Journal of Neuroendocrinology, 2002, Vol. 14, 442–449

Response to Male Odours in Progestin Receptor- and Oestrogen Receptor-Containing Cells in Female Rat Brain

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Key words: progestin receptor, oestrogen receptor, hypothalamus, amygdala, bed nucleus of stria terminalis.

Abstract

Sensory cues from male rats, such as odours and vaginal-cervical stimulation (VCS), play a modulatory role in female rat sexual behaviour. For example, exposure to male odours and VCS appears to be at least partially responsible for increases in sexual behaviour following repeated mating of oestradiol-primed female rats. Although there is evidence that VCS influences sexual behaviour via a ligand-independent progestin receptor (PR)-dependent mechanism, the mechanism by which odours influence sexual behaviour is not known. We tested the hypothesis that, similar to VCS, the effects of male odours on sexual behaviour are mediated by progestin receptors. Female rats were injected with the progestin antagonist, RU486, or oil vehicle and were then exposed to male-soiled bedding or clean bedding. Although exposure to male-soiled bedding resulted in higher levels of Fos immunoreactivity in brain areas associated with female sexual behaviour, the progestin antagonist did not reduce this effect. Furthermore, there was minimal coexpression of odour-induced Fos and progestin receptors in brain areas associated with female sexual behaviour. Together, these results suggest that the effects of male odours are not mediated by a PR-dependent mechanism. Therefore, we tested the hypothesis that oestrogen receptor (ER)-containing cells are involved in the effects of olfactory cues. Although there was virtually no coexpression of ERβ and odour-induced Fos in brain areas associated with female sexual behaviour, exposure to male odours slightly increased the number of cells coexpressing ERα and odour-induced Fos in the posterodorsal medial amygdala. Although, these results do not support the hypothesis that the effects of odours are mediated by a PR-dependent mechanism, they suggest that integration of male odours and hormonal cues may occur in ERα-containing cells in the posterodorsal medial amygdala.

In oestrous cycling female rats, sexual behaviour is induced by an increase in oestradiol followed by an increase in progesterone (1, 2). Sexual behaviour is prevented by treatment with oestrogen or progestin antagonists, suggesting that the behaviour is mediated by activation of intracellular steroid receptors (3). Although steroid hormones are critical for the induction of female sexual behaviour, sensory cues received during mating, such as vaginal-cervical stimulation (VCS) and male odours, also modulate the behaviour. For example, VCS from intromissions and ejaculations increases return latencies during paced mating (4) and accelerates oestrous termination (5, 6). In addition, male olfactory cues seem to enhance sexual receptivity, because removal of the vomeronasal organ reduces sexual receptivity in female rats on the day of proestrus (7, 8), as well as in ovariectomized rats treated with oestradiol and progesterone (8).

Sexual receptivity is also enhanced in the absence of progesterone when oestradiol-primed female rats are repeatedly mated with male rats (9–15). Sensory cues received during mating also contribute to this effect. For example, preventing VCS from intromissions and ejaculations prevents increases in sexual receptivity following repeated mating (16). Similarly, vomeronasal organ lesions reduce sexual receptivity following repeated mating (12, 15).

Increases in sexual receptivity following repeated mating in the absence of circulating progesterone are prevented by injection of progestin antagonists (e.g. RU486 and ZK98299) suggesting that increases in sexual behaviour following

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repeated mating are at least partially mediated by activation of progestin receptors (PRs) in the absence of progesterone (ligand-independent activation) (9). Indeed, VCS is believed to regulate sexual behaviour via this mechanism. VCS induces Fos expression in PR-containing cells in the ventromedial hypothalamus (VMH) and medial preoptic area (MPO) (17), and this expression is reduced by injection of progestin antagonists in the absence of progesterone, suggesting the VCS causes ligand-independent activation of PRs (9).

Unlike VCS, the mechanism by which odours influence sexual behaviour is not clear. It is possible that odours, such as VCS, modulate sexual behaviour by activating PRs in brain areas associated with female sexual behaviour. Indeed, the accessory olfactory pathway includes several brain areas associated with the display of female sexual behaviour, including the bed nucleus of the stria terminalis (BST), medial amygdala (MEA), VMH, and MPO (18). Furthermore, male odours induce a significant increase in Fos expression in the BST and MEA and a trend toward an increase in the VMH (19). As these areas contain high concentrations of PRs (20), we hypothesized that odours cause ligand-independent activation of PRs in these areas and thereby regulate female sexual behaviour.

Odor-induced Fos expression should be reduced by administration of a progestin antagonist if, similar to VCS (9), odours cause ligand-independent activation of PRs. Furthermore, it would be predicted that, similar to VCS-induced Fos (17), odour-induced Fos expression should occur in neurones containing PRs. Therefore, in Experiment 1, we tested the hypothesis that a progestin antagonist, RU486, would reduce odour-induced Fos expression in brain areas associated with female sexual behaviour. In Experiment 2, we determined if cells expressing odour-induced Fos coexpress PRs.

Some studies suggest that odours may influence sexual behaviour via an oestrogen receptor (ER)-dependent mechanism. For example, male odours increase ER binding in female prairie voles (21) and in male rats that have been feminized by castration and injection of oestradiol benzoate (22). If oestrogen receptors (ERs) are involved in the effects of odours on female sexual behaviour, odours of male rats could be expected to specifically activate neurones containing ERs. Therefore, in Experiment 3, we tested the hypothesis that odour-induced Fos is expressed in ERα-containing neurones. Although oestrogenic stimulation of female sexual behaviour is generally thought to result from activation of ERα, a recent study showed that intromissions specifically activate ERβ-containing cells in the posterodorsal MEA (MEApd), suggesting that ERβ-containing cells could also contribute to behavioural changes following mating stimulation (Greco *et al.*, unpublished). Therefore, in Experiment 4, we also explored the possibility that odour-induced Fos is expressed in ERβ-containing neurones.

Methods

Animals

Female Sprague-Dawley rats, weighing 175–200 g, obtained from Charles River Breeding Laboratories, Inc. (Wilmington, MA, USA) were group housed in a 14 : 10 h light/dark cycle. They received a pelleted certified rodent feed (Purina Lab Diet 5001, Ralston Purina, St Louis, MO, USA) and water *ad libitum* prior to surgery. At least 1 week after arrival, the rats were bilaterally ovariectomized and adrenalectomized under 2.5 mg/kg acepromazine, 10 mg/kg xylazine and 50 mg/kg ketamine. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts, Amherst. Ovariectomized/adrenalectomized rats were used in the present experiments to ensure the removal of all circulating progesterone. Following surgery, the rats received Purina Lab Diet 5015, water and a sodium chloride spool *ad libitum* for the remainder of the study. Purina Lab Diet 5015 was used because it has a higher fat content than Purina Lab Diet 5001 and seems to prevent some of the weight loss associated with adrenalectomy.

One week after surgery, the rats were injected s.c. with 2 µg oestradiol benzoate (dissolved in 0.1 ml sesame oil). Forty-four hours later, they were injected s.c. with 500 µg progesterone (dissolved in 0.1 ml sesame oil containing 5% benzyl alcohol and 15% benzyl benzoate). Beginning 1 week prior to perfusion, each female was placed in a small plastic tub (similar to the tubs used during exposure to the experimental treatment) containing clean bedding for 30 min daily. This exposure was intended to decrease Fos expression resulting from handling and changes in environment on the day of perfusion.

Perfusion

Rats were deeply anaesthetized with sodium pentobartibal (89 mg/kg) and chloral hydrate (425 mg/kg). The chest cavity was opened, and the descending aorta and ascending vena cava were clamped. The left ventricle was injected with 5000 U of sodium heparin dissolved in 1 ml of saline (0.15 M). A small incision was made in the right atrium, and a cannula was inserted into the left ventricle. Seventy-five ml of saline preceded the flow of fixative through the cannula. The brains were removed, placed into 0.1 M sodium phosphate buffer (pH 7.2) containing 20% sucrose for 48 h at 4 °C, and then sectioned at 40 µm on a freezing microtome. Sections were placed in a cryoprotectant solution at −20 °C until processed for immunocytochemistry.

Immunocytochemistry

For each animal, one set in four sections was immunostained. Sections were removed from the cryoprotectant solution and rinsed three times for 5 min each in Tris-buffered saline (TBS; pH 7.6). The sections were incubated in 1% sodium borohydride in TBS for 10 min to remove residual aldehydes followed by four, 5-min rinses in TBS. The sections were then incubated in 1% H_2O_2, 20% normal goat serum, and 1% bovine serum albumin for at least 20 min to reduce nonspecific staining and endogenous peroxidase activity. Sections were then incubated in primary antibody diluted in TBS containing 0.1% gelatin, 0.02% sodium azide and 0.02% Triton X-100 (Gel TBS), 0.5% Triton X-100 and 1% normal goat serum for 72 h at 4 °C. Following three, 5-min rinses in Gel TBS, the sections were incubated in secondary antibody diluted in Gel TBS for 90 min. Following an additional three, 5-min rinses in TBS, sections were incubated with Avidin DH: biotinylated horseradish peroxidase H complex (1 : 100 in TBS; Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, CA, USA) for 90 min. Following three, 5-min rinses in TBS, sections were treated with 0.05% diaminobenzidine and 0.05% H_2O_2 in TBS for 3 min. The sections were then rinsed a final three times in TBS for 5 min each. The sections were mounted on glass slides and allowed to dry, after which they were coverslipped with DePeX mounting medium (BDH Laboratory Supplies, Poole, UK).

In order to control for nonspecific staining, additional sections were incubated in solutions in which only the primary antibody was present or in solutions in which only the secondary antibodies were present. No nonspecific staining was observed (data not shown).

Immunofluorescence

The technique for immunofluorescent staining differed from the standard immunocytochemistry technique in that the sections were not incubated in a 1% sodium borohydride solution. In addition, sections were incubated in a cocktail of primary antibodies for 48 h at 4 °C. Following three, 5-min rinses in Gel TBS, the sections were incubated in a cocktail of fluorescent secondary antibodies for 90 min at room temperature. The sections were then rinsed an additional three times for 5 min each in TBS and mounted on glass slides and coverslipped with Vectashield mounting medium (Vector Laboratories.) immediately after immunocytochemistry.

Some sections were incubated in solutions in which the primary antibodies were omitted or in solutions in which one primary antibody and both

secondary antibodies were present in order to control for nonspecific immunofluorescent staining, cross-immunoreactivity and 'bleed through' of the fluorochromes. No nonspecific immunofluorescent staining, cross immunostaining or 'bleed through' was observed (data not shown).

Experiment 1

Treatment

Ten days after the initial exposure to the hormones, the rats received 2 μg oestradiol benzoate followed 45 h later by one of the following three treatments: (i) injection of 5 mg RU486 s.c. (dissolved in 0.4 ml sesame oil containing 5% benzyl alcohol and 15% benzyl benzoate) followed 1 h later by exposure to male-soiled bedding for 2 h; (ii) injection of oil vehicle s.c. followed 1 h later by exposure to male-soiled bedding for 2 h; (iii) injection of oil vehicle s.c. followed 1 h later by exposure to clean bedding for 2 h. Females receiving RU486 and exposed to clean bedding were not included in this experiment, because a previous study found that RU486 did not increase Fos expression in brain areas associated with female sexual behaviour in females receiving control treatments (Auger and Blaustein, unpublished observations). Male-soiled bedding was collected from cages of sexually experienced male rats after 3 days of use. Exposure to bedding took place in small plastic tubs. The rats were perfused immediately after removal from the tubs with 350 ml of 2% acrolein in 0.1 M sodium phosphate buffer (pH 7.2). Sections were immunostained for Fos protein using a rabbit polyclonal c-fos antiserum (1 : 140 000; Ab-5, Calbiochem-Novabiochem Corp., San Diego, CA, USA) and 3 μg/ml of biotinylated goat anti-rabbit immunoglobulin G (Vector Laboratories).

Data analysis

A Leitz Dialux 20 microscope (Ernst Leitz Wetzler GMBH, Wetzlar, Germany) was fitted with a MTI CCD72 camera (DAGE MTI, Michigan City, IN, USA) connected to a Macintosh G3 computer. The NIH Image program (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/) was used for image analysis. Prior to tissue examination, the microscope was adjusted for Kohler illumination using a ×10 objective. A microscope slide with a black dot was placed under the microscope and viewed on the monitor. The camera gain and black levels were adjusted, so that black was at the maximum grey level of 240 pixel density, and a clear portion of the slide was within single pixel digits. This allowed the grey level range of Fos immunoreactivity to be from 1 to 240 pixel density. Once the camera gain and black levels were adjusted, they remained calibrated for all sections to be analysed through a given area. The density threshold option was then set to a maximum of 4 SD above the mean pixel density. A new background mean and standard deviation were then determined with this setting, so that foreground immunostaining no longer factored into the average background pixel density. This procedure determined the mean pixel density of the background area excluding the contributions of foreground immunoreactivity. Representative sections from brain areas in which odours have been previously shown to induce increases in Fos expression [BST, MEApd and caudal ventrolateral VMH (cVMHvl)] (19) were matched using the according to Swanson (23) (Fig. 1). One carefully matched section for each area per animal was analysed using the above procedure with the experimenter blind to treatment condition. Data from each brain area were analysed with a one factor ANOVA. Significant effects were analysed with Newman–Keuls post-hoc comparisons.

Experiment 2

Treatment

Ten days after the initial exposure to the hormones, the females received 2 μg oestradiol benzoate. Forty- six hours later, the females were exposed to either male-soiled bedding or clean bedding as described in Experiment 1. The rats were perfused immediately after removal from the bedding with 250 ml of 4%

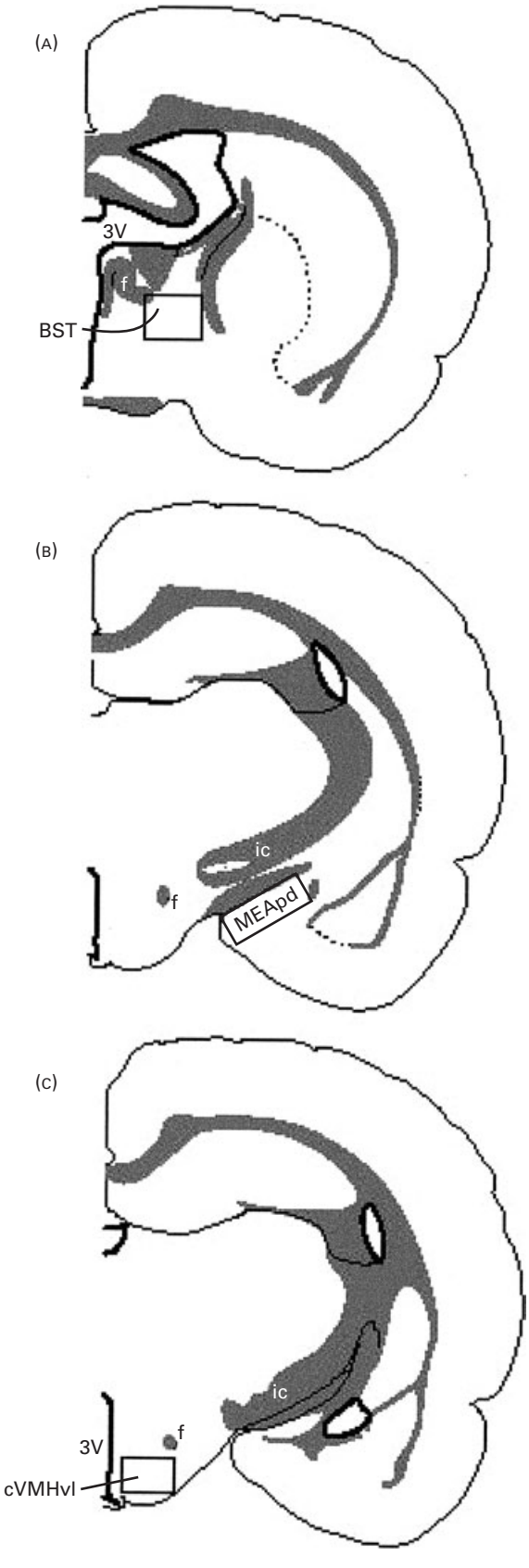

FIG. 1. Schematic drawings of brain sections matched for analysis according to Swanson (23). Boxes show areas in which Fos-immunoreactive cells were counted. (A) BST, bed nucleus of the stria terminalis. (B) MEApd, posterodorsal medial amygdala. (C) cVMHvl, caudal ventrolateral ventromedial nucleus of the hypothalamus. f, fornix; ic, internal capsule; 3V, third ventricle.

paraformaldehyde in 0.2 M sodium phosphate buffer (pH 7.2). Sections were immunostained for Fos protein and PR protein using a rabbit polyclonal c-fos antiserum (1 : 5000; Ab-5, Calbiochem-Novabiochem Corp.) and mouse monoclonal PR antibody (1 : 1000; MAB462, Chemicon International Inc., Temecula, CA, USA), followed by fluorescein-conjugated goat anti-rabbit secondary antibody (10 μg/ml; Jackson Immunoresearch Laboratories, West Grove, PA, USA) for detection of Fos immunoreactivity and cyanine 3-conjugated goat anti-mouse secondary antibody (5 μg/ml; Jackson Immunoresearch Laboratories) for detection of PR immunoreactivity.

Immunofluorescence data analysis

One day after mounting, sections were analysed using the NIH Image program. Digitized pictures of the same microscopic field were captured with two different pass-band filters, specific for fluorescein and cyanine-3. The images were superimposed and the number of single and double-labelled cells were counted by eye. Representative sections from brain areas in which odours induce significant increases in Fos expression (BST and MEApd) (19) were matched according to Swanson (23). The cVMHvl was not analysed, because virtually no Fos-immunoreactive cells were visible in this particular experiment. One section per area per animal was counted. Capturing of images from each treatment was randomized to prevent immunofluorescent fading of a particular treatment group. In addition, the experimenter was blind to the treatment condition. Data from each brain area were analysed with t-tests.

Experiment 3

Sections from Experiment 2 were immunostained and analysed for Fos protein and ERα protein as described in Experiment 2; however, a mouse monoclonal ERα antibody (1 : 500; 1D5, Dako, Carpinteria, CA, USA) replaced the mouse monoclonal PR antibody.

Experiment 4

Sections from Experiment 2 were immunostained and analysed for Fos protein and ERβ protein as described in Experiment 2; however, a mouse monoclonal ERβ antibody (2 μg/ml; hERβNT-221.3, Ligand Pharmaceuticals, Inc., San Diego, CA, USA) directed against a synthetic peptide corresponding to the 14 amino acid *N*-terminal sequence of the human ERβ (1–485 form) replaced the mouse monoclonal PR antibody.

Results

Experiment 1

The distribution of odour-induced Fos immunoreactivity was consistent with previous reports. Fos immunoreactivity was present in neurones in the main and accessory olfactory bulbs, the BST, MEApd and VMH (19). A one-factor ANOVA revealed a significant effect of treatment ($F = 6.74$; $P < 0.05$). Post-hoc comparisons revealed significantly more Fos-immunoreactive cells in the BST ($P < 0.05$) and MEApd ($P < 0.05$) of the soiled bedding-exposed females treated with either vehicle or RU486 than in those of the clean bedding-exposed females (Fig. 2). There were also significantly more Fos-immunoreactive cells in the cVMHvl ($P < 0.05$; data not shown); however, there were only a total of five cells. There were no significant differences between soiled bedding-exposed females treated with RU486 or vehicle in any brain area analysed.

Experiment 2

The distribution of PR immunoreactivity was consistent with previous reports. PR immunoreactivity was present in the BST, MEApd, MPO, the anterior, lateral and ventral portions of the hypothalamus, and the arcuate nucleus (20). t-tests revealed that the number of Fos-immunoreactive cells in the BST ($t = 2.95$, $P < 0.05$) and MEApd ($t = 2.57$, $P < 0.05$) was greater in the soiled bedding-exposed group than in the clean bedding-exposed group (Table 1). There were no significant differences between groups in number of PR-immunoreactive cells in either brain area. There was virtually no coexpression of Fos and PR immunoreactivity within cells (Fig. 3).

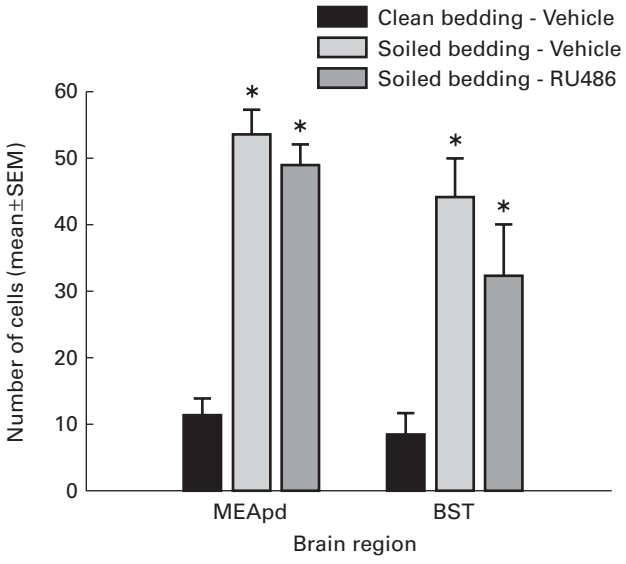

FIG. 2. Mean ± SEM number of Fos-immunoreactive cells following treatment with vehicle and exposure to clean bedding (clean bedding, vehicle), treatment with vehicle and exposure to male-soiled bedding (soiled bedding, vehicle), or treatment with RU486 and exposure to male-soiled bedding (soiled bedding, RU486). *Statistically significant difference from females treated with vehicle and exposure to clean bedding.

Experiment 3

The distribution of ERα immunoreactivity was also consistent with previous findings. ERα-immunoreactive neurones were found in the BST, MPO, anterior, lateral and ventral portions of the hypothalamus, the arcuate, and the central, cortical and medial amygdala (24). t-tests revealed that the number of Fos-immunoreactive cells in the BST ($t = 3.59$, $P < 0.01$) and MEApd ($t = 5.6$, $P < 0.001$) was greater in the soiled bedding-exposed group than in the clean bedding-exposed group (Table 1). There were no differences in the number of ERα-immunoreactive cells between groups in either area. In the MEApd, there were significantly more cells coexpressing Fos and ERα immunoreactivity in the soiled bedding-exposed females than in the clean bedding-exposed females (Figs 4 and 5). Although the percentages of Fos-immunoreactive cells coexpressing ERα were not significantly different between the two groups, there was a slight increase in the percentage of Fos-immunoreactive cells coexpressing ERα in the females exposed to soiled bedding (17%) compared to those exposed to clean bedding (12%). There was minimal coexpression in the BST.

TABLE 1. Mean ± SEM Number of Immunoreactive Cells in Bed Nucleus of the Stria Terminalis (BST) and Posterodorsal Medial Amygdala (MEApd) Sections in Experiments 2–4.

		BST		MEApd	
Experiment	Type of immunoreactive cells	Clean bedding-exposed	Soiled bedding-exposed	Clean bedding-exposed	Soiled bedding-exposed
2	Fos	12.60 ± 3.68	28.57 ± 3.72*	15.33 ± 1.89	44.00 ± 10.12*
	PR	14.40 ± 5.77	8.86 ± 1.90	3.67 ± 1.12	1.43 ± 0.61
	PR and Fos	0.40 ± 0.25	1.14 ± 0.14	0.33 ± 0.21	0.29 ± 0.18
3	Fos	10.17 ± 2.21	24.57 ± 3.18*	6.83 ± 2.09	32.29 ± 3.79*
	ERα	27.50 ± 5.18	24.43 ± 3.08	40.83 ± 4.36	39.00 ± 2.35
	ERα and Fos	0.67 ± 0.33	1.20 ± 0.42	0.83 ± 0.48	5.57 ± 1.73*
4	Fos	10.33 ± 2.58	28.00 ± 3.19*	12.50 ± 1.75	36.86 ± 6.25*
	ERβ	0.50 ± 0.34	1.57 ± 0.65	45.17 ± 9.28	32.00 ± 4.28
	ERβ and Fos	0.17 ± 0.17	0.29 ± 0.18	1.50 ± 0.22	1.57 ± 0.37

*Statistically significant difference between soiled bedding-exposed and clean bedding-exposed females in a given brain area.

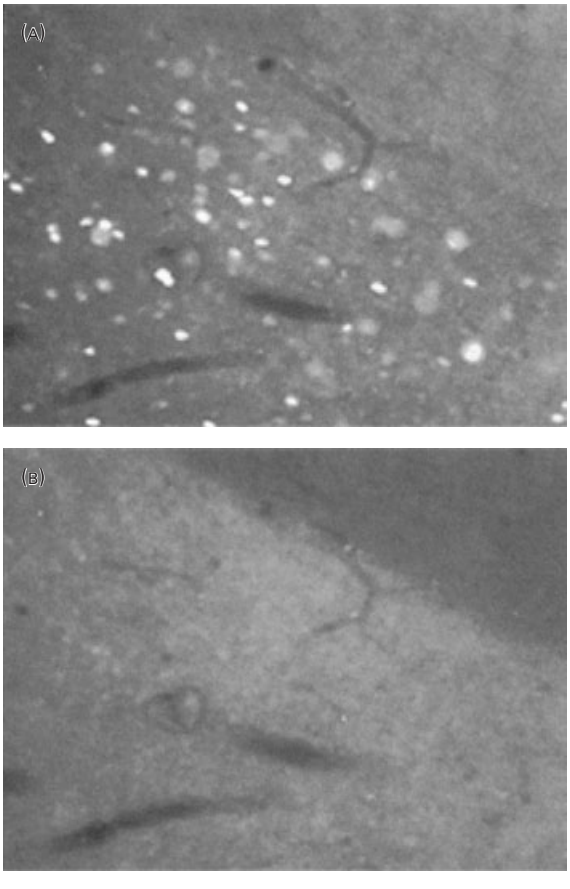

FIG. 3. Photomicrographs of (A) Fos-immunoreactive cells and (B) progestin receptor-immunoreactive cells in the same field within the posterodorsal medial amygdala of a female exposed to male-soiled bedding.

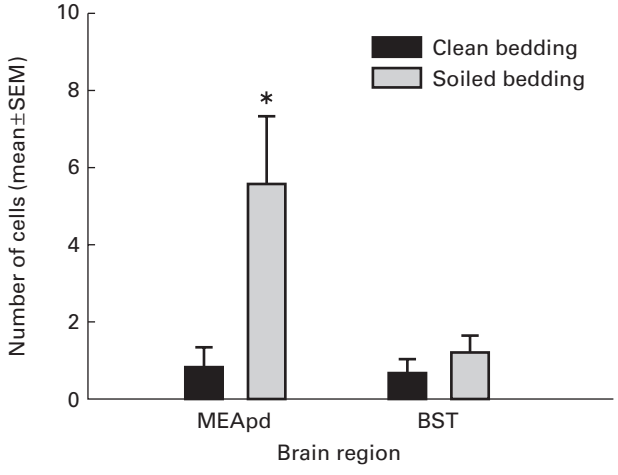

FIG. 4. Mean ± SEM number of cells coexpressing Fos and oestrogen receptor (ER)α immunoreactivity following exposure to clean bedding or male-soiled bedding. *Statistically significant difference from clean bedding-exposed females.

Experiment 4

ERβ immunoreactivity was found in the BST, periventricular zone of the preoptic area, paraventricular nucleus, supraoptic nucleus and the MEApd. Furthermore, ERβ-immunoreactive cells were more abundant in the MEApd than in the BST. These findings are consistent with previous studies of the distribution of ERβ (25–27). t-tests revealed that the number of Fos-immunoreactive cells in the BST ($t = 4.21$, $P < 0.01$) and MEApd ($t = 3.49$, $P < 0.01$) was greater in the soiled bedding-exposed group than in the clean bedding-exposed group (Table 1). There were no differences in the number of ERβ-immunoreactive cells between groups in either area. There were virtually no cells coexpressing Fos and ERβ immunoreactivity in either brain area (Fig. 6).

Discussion

In each experiment, exposure of female rats to male odours in the form of male-soiled bedding caused a significant increase in Fos expression in the BST and MEApd. There was also a

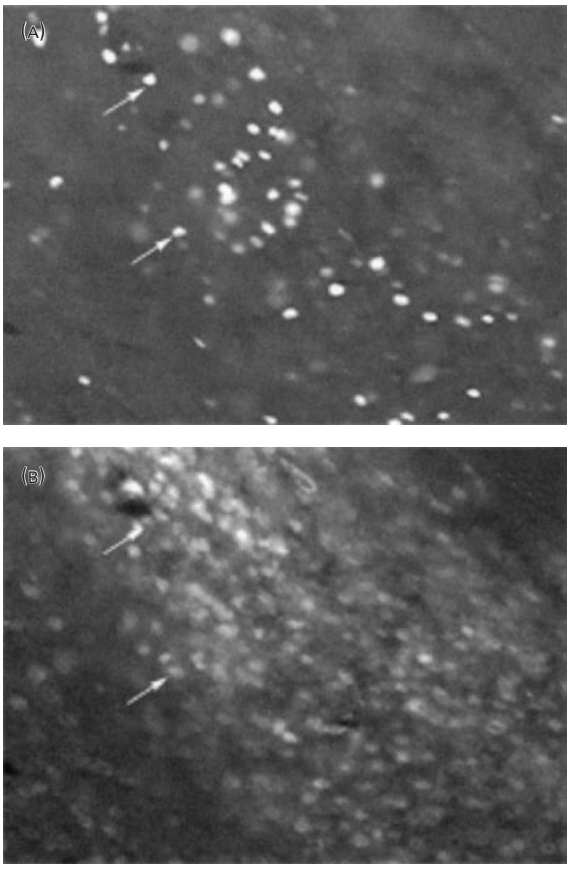

FIG. 5. Photomicrographs of (A) Fos-immunoreactive cells and (B) oestrogen receptor α-immunoreactive cells in the same field within the posterodorsal medial amygdala of a female exposed to male-soiled bedding. Arrows point to examples of cells containing both Fos and ERα immunoreactivity.

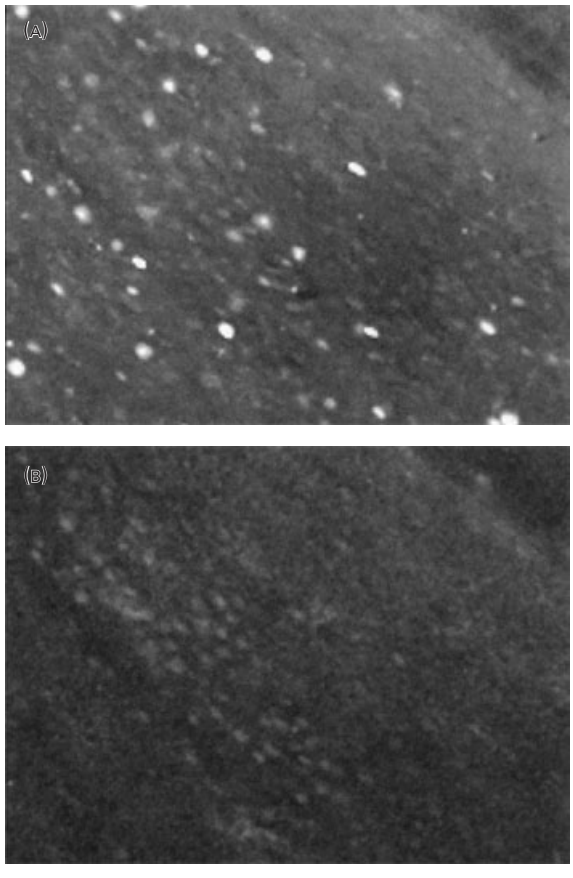

FIG. 6. Photomicrographs of (A) Fos-immunoreactive cells and (B) oestrogen receptor β-immunoreactive cells in the same field within the posterodorsal medial amygdala of a female exposed to male-soiled bedding.

significant increase in Fos expression in the cVMHvl in Experiment 1; however, as there was only a total of five cells in this area, the cVMHvl was not analysed in the subsequent experiments. These findings suggest that exposure to male odours causes a neuronal response in some brain areas associated with female sexual behaviour. The results are consistent with the findings of Dudley *et al.* (19), who found that male-soiled bedding caused a significant increase in Fos expression in the BST and MEA and a trend toward an increase in the VMH.

In Experiment 1, odour-induced, unlike VCS-induced (9), Fos expression was not reduced by injection of the progestin antagonist, RU486. Furthermore, in Experiment 2, we found that although odour-induced Fos expression and PRs were present in the same brain areas, they were not coexpressed in the same cells. Taken together, Experiments 1 and 2 strongly suggest that, unlike the case with VCS in some cells, the neuronal effects of odours are not mediated by a PR-dependent process.

Male odours (19) and VCS (28) induce a neuronal response in similar brain areas, such as the BST, MEApd and, to a lesser extent for odours, in the cVMHvl. However, the current findings suggest that odours and VCS activate distinct populations of steroid receptor-containing cells most likely via distinct molecular pathways. In some neuroanatomical areas, many cells in which VCS induces Fos expression contain PRs (17, 29), as well as ERs (28, Greco *et al.*, unpublished observations). Furthermore, VCS-induced Fos expression is reduced in some cells by injection of RU486 in the absence of circulating progesterone (9, 29), suggesting that VCS causes activation of PRs. In contrast, the current findings suggest that odours do not cause activation of PRs, nor do they induce Fos expression in PR-containing cells.

These differences may account for distinct behavioural outcomes of exposure to odours and VCS. For example, there is a slight increase in sexual behaviour following repeated mating in females with vomeronasal organ lesions (15), but we observed no increase in sexual behaviour in the absence of VCS (16). Furthermore, odour exposure alone is not sufficient to induce sexual behaviour (unpublished observations), whereas VCS alone will induce a low level of sexual behaviour (16). In addition, male odours regulate mate selection and approach behaviour (30–32), while VCS induces pseudopregnancy (33), accelerates oestrous termination (5, 6) and influences the pacing of mating by the female (4). It is likely that the activation of distinct neuronal pathways accounts for these differences.

In Experiment 3, exposure to male odours caused a significant increase in cells coexpressing Fos and ERα immunoreactivity in the MEApd, but not in the BST. Although ERβ-containing cells and odour-induced Fos expression were present in the same brain areas (Experiment 4), they were not coexpressed in the same cells. Although it remains to be tested whether the slight increase in cells coexpressing Fos and ERα immunoreactivity is of functional importance in mediating the effects of odours on female sexual behaviour, previous findings suggest that the MEApd is important for the integration of sensory information relevant for sexual behaviour. For example, lesions of the MEA decrease the display of lordosis (34, 35) and prevent the occurrence of pseudopregnancy following mating stimulation (36). Furthermore, the MEA appears to be part of a complex neural network involved in the regulation of female sexual behaviour. The MEA not only receives input from the accessory olfactory bulbs (18), but also receives it from other areas relevant to the display of female sexual behaviour, such as the VMH (37, 38) and midbrain central grey (37). Indeed, other sensory cues associated with mating, such as VCS, induce Fos expression in the MEApd (28, 39, Greco *et al*., unpublished observations). Furthermore, the MEA appears to respond directly to steroid hormones, as oestradiol priming increases spontaneous neuronal firing (37). The MEA projects to other brain areas believed to regulate the display of female sexual behaviour, such as the BST, MPO and VMH (40). Therefore, it is possible that the MEA serves as a site of integration for sensory cues, including olfactory cues, and hormonal stimulation which regulate female sexual behaviour.

Different types of mating stimulation appear to differentially activate ERα- and ERβ-containing cells in the MEApd, further supporting the idea that it is a site of sensory integration. The MEApd contains a high concentration of both ERα- and ERβ-containing cells. Fos immunoreactivity induced by 15 mounts by a male rat is distributed throughout all ER-containing cells in the MEApd; however, Fos induced by 15 intromissions is coexpressed with either ERα or both ERα and ERβ in the dorsal MEApd and almost exclusively with ERβ in the ventral MEApd (Greco *et al*., unpublished observations). In contrast, Experiments 3 and 4 suggest that odours activate some ERα containing cells in both the dorsal and ventral aspects of the MEApd. Taken together, these studies suggest that different types of reproductively relevant sensory cues, which lead to distinct behavioural outcomes, may differentially activate ERα- and ERβ-containing neurones.

Although some previous studies suggest that odours may regulate sexual behaviour by up- regulation of ERs (21, 22), the findings of Experiment 3 suggest that this is not the case, because females exposed to soiled bedding did not have more ER-immunoreactive cells than did clean bedding-exposed females. However, we cannot rule out the possibility that a slight increase in ERs may not have been detected with the immunofluorescent technique used in the current study.

Although the use of male-soiled bedding is a common means of exposing female rats to male odours (19, 21, 22), it is important to consider the possibility that the odours present during mating may not be identical to the odours present in male-soiled bedding. Therefore, we cannot completely rule out the possibility that some types of odours may influence behaviour through a PR-dependent mechanism; however, the types of odours present in male-soiled bedding do not appear to activate PR-immunoreactive cells.

In conclusion, the results of Experiments 1 and 2 suggest that, unlike VCS, odours do not mediate their effects on female sexual behaviour via a PR-dependent mechanism. The results of Experiments 3 and 4 suggest that odours slightly increase the coexpression of Fos and ERα, but not ERβ, in the MEApd. It remains to be tested whether this relationship is of functional importance in mediating the effects of odours on female sexual behaviour.

Acknowledgements

This work was supported by NS19327 (J.D.B.), Senior Scientist Award MH01312 (J.D.B.), NRSA fellowship MH12474 (A.L.B.) and NRSA fellowship HD40410 (M.E.B.) from the National Institutes of Health. We thank Robin Lempicki and Beth Lux for their technical assistance. We also thank Ligand Pharmaceutical for supplying the mouse monoclonal ERβ antibody used in these experiments.

Accepted 27 February 2002

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