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Response of ERα-IR and ERβ-IR cells in the forebrain of female rats to mating stimuli

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Abstract

Sexual behavior in female rats depends on the action of estradiol on estrogen receptors (ERs) found in particular brain regions. While hormonal regulation of female sexual behavior requires ERα, the possible functions of ERβ remain to be clarified. Mating stimulation has several behavioral and physiological consequences and induces Fos expression in many brain areas involved in the regulation of reproductive behavior and physiology. In addition, some cells in which mating induces Fos expression coexpress ERα. To determine whether cells in which Fos is induced by a particular mating stimulus coexpress ERα, ERβ, or both, we used a triple-label immunofluorescent technique to visualize ERα-, ERβ-, and mating-induced Fos-immunoreactivity (Fos-ir) in neurons in which mating stimulation reliably increases Fos expression. Ovariectomized, hormone-primed rats were either unmated, received 15 mounts, or received 15 intromissions. In the rostral medial preoptic area, Fos-ir was induced by mounts alone primarily in cells coexpressing ERα-ir, while Fos-ir was induced by intromissions mainly in cells coexpressing both ERα-ir and ERβ-ir (ERα/ERβ-ir). In the dorsal part of the posterodorsal medial amygdala, Fos-ir was induced by intromissions in cells coexpressing ERα-ir and ERα/ERβ-ir. However, in the ventral part of the posterodorsal medial amygdala, Fos-ir was induced by intromissions primarily in cells coexpressing only ERβ-ir. These data suggest that qualitatively different sexual stimuli may be integrated through distinct ER-containing circuits in the rostral medial preoptic area and posterodorsal medial amygdala. The diversity in coexpression of type of ER in cells in different brain areas after various mating stimuli suggests a role for both ERα and ERβ in the integration of hormonal information and information related to mating stimuli.

Keywords: c-Fos; Fos; Medial amygdala; Preoptic area; Mating stimulation; Sexual behavior; Intromissions; Vaginocervical stimulation; ERα; ERβ; Triple-label immunofluorescence

In female rats, hormonal induction of sexual receptivity is dependent on the binding of estradiol to intracellular receptors in the brain (Blaustein and Erskine, 2002; Blaustein and Olster, 1989). Two forms of estrogen receptors (ERs), ERα and ERβ, have been found in various brain regions of female rats (Kuiper et al., 1997; Shughrue, et al. 1997). Both ERs share high homology of sequences and equal affinity for estradiol, and both show overlapping distributions in female rat brain (Shughrue et al., 1997; Shughrue and Merchenthaler, 2001; Shughrue et al. 1998). The greatest concentrations of both ERα and ERβ are found in the rostral medial preoptic area (rMPO), the bed nucleus of the stria terminalis (BNST), and the posterodorsal medial amygdala (MEApd). ERα-ir and ERβ-ir are coexpressed in populations of cells in these brain regions of female rats (Greco et al., 2001; Shughrue et al., 1998).

Although both forms of ER are present in the female rat and mouse brain, work with ER gene-disrupted strains of mice suggests that estrogenic stimulation of rodent sexual receptivity is dependent on the binding of estradiol to ERα. ERα-knockout female mice (ERαKO, recently renamed ERα-Neo KO; Pendaries et al., 2002) do not display sexually receptive behaviors in response to administration of estradiol or estradiol and progesterone. While the essential role of ERα is well understood, the role of ERβ in female rodent sexual behavior has not yet been clarified. ERβ gene-disrupted female mice (ERβ-knockout; ERβKO) dis-

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play typical sexual behavior and can successfully reproduce (Ogawa et al., 1999). However, these mice show a longer period of sexual receptivity during the estrous cycle contrasted with wild-type mice (Ogawa et al., 1999), suggesting that ERβ may play a more subtle role than ERα in the regulation of sexual receptivity.

During mating, female rats receive many types of sensory information (somatosensory, olfactory, and auditory) from males. Somatosensory stimulation received during mating, especially vaginal–cervical stimulation (VCS) received during intromissions and ejaculations, is though to be particularly important for inducing changes in sexual behavior (Bennett et al., 2001; Erskine, 1985; Yang and Clemens, 1997) and reproductive functioning (Blaustein and Greco, 2002; Erskine, 1995; Gunnet and Freeman, 1983). The effects of mating are dependent on the quality of the stimulus, such that VCS associated with intromissions and ejaculations has a greater effect on mating induced behavioral and endocrine changes than mounts in the absence of intromissions (Bennett et al., 2001; Coopersmith et al., 1996b; Reading and Blaustein, 1984).

Mating stimuli have effects on immediate-early gene expression that are also dependent on the particular qualities of the stimulus. Mating-induced changes in expression of Fos-immunoreactivity (Fos-ir) are reliably observed in brain regions such as the rMPO, MEApd, BNST, and the ventromedial hypothalamus (VMH) (Coolen et al., 1996; Erskine, 1993; Erskine and Hanrahan, 1997; Flanagan-Cato and McEwen, 1995; Pfaus et al., 1993; Polston and Erskine, 1995; Rowe and Erskine, 1993; Tetel et al., 1993, 1994; Wersinger et al., 1993). In these brain regions, more Fos-ir cells are observed in rats following intromissions and ejaculations as contrasted with rats receiving mounts alone or no stimulation, suggesting that VCS is crucial for Fos induction following mating.

Many Fos-ir cells in the rMPO, BNST, MEApd, and VMH coexpress ERα-ir (Tetel et al., 1994), suggesting that hormonal and sensory information important for female reproduction may be integrated within populations of forebrain ER neurons. However, it has yet to be determined whether cells responsive to mating stimuli also contain ERβ-ir, which could suggest that ERα- and ERβ-expressing neurons may both play a role in the integration of sensory stimulation received during mating.

In the present study, we used a triple-label immunofluorescent technique to determine whether ERα and/or ERβ are expressed in cells that respond to particular types of mating stimulation. We examined brains of female rats that were placed in the testing arena without males, that received mounts without intromissions, or that received mounts with intromissions. The neuroanatomical areas that were analyzed were chosen based on their response to mating stimulation and the abundance of ERα- and ERβ-immunoreactive cells.

Materials and methods

Animals

Female Sprague–Dawley rats (175–200 g; Charles River Breeding Laboratories, Wilmington, MA) were grouped-housed for 1 week in temperature-controlled rooms, on a 14:10 light:dark cycle with food and water available ad libitum. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts–Amherst. All rats were ovariectomized after *ip* injections of a cocktail of xylazine (5 mg/kg), ketamine (26 mg/kg), and acepromazine (0.9 mg/kg). One week later, they (n = 18) were injected *sc* with 5 μg of estradiol benzoate, followed 44 h later with 500 μg of progesterone. They were tested 4 h after progesterone injections.

Sexual behavior

Female rats were placed into one of three treatment groups (n = 6 to 7 in each). One group was unmated (Unmated group); one group received 15 mounts without intromission (Mounts group); and one group received 15 intromissions and an uncontrolled number of mounts (Intromissions + Mounts group). Females receiving intromissions also received ejaculations, which were scored as intromissions. During testing, animals from each group were placed concurrently in separate testing arenas (apparatus described by Erskine (1989)). Although the males were removed from the arena as each animal reached its criterion (i.e., either 15 mounts or 15 intromissions with mounts), the three rats remained in their arenas until testing of the three animals was completed. The vaginas of the rats in the Mounts group were covered with cloth tape during testing to prevent intromissions, as we and others have done previously (Bennett et al., 2001; Tetel et al., 1993). Lordosis responses were scored on a 4-point scale (0–3; Hardy and Debold, 1971), and the percentage of receptive responses (lordosis quotient = (number of lordosis responses/number of mounts) × 100) was recorded.

Perfusions

One hour after initiation of behavioral testing, animals received a lethal dose of sodium pentobarbital (89 mg/kg) + chloral hydrate (425 mg/kg) ip, and they were perfused with 0.9% physiological saline (25 ml) for 1 min followed by 4% paraformaldehyde (25 ml/min) for 10 min. The brains were removed from the cranium, and they were placed into 0.1 M sodium phosphate buffer (pH 7.2) containing 20% sucrose for 48 h. Thirty-five-micrometer sections from the preoptic area to the midbrain region were cut on a freezing microtome, and the sections were placed into a cryoprotectant solution.

Triple-label immunofluorescence for ERβ-ir, ERα-ir, and Fos-ir

For all animals, brain sections containing the preoptic area and the medial amygdala were selected. They were removed from cryoprotectant and rinsed three times for 5 min each in Tris-buffered saline (0.05 M TBS, pH 7.6). Sections were placed into 1 % H_2O_2, 20 % normal donkey serum, and 1 % bovine serum albumin for 20 min. Sections were then incubated for 2 days at 4°C in a solution containing the rabbit polyclonal anti-rat ERα antiserum, C1355 (1:30,000; gift from M. Shupnik, University of Virginia) raised against the last 15 amino acid C-terminal sequence of the rat ERα, the sheep polyclonal fos antiserum (1:200; Genosys) raised from a conserved region of the mouse and human Fos protein, and the mouse monoclonal ERβ antiserum, (2 μg/ml, hERβNT-221.3; Ligand Pharmaceuticals, San Diego, CA) raised against a synthetic peptide corresponding to the 14 amino acid N-terminal sequence of the human ERβ (1-485 form), in a buffer containing TBS, 0.1% gelatin, 0.02% sodium azide, 0.5% Triton X-100, and 1% normal donkey serum (TBS-gel). Following three washes in TBS-gel buffer, the sections were incubated in a solution containing the coumarin-conjugated donkey anti-rabbit secondary antiserum for ERα-ir (10 μg/ml; Jackson Immunoresearch, West Grove, PA) and the fluorescein-conjugated donkey anti-sheep secondary antiserum for Fos-ir (10 μg/ml; Jackson Immunoresearch) for 90 min at room temperature. The sections were then rinsed three times for 5 min each in TBS-gel and incubated in a solution containing the sheep IgG (1:2000, Jackson Immunoresearch) and the rabbit IgG (1:2000, Vector Laboratories, Burlingame, CA) for 60 min at room temperature using a multiple-bridge technique to intensify the immunofluorescent signal for ERα and Fos (Blaustein and Turcotte, 1989). The sections were again rinsed three times for 5 min each in TBS-gel and incubated in a solution containing the fluorescein donkey anti-sheep (10 μg/ml), the coumarin donkey anti-rabbit (10 μg/ml), and the cyanine 3-conjugated donkey anti-mouse (for ERβ-ir; 12 μg/ml, Jackson Immunoresearch) secondary antisera for another 90 min at room temperature. After three final rinses for 5 min each in TBS, the sections were mounted onto slides, air-dried, and coverslipped with Vectashield mounting medium (Vector Laboratories).

To control for nonspecific immunofluorescent staining, cross-immunoreactivity, and "bleed through" of the fluorochromes, other sections were incubated either in solutions in which the primary antibodies were omitted but the secondary antibodies were present or in solutions in which only one primary antibody was present and the three secondary antibodies and the IgGs were present. No nonspecific immunofluorescent staining, cross-immunostaining, or bleed through was observed.

Data analysis

Quantification of triple-labeled immunofluorescent cells

Using the NIH Image computer analysis system (developed at the National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/), digitized pictures of the same microscopic field were captured with three different band-pass filters, specific for fluorescein (A:492; E:520), coumarin (A:350; E:450), and cyanine 3 (A:550; E:570). The images were superimposed, and the number of single-, double-, or triple-labeled cells was counted by eye in the rMPO and in the dorsal and ventral parts of the posterodorsal nucleus of the medial amygdala (dMEApd and vMEApd, respectively; Fig. 1). Because the rMPO and MEApd have mostly been previously reported to express different levels of Fos-ir depending the type of mating stimulation, we chose to study these two particular brain areas. Brain areas were selected based on the atlas of Swanson: rMPO: −0.46 from bregma; MEApd: −2.85 from bregma (Swanson, 1998). The distinction between the dMEApd and the vMEApd was based on the characteristic presence of the ERβ-ir cluster of neurons in the vMEApd. The quantification was performed with a 20× objective (surface area photographed and quantified: 450 μm × 340 μm). To avoid the problem of overlapping cells that may arise from superimposed images, pictures were taken within the same focal plan and only cells in which an immunoreactive cell nucleus was clearly distinguishable were counted. This approach induces an overall underestimation of cell counts for each immunoreactivity.

Statistical analysis

One-way ANOVA was performed to determine statistical differences in means of single double-, and triple-labeled cells between groups. Statistically significant results, at $p \leq 0.05$, were followed by a *post hoc* Bonferroni corrected paired *t* test.

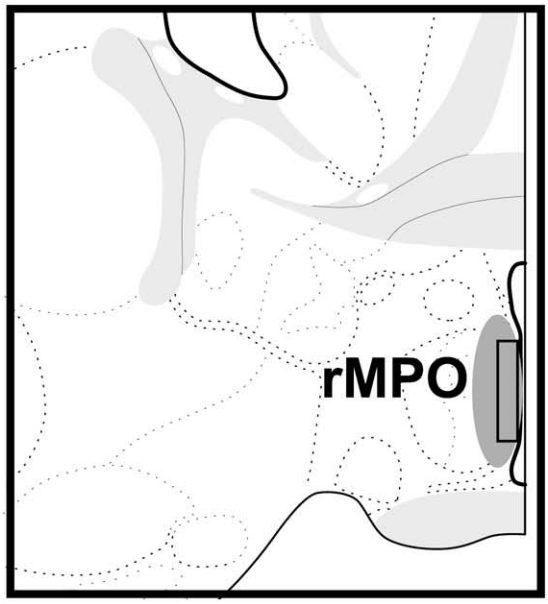
Results

Sexual behavior

All females in the Mounts and Intromissions + Mounts groups displayed maximal levels of sexual receptivity (mean LQ = 100 ± 0), so there were no group differences in levels of sexual receptivity. Sexual receptivity was not assessed in the Unmated group. In the Intromissions + Mounts group, females received 15 intromissions and a mean of 10 ± 1.8 mounts. The maximum duration of the behavioral test was on average 15 min to reach 15 intromissions.

Distribution of ERα-ir, ERβ-ir and Fos-ir

The distribution of ERα-ir was consistent with previous reports (Mufson et al., 1999; Sar and Parikh, 1986). ERα-ir was present in neurons of the periventricular zone of the preoptic area, the rMPO, the preoptic area, the dorsal and ventral BNST, the anterior, lateral, and ventral portions of the



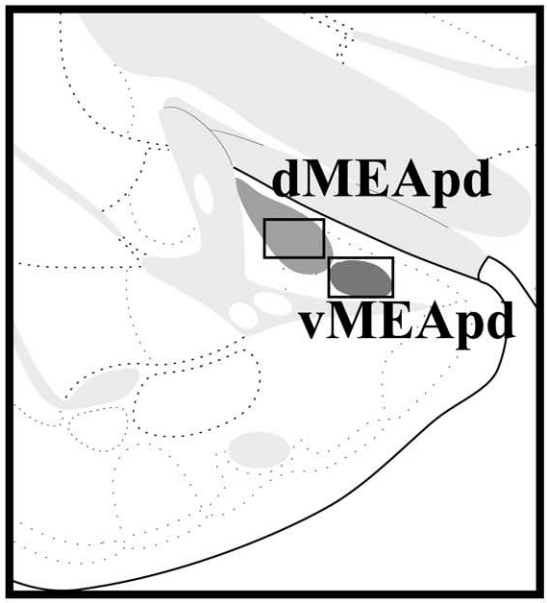


Fig. 1. Schematic figure showing neuroanatomical areas analyzed: the rostral part of the preoptic area (rMPO) and the dorsal (dMEApd) and ventral (vMEApd) parts of the posterodorsal medial amygdala. The boxes indicate areas of quantification. Adapted from Swanson (1998).

hypothalamus, the central, cortical, and medial amygdala, the arcuate nucleus, and, to a lesser extent, the hippocampus. In the MEApd, ERα-ir cells were evenly distributed in the dorsal part of the MEApd (dMEApd; Fig. 2) but in the ventral part of the MEApd (vMEApd) fewer ERα-ir neurons were present (Fig. 2). The number of ERα-ir cells in the rMPO and the MEApd did not differ among treatment groups (Table 1).

The distribution of ERβ-ir was also consistent with previous reports for ERβ-mRNA distribution (Shughrue et al., 1997) and protein (Greco et al., 2001; Li et al. 1997; Shughrue and Merchenthaler, 2001). Strongly labeled ERβ cells were found in neurons of the anteroventral periventricular nuclei, the rMPO, the rostrocaudal parts of the dorsal and ventral nuclei of the BNST, the magnocellular paraventricular nucleus, the anterior and caudal supraoptic nucleus, and the MEApd. In the MEApd, ERβ-ir neurons

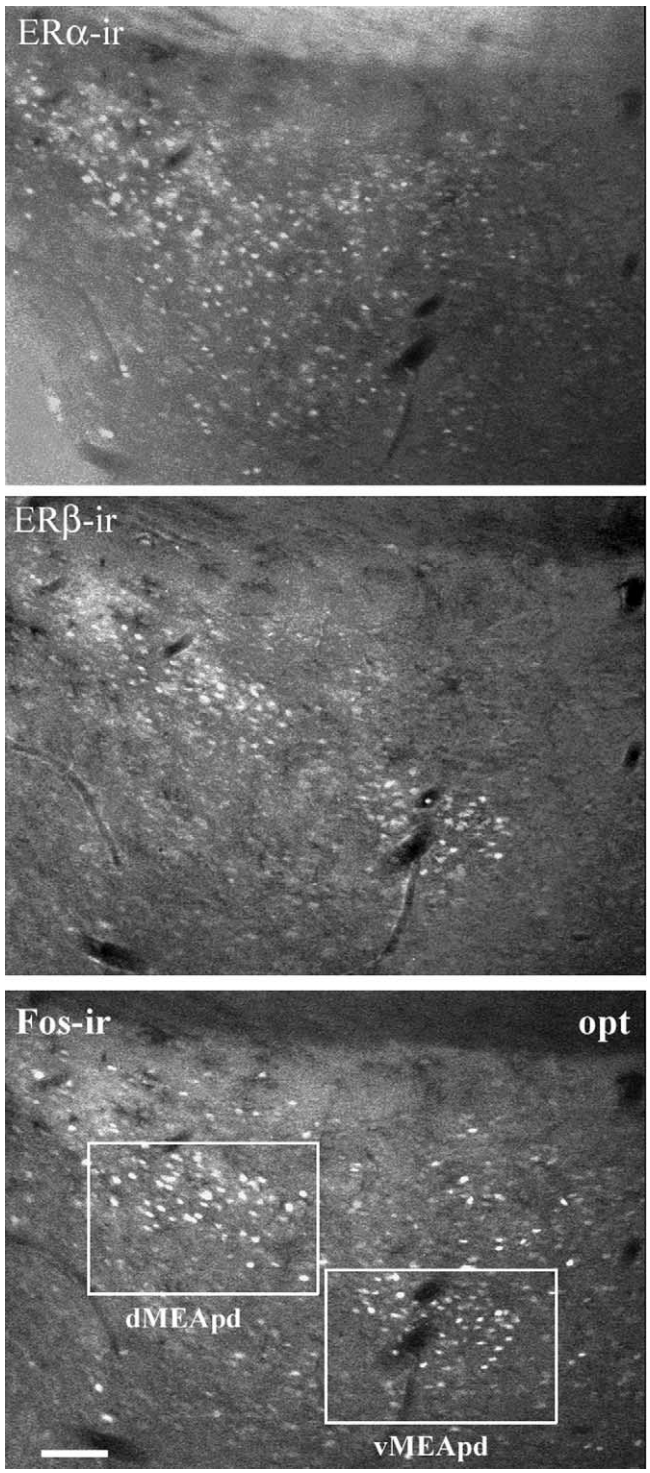


Fig. 2. Photomicrographs taken with a 10× objective of ERα-ir (top), ERβ-ir (middle), and Fos-ir (bottom) induced by 15 intromissions in the MEApd. Boxes indicate the dMEApd and the vMEApd. Bar represents 100 μm.

Table 1
Numbers of ERα-, ERβ-, and ERα/ERβ-ir cells in the rMPO, dMEApd, and vMEApd of unmated, mounts, or intromissions + mounts groups of female rats

Group	ERα-ir	ERβ-ir	ERα/ERβ-ir
rMPO			
U	242.6 ± 5.6	35.4 ± 3.8	32.3 ± 4.4
M	209.2 ± 18.4	29.2 ± 6.4	26.8 ± 5.7
I + M	208.8 ± 33.5	42.7 ± 4.3	38.1 ± 3.2
dMEApd			
U	123.7 ± 19.6	36.3 ± 6.3	17.4 ± 3.5
M	127.9 ± 14.6	43.1 ± 6.4	19.4 ± 2.9
I + M	129.4 ± 24.4	37.5 ± 9.4	18.2 ± 2.9
vMEApd			
U	4.5 ± 0.7	16.8 ± 1.9	2.0 ± 0.3
M	9.0 ± 2.7	20.1 ± 4.5	4.8 ± 1.5
I + M	6.4 ± 2.1	21.0 ± 2.4	3.8 ± 1.7

Note. Values are means ± SEM. rMPO, Preoptic area; dMEApd, dorsal posterodorsal medial amygdala group; vMEApd, ventral posterodorsal medial amygdala group. U, unmated; M, mounts, I + M, intromissions + Mounts. U, $n = 5$, M, $n = 6$, I + M, $n = 6$.

were present in a dorsal and a ventral cluster located along the optic tract (Figs. 2 and 3). Some ERβ-ir cells were also found in the medial preoptic area and the anterior medial amygdala nuclei, and a few were observed in the hippocampus. The number of ERβ-ir cells in the rMPO and the MEApd did not differ among treatment groups (Table 1).

The presence of mating-induced Fos-ir in neurons of the rMPO and MEApd agrees with previous reports (Erskine and Hanrahan, 1997; Pfaus et al., 1993; Polston and Erskine, 1995; Tetel et al., 1993). In the rMPO, Fos-ir neurons induced by 15 intromissions formed a cluster along the periventricular zone of the preoptic area. In the medial amygdala, Fos-ir neurons induced by 15 intromissions formed a band of neurons in the dMEApd and a round cluster in the vMEApd (Figs. 2 and 3).

Mating stimulation caused statistically significant changes in Fos-ir in the rMPO ($F(2, 14) = 1.84$, $p \leq 0.05$), the dMEApd ($F(2, 15) = 10.86$, $p \leq 0.05$), and the vMEApd ($F(2, 13) = 21.05$, $p \leq 0.05$). In the rMPO, the number of Fos-ir neurons in the Intromissions + Mounts group was significantly greater than in Unmated group ($p \leq 0.05$). Although there was a trend of an increase in the Mounts group, it did not differ significantly from the Intromission + Mounts or Unmated groups (Fig. 4A). In the dMEApd and the vMEApd, the number of Fos-ir neurons in the Intromissions + Mounts group was significantly greater than-that observed for either the Mounts or the Unmated groups ($p \leq 0.05$, Fig. 4A).

Coexpression of ERα-ir and ERβ-ir

Coexpression of ERα- and ERβ-ir was observed in cells of the rMPO, the dMEApd, and the vMEApd. The number of cells containing both ERα- and ERβ-ir did not vary among treatment groups in any of these areas (Table 1). In the rMPO, 13 to 20% of ERα-ir cells coexpressed ERβ-ir, and 90 to 97 % of the ERβ-ir cells expressed ERα-ir. In the dMEApd, 13 to 15 % of ERα-ir cells coexpressed ERβ-ir, and 48 to 53 % of ERβ-ir cells coexpressed ERα-ir. In the vMEApd, 47 to 66 % of ERα-ir cells expressed ERβ-ir and 11 to 26 % of ERβ-ir cells expressed ERα-ir (although the actual number of double-immunostained cells was quite small; 2–3.8).

Coexpression of Fos-ir with ERα-ir, ERβ-ir, or ERα/ERβ-ir

Mating-induced Fos-ir was coexpressed in cells expressing either ERα-ir only, ERβ-ir only, or both receptors (ERα/ERβ-ir) and varied as a function of mating stimulation. In the rMPO, the number of Fos-ir neurons coexpressing ERα-ir only increased significantly in the Mounts group ($n = 6$) in comparison to the Unmated group ($n = 5$; $F(2, 13) = 7.3$, $p \leq 0.05$, Fig. 4A). Although there was a trend of an increase, the Intromissions + Mounts group ($n = 5$) did not differ significantly from either the Unmated or Mounts groups. In contrast, the number of Fos-ir cells coexpressing ERα/ERβ-ir increased significantly in the Intromissions + Mounts group contrasted with the other groups ($F(2, 14) = 11.4$, $p \leq 0.05$, Fig. 4A). There was no consistent difference in the number of Fos-ir cells coexpressing ERβ-ir only among the three groups (Fig. 4A). Expressed as a percentage of Fos-ir cells coexpressing each of the ERs, in the Mounts group, 49% of Fos-ir neurons coexpressed ERα only and, in the Intromissions + Mounts group, 32% of Fos-ir neurons coexpressed ERα-ir and 33% coexpressed ERα/ERβ-ir.

In the dMEApd, the numbers of Fos-ir neurons coexpressing ERα-ir only ($F(2, 15) = 6.5$, $p \leq 0.05$, Fig. 4B) and ERα/ERβ-ir ($F(2, 15) = 22.3$, $p \leq 0.05$, Fig. 4B) increased in the Intromissions + Mounts group ($n = 6$) contrasted with the two other groups (Unmated, $n = 7$; Mounts, $n = 5$). In contrast, there was no difference in the number of Fos-ir neurons coexpressing ERβ-ir only among the three groups (Fig. 4B). Expressed as a percentage of Fos-ir cells coexpressing each of the ERs, in the Intromissions + Mounts group, 33% of Fos-ir neurons coexpressed ERα-ir and 19% coexpressed ERα/ERβ-ir.

In the vMEApd, the only consistent change observed was in the number of neurons in which Fos-ir was coexpressed with ERβ-ir, which increased in response to Intromissions + Mounts ($n = 5$; $F(2, 12) = 59.57$, $p \leq 0.05$, Fig. 4C) contrasted with the Mounts ($n = 5$) and Unmated ($n = 5$) groups. There were no differences in the number of Fos-ir neurons coexpressing only ERα-ir or ERα/ERβ-ir among the groups (Fig. 4C). The majority of neurons in which Fos-ir was induced by Intromissions and Mounts coexpressed ERβ-ir only (57 % of Fos-ir cells).

Coexpression of ERβ-ir with Fos-ir, ERα-ir, or Fos/ERα-ir

The number of ERβ-ir cells coexpressing Fos-ir only, ERα-ir, or Fos-ir and ERα-ir (Fos/ERα-ir) varied as a func-

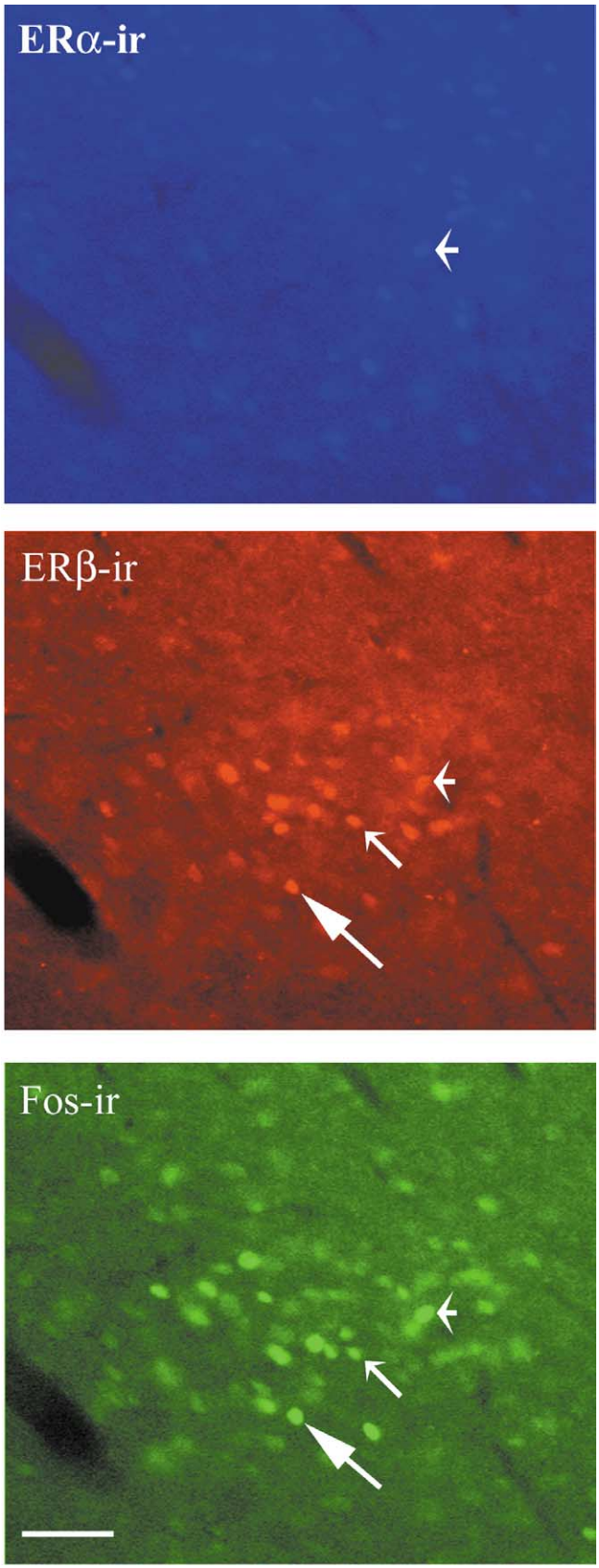


Fig. 3. Pseudocolored photomicrographs taken with a 20× objective of ERα-ir (top). ERβ-ir (middle), and Fos-ir (bottom) induced by 15 intromissions in the vMEApd. White arrows indicate either examples of cells coexpressing ERβ-ir and Fos-ir (large and mid-size arrows) or example of cells coexpressing ERα-ir, ERβ-ir, and Fos-ir (small arrows). Bar represents 50 μm.

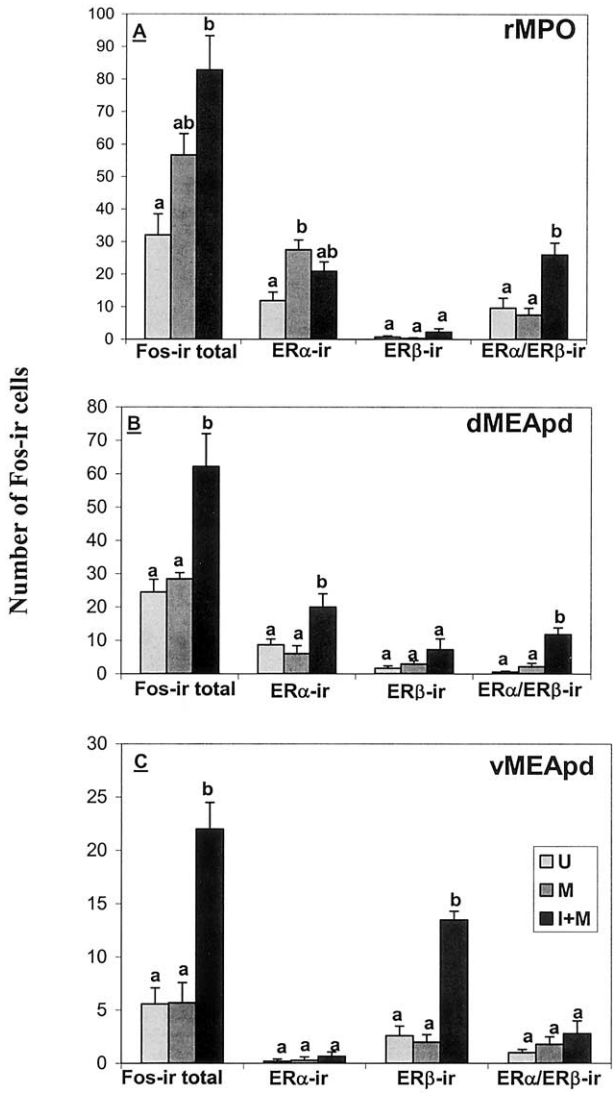


Fig. 4. Mean number (± SEM) of cells expressing Fos-ir in the rMPO (A), the dMEApd (B), and the vMEApd (C) (left) and mean number (± SEM) of cells expressing Fos-ir that coexpress ERα-ir only, ERβ-ir only, or ERα/ERβ-ir following different types of mating stimulation: Unmated (U), Mounts (M), and Intromissions + Mounts (I + M). Different letters above bars indicate statistically significant differences between groups. Bonferroni corrected *t* tests were used and differences were considered significant at $p < 0.05$.

tion of mating stimulation. In the rMPO, the number of ERβ-ir cells coexpressing Fos/ERα-ir increased significantly in the Intromissions + Mounts group in comparison to the Unmated and the Mount groups ($F(2, 14) = 11.4$, $p \leq 0.05$, Table 2). There was no difference in the number of ERβ-ir cells coexpressing Fos-ir only or ERα-ir only among the three groups (Table 2). Expressed as a percentage of ERβ-ir cells coexpressing Fos-ir, ERα-ir, or Fos/ERα-ir, in the Intromissions + Mounts group, 5% of ERβ-ir neurons

Table 2
Numbers of ERβ-ir cells coexpressing ERα-ir, Fos-ir, and Fos/ERα-ir in the rMPO, dMEApd, and vMEApd of unmated, mounts, intromissions + mounts groups of female rats

Group	ERα-ir	Fos-ir	Fos/ERα-ir
rMPO			
U	22.6 ± 1.9 ($n = 4$)	0.7 ± 0.4 ($n = 5$)	9.7 ± 3.0[a] ($n = 5$)
M	19.3 ± 6.4 ($n = 6$)	0.3 ± 0.2 ($n = 5$)	7.5 ± 2.0[a] ($n = 5$)
I + M	12.1 ± 0.8 ($n = 6$)	2.3 ± 1.1 ($n = 6$)	26.0 ± 3.7[b] ($n = 6$)
dMEApd			
U	16.7 ± 3.3[a] ($n = 7$)	1.7 ± 0.6 ($n = 7$)	0.6 ± 0.3[a] ($n = 7$)
M	17.2 ± 2.2[a] ($n = 5$)	3.0 ± 1.0 ($n = 4$)	2.2 ± 1.0[a] ($n = 5$)
I + M	6.3 ± 1.3[b] ($n = 6$)	7.3 ± 3.2 ($n = 6$)	11.8 ± 2.0[b] ($n = 5$)
vMEApd			
U	1.0 ± 0.3 ($n = 5$)	2.6 ± 0.9[a] ($n = 4$)	1.0 ± 0.3 ($n = 4$)
M	3.0 ± 1.2 ($n = 5$)	2.0 ± 0.7[a] ($n = 5$)	0.8 ± 0.7 ($n = 5$)
I + M	1.0 ± 0.5 ($n = 5$)	13.5 ± 0.8[b] ($n = 4$)	2.8 ± 1.2 ($n = 5$)

Note. Values are means ± SEM. See Table 1 note for definitions. Different letters indicate statistically significant differences between groups. Bonferroni corrected *t* tests were used and differences were considered significant at $p < 0.05$.

coexpressed Fos-ir, 30.3% coexpressed ERα-ir only, and 59.7% coexpressed Fos/ERα-ir.

In the dMEApd, the number of ERβ-ir cells coexpressing Fos-ir only did not change significantly among the three groups. However, the number of ERβ-ir cells coexpressing ERα-ir only decreased significantly in the Intromissions + Mounts group ($F(2, 15) = 5.5$, $p \leq 0.05$, Table 2) in comparison to the Unmated and Mounts groups. In contrast, the number of ERβ-ir coexpressing Fos/ERα-ir increased significantly ($F(2, 14) = 22.3$, $p \leq 0.05$, Table 2) in comparison to the Unmated and the Mounts groups. Expressed as a percentage of ERβ-ir cells coexpressing Fos-ir, ERα-ir, or Fos/ERα-ir, in the Intromissions + Mounts group, 16% of ERβ-ir neurons coexpressed Fos-ir, 19% coexpressed ERα-ir, and 29.8% coexpressed Fos/ERα-ir.

In the vMEApd, the number of ERβ-ir coexpressing Fos-ir only was dramatically increased in the Intromission + Mounts group ($F(2, 10) = 59.2$, $p \leq 0.05$, Table 2) in comparison to the Unmated and the Mounts groups. There were no significant changes in the number of ERβ-ir cells coexpressing either ERα-ir only or Fos/ERα-ir among the three groups. Expressed as a percentage of ERβ-ir cells coexpressing ERα-ir, Fos-ir, or Fos/ERα-ir, in the Intromissions + Mounts group, 68.9% of ERβ-ir neurons coexpressed Fos-ir, 2.3% coexpressed ERα-ir, and 7.3% coexpressed Fos/ERα-ir.

Discussion

Results of the present study demonstrate that mounts and intromissions can differentially activate ERα- and ERβ-containing neurons in the preoptic area and medial amygdala of female rats. We observed an increase in mating-induced Fos-ir in the MEApd following 15 intromissions in contrast to 15 mounts or no stimulation. While in the dMEApd, ERα-ir was primarily coexpressed with intromission-induced Fos-ir, in the vMEApd, ERβ-ir was primarily coexpressed with intromission-induced Fos-ir. In fact, in the vMEApd, 57% of intromission-induced Fos-ir cells coexpressed ERβ-ir only, which represented about 69% of the ERβ-ir population. In the rMPO, although mounts alone increased Fos-ir expression in ERα-ir only cells, intromissions increased Fos-ir expression in cells coexpressing both ERα only and ERα and ERβ-ir (ERα/ERβ-ir).

The distributions of ERα and ERβ reported in this study are consistent with previous reports of mRNA and protein distributions (Greco et al., 2001; Li et al., 1997; Mufson et al., 1999; Sar and Parikh, 1986; Shughrue and Merchenthales, 2001). In the present study, we found large populations of ERα-ir neurons in hypothalamic and limbic regions, such as the rMPO and the MEApd (Lonstein et al., 2000) and, as previously noted for ERα-mRNA, the distribution of ERα-ir in the MEApd was heterogeneous (Osterlund et al., 1998). The dMEApd has a considerably higher density of ERα-ir cells than the vMEApd (Fig. 2). We identified strongly labeled ERβ neurons in the preoptic area, the BNST, the supraoptic nucleus, the PVN, and the MEApd (Greco et al., 2001). Of particular interest was a dorsoventral band of cells in the MEApd paralleling the optic tract (Fig. 2), which matched very well the distributions of ERβ-ir and mRNA in that region (Shughrue et al., 1997; Shughrue and Merchenthales, 2001). Thus, in the MEApd, the distributions of ERα and ERβ are overlapping, but not identical. This differential, subregional expression of ERα and ERβ in the MEApd has also been observed by *in situ* hybridization (Osterlund et al., 1998).

In the present study, we also observed different levels of coexpression of both ERs in the rMPO and the MEApd. ERβ-mRNA and ERα-ir have been reported to be coexpressed within cells in the rMPO and the MEA of female rats (Shughrue et al., 1998), and we observed a high level of coexpression of ERα- and ERβ-ir in cells of the preoptic area (over 90% of ERβ-ir cells coexpressed ERα-ir) and the dorsal MEApd (over 50% of ERβ-ir cells coexpressed ERα-ir; Table 1) (Greco et al., 2001). In contrast, in the vMEApd, few ERα-ir cells were observed, so few of the ERβ-ir cells in this area coexpressed ERα-ir (Figs. 2 and 3; Table 1).

The ERβ antibody used in this experiment is specific for ERβ, as determined by the recognition of only ERβ in Western blots and by the elimination of immunostaining after antibody preadsorption with its specific peptide (Greco et al., 2001). Although we generally observed ERβ-ir in fewer brain regions and cells than expected based on work done with a different antibody (Shughrue and Merchenthales, 2001) or with in situ hybridization for ERβ mRNA, the ERβ immunostaining in the present study corresponded to areas of highest intensity of ERβ immunostaining (Shughrue and Merchenthales, 2001) and ERβ mRNA (Shughrue et al., 1997). Thus the number of cells that we report that coexpress ERβ-ir may represent an underestimate of the overall number. However, the percentages of ERβ-ir cells coexpressing ERα-ir are similar to values previously re-

ported for coexpression of ERα-ir in ERβ mRNA-containing cells in the female rat brain (Shughrue et al., 1997).

The distribution and pattern (Figs. 2 and 4) of mating-induced Fos-ir in the preoptic area and the medial amygdala observed in this study are consistent with previous reports showing that VCS received *via* intromissions and ejaculations is sufficient to induce significant increases in Fos-ir expression in these areas, while stimulation received during mounts alone typically does not increase overall Fos-ir expression above unmated controls (Coolen et al., 1996; Erskine and Hanrahan, 1997; Polston and Erskine, 1995; Rowe and Erskine, 1993; Tetel et al., 1993). We observed clusters of Fos-ir neurons in the rMPO and in a dorsal and a ventral part of the MEApd (Fig. 2) that have also been reported in females after critical amounts of intromissions have been received (for induction of pseudopregnancy), suggesting that they may represent summation of mating stimuli in these areas (Erskine and Hanrahan, 1997).

The preoptic area and the MEApd have been proposed to be important centers for the integration of sensory information relevant for the behavioral and neuroendocrine effects of VCS (Blaustein and Erskin, 2002; Coopersmith et al. 1996a; Polston and Erskine, 1995). That is, they have been reported to show Fos response to different amounts of intromissions, and they respond differentially to intromissions vs mounts (Polston and Erskine, 1995). Moreover, it is well known that both the preoptic area and the MEApd belong to a complex neural network involved in the integration of chemosensory, somatosensory, and visceral information related to sexual stimuli (Pfaff et al., 1994). Furthermore, it is now clear that the MEA are organized into a dorsal (i.e., the posterodorsal MEA) and a ventral (i.e., the anterodorsal, anteroventral, and posteroventral MEA) subdivisions, which have distinctive projection sites (Canteras et al., 1995, 1996) and which may play different functions in the integration of sensory information. However, to our knowledge no information is yet available about possible differences in the neuroanatomical connections of the dorsal versus the ventral aspects of each area that would provide suggestions about the function in sexual behavior of each of the clusters of Fos-ir cells described in this paper (Figs. 2 and 3).

As mentioned above, VCS received during mating has a major behavioral and neuroendocrine impact on female reproduction. Indeed, the receipt of VCS during mating accelerates the rate of estrous termination (Bennett et al., 2001; Coopersmith and Erskine, 1994; Coopersmith et al., 1996b; Erskine et al., 1989; Hardy and DeBold, 1972; Reading and Blaustein, 1984), and it induces twice-daily prolactin surges and estrous acyclicity associated with pregnancy or pseudopregnancy (Erskine, 1995; Gunnet and Freeman, 1983; Smith and Neill, 1976; Terkel and Sawyer, 1978). Preventing the receipt of VCS during mating prevents the acceleration of estrous termination and induction of pseudopregnancy observed following mating. A minimum threshold level of VCS (10–15 intromissions) is required to accelerate estrous termination and induce pseudopregnancy (Coopersmith et al., 1994, 1996b; Erskine, 1995; Erskine et al., 1989; Gunnet and Freeman, 1983; Hardy and Debold, 1972; Pfaus et al., 2000; Reading and Blaustein, 1984; Smith et al., 1975; Terkel and Sawyes, 1978), suggesting that some form of central processing and integration of sensory, sexual information is required.

Although it had been shown earlier that VCS-induced Fos-ir is coexpressed with ERα and progestin receptors in cells in some brain areas (Auger et al., 1996; Blaustein and Greco, 2002; Tetel et al., 1994), we now report that the forms of ER expressed in neurons that respond to mating stimulation vary depending on the type of mating stimulation received (i.e., intromissions or mounts) and particular populations of cells studied. For example, in the rMPO, statistically significant increases in overall Fos expression were not seen in response to mounts alone (Fig. 4A). Interestingly, although mounts alone did not significantly increase the number of Fos-ir neurons in the rMPO, when those coexpressing ERα-ir only were considered, mounts alone did in fact increase the number of Fos-ir cells. While the mount-induced increase in Fos-ir occurs primarily in ERα-ir only cells, intromissions induce Fos-ir primarily in cells coexpressing ERα and ERβ-ir (Fig. 4A). This suggests that, in the rMPO, mounts alone preferentially induce response in neurons expressing ERα only, while mounts + intromissions induce response in neurons expressing ERα only as well as in those coexpressing ERα and ERβ. Therefore, an increase in mating stimulation may recruit populations of cells containing different forms of ERs. In contrast, in the dMEApd, while mounts alone do not induce Fos expression, intromissions induce Fos expression primarily in neurons coexpressing ERα-ir only and ERα/ERβ-ir (Fig. 4B). Similarly, in the vMEApd, intromissions were required to induce Fos expression. However, in contrast to the rMPO and the dMEApd, cells containing Fos-ir in response to intromissions coexpress primarily ERβ-ir alone (Fig. 4C). This represented over 50% of the Fos-ir cell population and about 69% of the ERβ-ir population of the vMEApd (Table 2). These data suggest that, in the vMEApd, cells containing ERβ, but not ERα, may respond selectively to mating stimulation that includes intromissions. Therefore, different types of sexual stimulation induce response of cells expressing different forms of ERs in these areas, and ERβ-ir cells may be more specifically involved in the integration of information related to intromissions.

The heterogeneity of forms of ER expressed in responsive neurons may lead to distinct cellular, neuroendocrine, or behavioral consequences. Indeed, ERβ shares a high level of sequence homology with ERα in some receptor domains, and both receptors bind estradiol with similar affinity (Kuiper et al., 1997). However, ERβ and ERα can exhibit different transcriptional activities in cotransfection experiments depending on the ligands and promoters tested (Paech et al., 1997; Zou et al., 1999).

Furthermore, ERβ and ERα have the ability to homodimerize and heterodimerize and to display different transcriptional activities dependent on the particular dimer

that is formed and the physiological conditions in which they are placed (Pettersson et al., 1997; Tremblay et al., 1999). For example, ERβ negatively modulates ERα transcriptional activity when estradiol concentration is low, but not high (Hall and McDonnell, 1999). Thus, cellular responses to estrogenic ligands may depend on the forms of ERs present and, when the two forms of ER are coexpressed, on the relative concentration of each subtype. Furthermore, the present results suggest that neurons containing different types of ERs may respond to qualitatively different mating stimuli.

The preoptic area and the MEApd have been proposed to be important centers for this integrative process due to their particular sensitivity to amounts of mating stimulation (Pfaus et al., 1996; Polston and Erskine, 1995). That is, they have been reported to show Fos response to different amounts of intromissions, and they respond differentially to intromissions vs mounts (Polston and Erskine, 1995). The present results support this idea in part and suggest further that, in these brain regions, responsive neurons expressing each form of ER may be involved in different functions.

It is interesting that, in one area, the vMEApd, only ERβ-ir cells expressed Fos, and they only expressed Fos in response to intromissions (Table 2). The possibility that ERβ has a role in the regulation of the timing of sexual behavior is suggested by work in ERβKO mice. In this ERβ-disrupted strain of mice, receptive behaviors appear to be normal (Ogawa et al., 1999), and these females can successfully reproduce (Krege et al., 1998). However, during the estrous cycle, ERβKO mice display receptive behavior for a longer period than wild-type controls. This suggests that ERβ may be involved in regulation of the duration of sexual receptivity (Ogawa et al., 1999), which in turn is influenced by mating stimulation (Bennett et al., 2002). Although species differences must be kept in mind, these results, together with the present data, suggest that ERβ-containing neurons (in the MEApd and perhaps other areas that were not analyzed in this study) may be involved in the regulation of the duration of female sexual behavior. This may include changes in duration in response to mating stimulation.

The results of the present study demonstrate that distinct ER-sensitive circuits may process different types of mating stimuli in female rats in a brain region-specific manner. More specifically, the integration of mating stimuli critically important for the regulation of female sexual behavior may be relayed through an ERβ-containing population of cells in the medial amygdala.

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