IR cells are confined to this region (Fig. 1C).

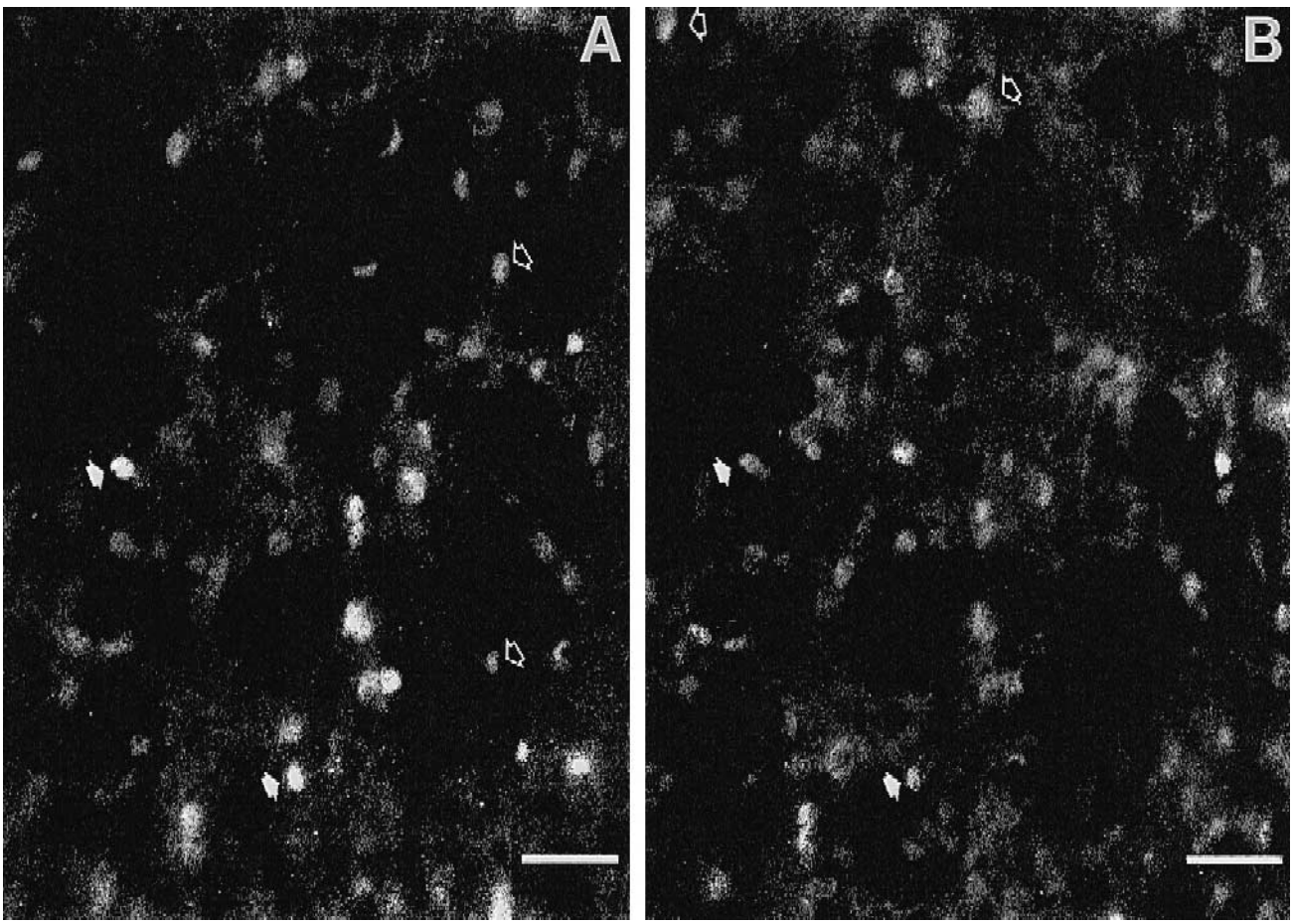

Fig. 3. Photomicrographs of Fos-IR cells (A) and PR-IR cells (B) in the same field within the MPOA. Solid white arrows point to examples of cells containing both Fos-IR (A) and PR-IR (B). Open arrows point to examples of cells containing only Fos-IR (A) or PR-IR (B). Scale bar = 50 μm.

3. Results

As reported previously [1,23], progesterone increased Fos-IR within the MPOA and in two levels of the VMH ($P < 0.05$; Fig. 2A). However, progesterone did not affect the number of cells expressing Fos-IR within the Arc. With the immunofluorescent technique described, PR-IR was observed only within the MPOA, Arc, and the VMH. While a small number of PR-IR cells was observed within the posterodorsal medial amygdala in sections immunostained using a diaminobenzidine technique, no immunostaining of PR-IR was observed using the less sensitive immunofluorescent technique. Thus, only the MPOA, the Arc, and the VMH were examined for colocalization of Fos-IR and PR-IR (Fig. 1A–C).

In the MPOA, there was a statistically significant increase in the number of Fos-IR cells containing PR-IR ($P < 0.05$, Fig. 2B and Fig. 3). Progesterone also increased the number of Fos-IR cells containing PR-IR in the rVMHVL ($P < 0.05$, Fig. 2B). In the rVMHVL-ORA, which includes the PR-IR containing area that begins within the rVMHVL and extends dorsally toward the fornix (Fig. 1B), progesterone induced a dramatic 7.4-fold increase in the number of Fos-IR cells colocalized with PR-IR ($P < 0.005$, Fig. 2B). In addition, progesterone increased the number of Fos-IR cells containing PR-IR within the cVMHVL ($P < 0.01$, Fig. 2B). However, progesterone did not increase the number of Fos-IR cells, nor did it increase the number of Fos-IR cells containing PR-IR within the Arc ($P > 0.05$, Fig. 2B).

4. Discussion

As reported previously [1,23], a behaviorally-effective progesterone treatment in estradiol-primed female rats increased the number of Fos-IR neurons within the MPOA and in two levels of the VMH (Fig. 2A). However, the progesterone treatment did not increase the number of Fos-IR neurons within the Arc. Due to lower sensitivity of the fluorescent technique, PR-IR was observed only within the MPOA, Arc, and the VMH, while tissue sections that were reacted with diaminobenzidine exhibited some PR-IR cells in the posterodorsal medial amygdala. Thus, only the MPOA, Arc, and two levels of the VMH were examined for colocalization of progestin receptors and Fos protein (Fig. 1A–C). It should be noted that, as the immunofluorescent technique for identifying PR-IR and Fos-IR neurons was not as sensitive as the diaminobenzidine technique, the percentages of Fos-IR neurons containing PR-IR are likely to be underestimates. However, the results do suggest that many neurons which respond to progesterone also contain progestin receptors.

In the MPOA, progesterone increased the total number of Fos-IR neurons (Fig. 2A). In addition, there was an increase in the number of Fos-IR neurons containing PR-IR in the MPOA after progesterone treatment (Fig. 2B and Fig. 3). Interestingly, 28% of the cells that expressed Fos-IR after progesterone treatment contained PR-IR, whereas 37% of the cells that expressed Fos-IR after estradiol treatment alone contained PR-IR. The observation that there is a greater percentage of Fos-IR neurons in the MPOA containing PR-IR before progesterone treatment suggests that progesterone-induces Fos-IR within a population of cells in that area that either lack progestin receptors or have insufficient levels of progestin receptors to be detectable by the immunofluorescent technique.

Progesterone treatment also increased Fos expression within PR-IR neurons in the VMH. We analyzed two rostral-caudal levels of the VMH, as the distribution of PR-IR neurons differs between these two levels. In the rVMHVL, progesterone also increased the number of Fos-IR cells containing PR-IR (Fig. 2B). The percent of Fos-IR neurons containing PR-IR also increased from 21% in EB + Oil controls to 32% in EB + P-treated animals in the rVMHVL. However, the PR-IR neurons within the rVMHVL are not confined to this nucleus and extend dorsally toward the fornix. We have referred to this area as the ovarian progestin receptor-containing area associated with the rVMHVL (rVMHVL-ORA; Fig. 1B). Within this neuroanatomical area defined on the basis of progestin receptor-containing neurons, progesterone increased the percentage of Fos-IR neurons containing PR-IR from 17% in controls receiving EB + Oil to 37% in animals receiving EB + P. While this is only a moderate increase in percentage, it represents a dramatic 7.4-fold increase in the actual number of Fos-IR neurons containing PR-IR (Fig. 2B). Progesterone also increased the number of Fos-IR neurons expressing PR-IR in a caudal section of the VMH. The percent of Fos-IR neurons containing PR-IR within the cVMHVL increased from 27% in EB + Oil controls to 35% in EB + P animals, a 3.9-fold increase in the number of Fos-IR neurons containing progestin receptors (Fig. 2B). At this level of the VMH, the PR-IR neurons are confined to the cVMHVL. In the Arc, progesterone did not increase the total number of neurons expressing Fos-IR or the number of Fos-IR neurons containing PR-IR (Fig. 2A,B). Thus, under the conditions used in this experiment, systemic treatment with progesterone after estradiol-priming induces Fos-expression in progestin-receptor containing neurons located within the MPOA and the VMH, but not in PR-IR neurons located within the Arc. The percentages of colocalization are not absolute, especially since the fluorescent technique is not as sensitive as the diaminobenzidine technique. Rather they suggest that some neurons which respond to progesterone with increased Fos expression also contain progestin receptors.

The increase of Fos expression within PR-IR neurons following progesterone treatment may be an indirect effect of progesterone, such as through synaptic connections [49], or direct, as this study shows that Fos protein and progestin receptors can be found within the same cell. Indeed,

estrogen response elements are known to exist on the 5′-flanking sequence [25] and 3′-flanking sequence [24] of the mouse c-*fos* oncogene. Alternatively, some effects of ovarian steroids on Fos expression could be mediated by membrane receptors for progesterone [21]. It is also possible that progesterone treatment increases Fos expression via direct and indirect pathways. This is illustrated by the recent finding that progesterone implants in the VMH increase Fos expression in the MPOA of estradiol-primed female rats [44]. Thus, it appears that progesterone treatment may increase Fos-IR directly, suggested by the colocalization of Fos protein and progestin receptors within the same cell, and indirectly at projection sites of progesterone-sensitive neurons. It is possible, but remains to be tested, that the increased Fos expression following progesterone treatment may be associated with the effects of progesterone on reproductive behavior. Alternatively, the progesterone-induced Fos expression within PR-IR neurons may not be involved in progesterone-facilitation of sexual behavior; rather, it may mediate other progestin receptor-dependent processes. However, the increased Fos expression within PR-IR neurons does suggest that they respond genomically to a behaviorally-effective dose of progesterone.

One pathway by which progesterone treatment may increase Fos-IR indirectly in PR-IR neurons is by modulating catecholamine transmission. For example, noradrenergic transmission in the hypothalamus, which is increased by steroid hormone treatment [20,45], has been shown to induce Fos expression in the rat brain [2–4]. Indeed, noradrenergic neurons have been found to project to areas in which we observed progesterone-induced Fos-IR [38]. Noradrenergic neurons are important in modulating gonadotropin-releasing hormone activity [31], steroid receptors [5,6,9], and reproductive behaviors [18]. Thus, it is possible that some of the Fos expression induced by progesterone may result from increased activity of the noradrenergic system in female rat brain.

In summary, treatment with a behaviorally-effective dose of progesterone in estradiol-primed female rats increases Fos expression within the MPOA and the VMH. Many of those progesterone-responsive neurons also contain progestin-receptors. Progesterone treatment could increase Fos-IR directly in some progestin receptor-containing neurons by binding to a steroid response element on the c-*fos* gene [24,25,39,48,49]. Alternatively, progesterone treatment may increase Fos expression indirectly, for example, through the noradrenergic system [2–4,27]. The steroid hormone-induced Fos protein may then regulate the expression of other gene products [16,41] involved in reproductive behaviors.

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