BIOLOGY OF REPRODUCTION **59,** 1454–1463 (1998)

Sex Difference and Testosterone Modulation of Pheromone-Induced Neuronal Fos in the Ferret's Main Olfactory Bulb and Hypothalamus[1]

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ABSTRACT

A carnivore, the ferret possesses a vomeronasal organ—accessory olfactory bulb (VNO-AOB) projection to the hypothalamus; however, little is known about its function. Pheromones in soiled bedding from estrous female ferrets or an artificial peppermint odor significantly augmented nuclear Fos protein immunoreactivity (Fos-IR), a marker of neural activation, in several main olfactory bulb (MOB) sites but not in the AOB of gonadectomized male and females. Testosterone propionate (TP) significantly augmented the MOB's neuronal Fos responses to estrous females' pheromones, but not to peppermint. Estrous odors, but not peppermint, also augmented neuronal Fos-IR in the medial preoptic area (mPOA) of female, but not male, subjects. Pheromones in soiled bedding from breeding male ferrets significantly augmented neuronal Fos-IR in the MOB and in the medial amygdala of gonadectomized, TP-treated male and female subjects. Again, male pheromones failed to influence neuronal Fos-IR in the AOB of either sex, and only females showed significant increases in neuronal Fos-IR in the lateral aspect of the ventromedial nucleus and mPOA. These results point to an essential role among higher mammals of the main olfactory epithelium-MOB projection to the hypothalamus in detecting and processing pheromones. Gonadectomized ferrets showed significant increases in sniffing behavior when placed on either female or male bedding. This occurred regardless of whether they had received TP or oil vehicle, suggesting that testosterone's facilitation of neuronal Fos responses to estrous females' odors in the MOB of both sexes cannot be attributed to increased scent gathering. Androgen receptor-IR was present in the MOB granule cell layer of male and female ferrets, raising the possibility that testosterone acts directly on these cells to augment their responsiveness to pheromones.

INTRODUCTION

Chemosensory cues (pheromones) derived from urine or from anal and genital sebaceous gland secretions contribute to heterosexual partner selection and subsequent reproductive success in many vertebrate species [1, 2]. In rodents, pheromones are often detected by sensory neurons located in the vomeronasal organ (VNO), which send their axons to the accessory olfactory bulb (AOB) (reviewed in [3, 4]). Pheromonal inputs are then conveyed to the hypothalamus via a pathway that includes subdivisions of the medial amygdaloid nucleus (MA) and the bed nucleus of the stria terminals (BNST), with further projections to the medial preoptic area (mPOA) and ventrolateral portion of the ventromedial hypothalamic nucleus (VLH) [5, 6]. Studies using hamsters [7, 8], rats [9, 10], and mice [11] have monitored increases in nuclear Fos protein immunoreactivity (Fos-IR) as a marker of pheromone-induced neuronal activation throughout the VNO-AOB olfactory pathway. Androgen and estrogen receptors are expressed in the MA, BNST, mPOA, and VLH of the rat [12] and hamster [13], allowing sex steroids to facilitate neuronal responses to olfactory inputs in these regions. Castration of male rats, hamsters, or mice eliminates their preference for estrous female odors [14–18]. In the male hamster, pheromonal and hormonal cues must be integrated in the MA and/or BNST in order for mating to occur [19]. The relevant pheromonal signals are usually detected by the VNO, although the main olfactory epithelium-main olfactory bulb (MOE-MOB) olfactory projection to the hypothalamus may also contribute to sexual arousal and performance [20].

Considerably less research on pheromonal communication has been carried out using carnivores or other nonrodent mammals. The available studies have generally implicated the MOE-MOB olfactory system in responses to pheromones. For example, the male pig, an ungulate, produces a steroidal pheromone, androsterone, that elicits approach behaviors as well as receptive immobility in estrous sows [21, 22]. Although the pig possesses a VNO and AOB, females' behavioral responses to androsterone seem to be mediated by the MOE-MOB projection to the forebrain [23]. In the ferret, a carnivore, pheromones derived from the urine as well as anal and sebaceous gland secretions attract heterosexual conspecifics [24]. A VNO and associated AOB have been identified in the ferret [25]. However, in the ferret as in other carnivores with a VNO and AOB [26, 27], the VNO and AOB are proportionately smaller than in rodent and ungulate species. The relatively small size of the ferret's VNO-AOB system raises the question of whether it is activated by pheromones. When gonadally intact, breeding male and female ferrets were either allowed to mate or exposed to odors from breeding animals of the opposite sex, significant increases in neuronal Fos-IR were seen in the granule cell layer of the MOB but not in the AOB [28]. Female subjects showed a significant induction of neuronal Fos-IR in central segments of the chemosensory pathway (i.e., mPOA and VLH) in response to male odors, whereas male subjects showed no such response to female odors. The absence of any odor-induced neuronal Fos response in the AOB does not definitively prove that this system is unresponsive to pheromonal signals. The presence of increased neuronal Fos-IR in the MOB suggests, however, that the main olfactory system is activated by pheromones. The observed [28] odor-induced increments in neuronal Fos-IR in the MA (both sexes), and in the BNST, VLH, and mPOA (females only) further suggest that these main olfactory inputs are conveyed to the hypothalamus, at least in females.

In our previous study [28], we observed a sex difference in hypothalamic Fos responses to different pheromones. In experiment 1 of the present study, we systematically compared the ability of pheromones from the same source

Accepted July 31, 1998.

Received June 23, 1998.

[1]This work was supported by NIH grant HD21094 and by NIMH Senior Research Scientist Award MH 00392.

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(soiled estrous female bedding) as well as a nonpheromonal odor, artificial peppermint, to augment neuronal Fos-IR throughout the chemosensory pathway to the hypothalamus in male and female ferrets. We previously compared neuronal Fos responses to pheromones in gonadally intact male and female ferrets in breeding condition [28]. Rigorous sex comparisons of neuroendocrine and behavioral functions are best carried out in gonadectomized subjects that receive either no replacement sex steroids or the same activational hormone treatment (reviewed in [29]). Therefore, in experiment 1 we asked whether concurrent exposure to a sex steroid (in this instance, testosterone propionate [TP]) is required in order for neuronal Fos responses to either odor stimulus to occur in gonadectomized male and female subjects. In experiment 2, we compared the ability of pheromones from a breeding male to augment Fos-IR in the chemosensory pathway of gonadectomized, TP-treated male and female subjects. Results obtained in experiment 1 showed that TP facilitated the ability of estrous female pheromones, but not peppermint odor, to stimulate neuronal Fos-IR in the MOB of both sexes. In experiment 3, we asked whether this effect of testosterone is associated with increased scent gathering by gonadectomized male and female ferrets that are exposed to pheromones. Finally, we asked whether either androgen or estrogen receptors are present in the ferret's MOB and are thus available to mediate the observed TP-dependent facilitation of MOB granule cell responsiveness to pheromones.

MATERIALS AND METHODS

Animals

Male and female Fitch ferrets, aged 12–19 wk, were purchased from Marshall Farms (North Rose, NY) and were housed individually in modified rabbit cages (24'' × 12'' × 18'') on a long-day (16L:8D) photoperiod. All ferrets were fed a diet of moistened Purina ferret chow and were given water ad libitum. Ferrets were gonadectomized under ketamine (95 mg/kg) and xylazine (12 mg/kg) anesthesia. All ferrets received daily s.c. injections of TP (5 mg/kg) dissolved in sesame oil. When administered to castrated male ferrets, this dose of TP elicits the full range of masculine sexual behaviors and sustains plasma levels of testosterone that are characteristic of gonadally intact male ferrets in breeding condition [30, 31]. We selected testosterone, instead of estradiol, as the activational sex steroid used in these experiments because a previous study [16] showed that TP treatment successfully augmented neuronal Fos responses to estrous odors in both male and female rats after gonadectomy. It is possible that estradiol treatment would have exerted a similar effect in rats and would have duplicated the facilitatory effects of TP on pheromone-induced neuronal Fos responses seen in the present study using ferrets. After 4 wk of TP treatment, all animals were given olfactory experience by being placed in a cage for 30–60 min on separate days with a male in breeding condition or with an estrous female. These experiments were conducted in accordance with the guidelines set forth by Guiding Principles for the Care and Use of Research Animals and were approved by Boston University IACUC (protocol #97–017).

Experimental Design

Experiment 1: Neuronal Fos-IR responses in the chemosensory pathway after exposure to soiled female bedding or peppermint odors. After receiving olfactory experience, groups of gonadectomized male (total n = 28) and female (total n = 24) ferrets either continued to receive daily TP injections or were given daily injections of oil vehicle. After 5 wk, subgroups of TP- and oil-treated males and females were exposed to one of three different stimulus conditions. Some ferrets were placed individually in a colony cage (24'' × 24'' × 18'') that contained commercial wood chip bedding previously soiled by an estrous female for two days. This bedding presumably emitted pheromones derived from urine and feces as well as anal and sebaceous gland secretions. Such soiled bedding had induced a neuronal Fos-IR response in the MOB of gonadally intact male and female ferrets in breeding condition [28]. Additional groups of subjects were placed in a plastic cage (24'' × 8'' × 8'') for 30 min, whereupon peppermint odor was pumped into the cage by passing air (60 mm Hg) over imitation peppermint extract (Burns Philp Foods, Inc., San Francisco, CA) suspended in sesame oil. Finally, other groups of ferrets were placed in a clean colony cage with clean wood chip bedding beneath the slatted floor. Each of these stimulus conditions prevailed for 1.5 h, whereupon subjects were killed with an overdose (100 mg/kg) of sodium pentobarbital (JA Webster, Leominster, MA) and were perfused via the heart with 4% paraformaldehyde.

Experiment 2: Neuronal Fos-IR responses in the chemosensory pathway after exposure to soiled bedding from a male in breeding condition. After they had received olfactory experience and had been used in a behavioral study (experiment 3), gonadectomized males (total n = 10) and females (total n = 7) received daily s.c. injections of TP (5 mg/kg) for 5 wk. Subgroups of males and females were then either placed in a colony cage that contained wood chip bedding previously soiled for two days by a male ferret in breeding condition or in a clean cage that contained clean wood chip bedding on top of the slatted floor. After 1.5 h, animals were killed with an overdose (100 mg/kg) of sodium pentobarbital and were perfused via the heart with 4% paraformaldehyde.

Experiment 3: Effects of chemosensory cues and testosterone treatment on scent-gathering behavior. After they had received olfactory experience, subgroups of gonadectomized male (total n = 10) and female (total n = 10) ferrets continued to receive daily s.c. injections of TP or oil vehicle. Beginning 4 wk later, ferrets were tested individually on three separate days in a colony cage for scent-gathering and locomotor activity. During each test, either clean wood chip bedding or wood chip bedding that had been soiled for two days by either an estrous female or a male in breeding condition was placed on top of the slatted cage floor. Each subject was tested for 30 min on each type of bedding but received only a single type of olfactory stimulus on a given test day. A video camera was positioned directly above the cage to record each test session. These video tapes were later used to score the duration of scent-gathering behavior (sniffing) and the locomotor activity (grid crossings) displayed by each subject. Sniffing behavior is easily recognized as stereotyped head bobbing and movement of the nares. In order to facilitate the measurement of ferrets' locomotor activity, white tape was placed on top of each test cage so as to divide it into 4 quadrants. An observer scored a grid crossing whenever a subject's snout crossed one of the quadrant boundaries. A single observer, who had no knowledge of either the hormonal treatments being received or the odor stimulus being presented to individual subjects, used Observer 3.0 software (Noldus Co., Wageningen, Holland) to record the number of grid crossings and the duration of sniffing for each test session.

These data were analyzed using a three-way ANOVA (sex × steroid treatment × olfactory stimulus), and post-hoc comparisons of group means were carried out using the Student-Neuman-Keuls procedure. The subjects from this behavioral study were later used in experiment 2.

Histology

The heart was exposed, and 1000 units of heparin (10 000 U/ml; Henry Schien, New York, NY) was injected into the left ventricle. Animals were perfused via the ascending aorta with approximately 50 ml of 0.1 M PBS, pH = 7.4, followed by 800 ml (for females) or 1000 ml (for males) of 4% paraformaldehyde in 0.1 m PBS at a pressure of 100 to 110 mm Hg. After perfusion, the brains were immediately removed and postfixed for 4 h in 4% paraformaldehyde. Brains were then cryoprotected in 30% sucrose/PBS at 4°C until they sank.

The right forebrain and olfactory bulb were frozen on dry ice and sectioned coronally at 30 μm using a Reichert-Jung SM2000R table-top sliding microtome. Every brain section was saved in tissue antifreeze (500 ml 0.1 M Tris-buffered saline [TBS], 300 ml ethylene glycol, 300 g sucrose, 10 g polyvinylpyrrolidone (M_r 40 000) and distilled H_2O to a volume of 1.0 L) and maintained at −20°C for later Fos or steroid receptor immunocytochemistry (ICC).

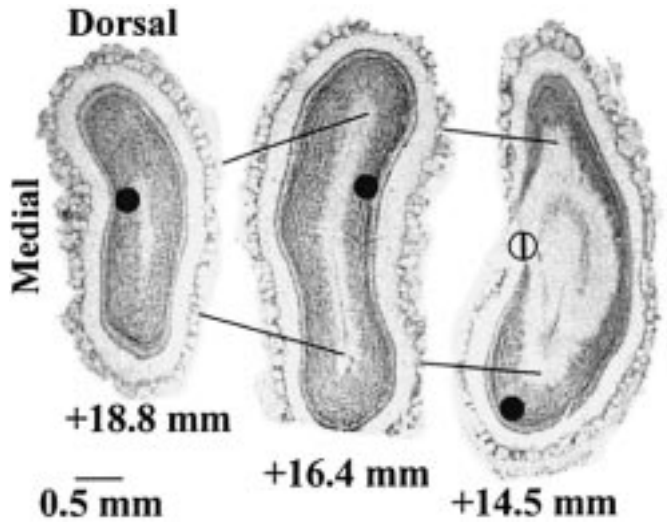

FIG. 1. Representative photomicrographs of coronal, Nissl-stained sections through a female ferret's rostral (+18.8 mm in front of the interaural line), central, and caudal olfactory bulb. Odor-induced increases in the number of Fos-IR cells were counted in the areas designated. The black circles show the location of the 0.1-mm² counting areas in the granule cell layer of the MOB. The black rectangle within the open circle in the caudal section shows the location of the 0.03-mm² counting area in the cell layer of the AOB.

ICC

Fos ICC was performed on every fourth section from the forebrain and olfactory bulb. One animal from each treatment group was included in each ICC run. Free-floating sections were incubated in 3% normal goat serum/1% H_2O_2/PBS for 1.5 h at room temperature and then transferred to primary Fos antiserum (DCH-1; 1:5000 in 0.4% Triton X-100/PBS; rabbit polyclonal antiserum raised against N-terminal amino-acids 2–17). Sections were incubated in Fos primary antiserum on a shaker for 16 h at room temperature. Sections were then rinsed four times with 0.1 M PBS at room temperature for 10 min, incubated with biotinylated goat anti-rabbit antibody (1:200; Vector Labs., Burlingame, CA) for 2 h, washed four times in 0.1 M PBS, incubated for 2 h in ABC (1:100; Vector Labs.), and washed 4 more times in 0.1 M PBS. Sections were then reacted with nickel diaminobenzidine (DAB) solution (Vector Labs.) for 7 min and washed 5 times in 0.1 M PBS. Sections were mounted on gelatin-coated slides and air-dried before being coverslipped with Permount (Fisher Scientific Co., Pittsburgh, PA). The specificity of the DCH-1 Fos-antibody in ferret brain has been demonstrated previously [28, 32].

Androgen receptor (AR) and estrogen receptor (ER) ICC were performed on alternate sections of olfactory bulb and forebrain from a subset of male and female ferrets used in experiment 1. Androgen receptor ICC was performed according to the protocol published by Kashon et al. [33] for ferret brain, using brain sections from two gonadectomized, TP-treated females and males in addition to one gonadally intact breeding male. Brain sections were preincubated with 0.5 M glycine in 0.1 M PBS for 30 min and blocked with 0.5% H_2O_2/4% normal goat serum in 0.1 M PBS for 30 min. Sections were then incubated in primary PG-21 AR antiserum [34] (1 μg/ml) for 48 h at 4°C, and biotinylated goat anti-rabbit secondary antibody (1:200; Vector Labs.) for 4 h followed by ABC (1:100) for 2 h; they were then reacted with nickel DAB solution for 4 min. Estrogen receptor ICC was performed on brain sections from three females and two males that had been gonadectomized and treated with oil vehicle [35]. Brain sections were prewashed in 0.05 M sodium meta-periodate for 15 min and sodium borohydrate for 15 min. Sections were then incubated in primary H222 ER antibody [36] (2 μg/ml), for 36 h at 4°C followed by biotinylated sheep anti-rat secondary antibody 1:200 (Amersham Life Science, Piscataway, NJ) for 2 h and ABC (1:100) for 2 h; sections were then reacted with nickel DAB solution for 7 min. We used gonadectomized TP-treated subjects for AR ICC and oil-treated subjects for ER ICC in order to maximize the likelihood of seeing any AR or ER immunoreactive cells in ferrets' olfactory bulbs.

Analysis of Neuronal Fos ICC

Slides were coded so that the investigator had no knowledge of the sex of individual subjects or the steroid treatments and olfactory stimuli that they had received. With the exception of the VLH, one section was chosen and counted for each brain region. Brain sections were viewed under a ×40 objective (×20 for BNST), and all Fos-IR cells present in standard areas of 0.1 mm² (1.0 mm² for the BNST) were traced onto blank paper with the aid of a camera lucida microscope attachment. The Fos-IR cells present in 5 adjacent brain sections that contained the VLH were also traced under a ×20 objective (0.21 mm² per section).

The number of Fos-IR cells was counted in three different regions of the MOB (Fig. 1). The most caudal area of these regions (+14.5 mm in front of the interaural line) was previously found to have numerous Fos-IR cells in ferrets of both sexes after they mated [28]. In addition, Fos-IR cells in a central (+16.4 mm in front of the interaural line) and a rostral (+18.8 mm) region of the MOB were chosen for analysis on the basis of a pilot study in which more Fos-IR cells were observed in 2 TP-treated females exposed to estrous bedding than in clean-cage control females from experiment 1. Fos-IR was counted in the gran-

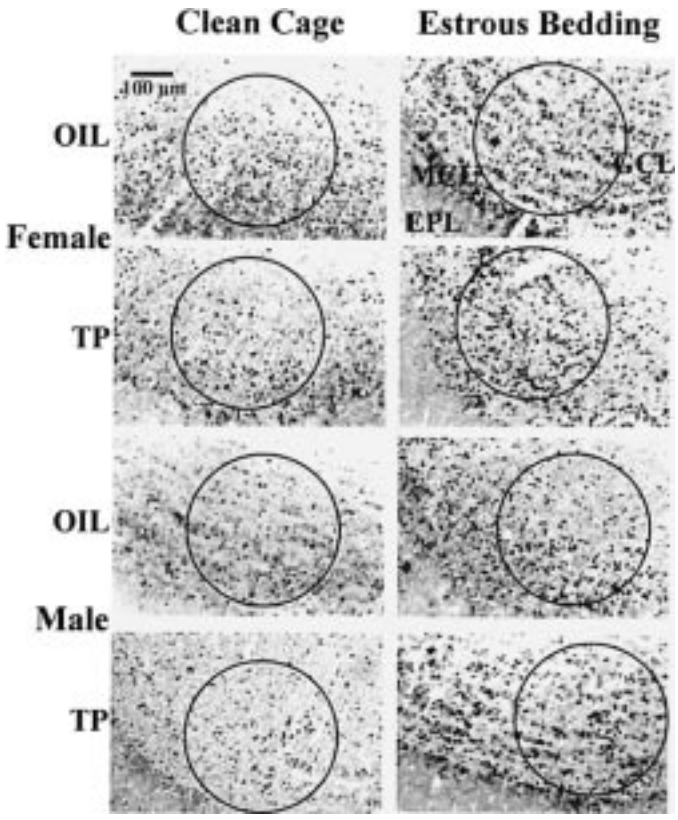

FIG. 2. Representative photomicrographs of Fos-IR cells in the caudal counting region (black circles) of the MOB from gonadectomized oil- and TP-treated female and male ferrets exposed to either a clean cage or to soiled estrous bedding in experiment 1. EPL, external plexiform layer; MCL, mitral cell layer; GCL, granule cell layer.

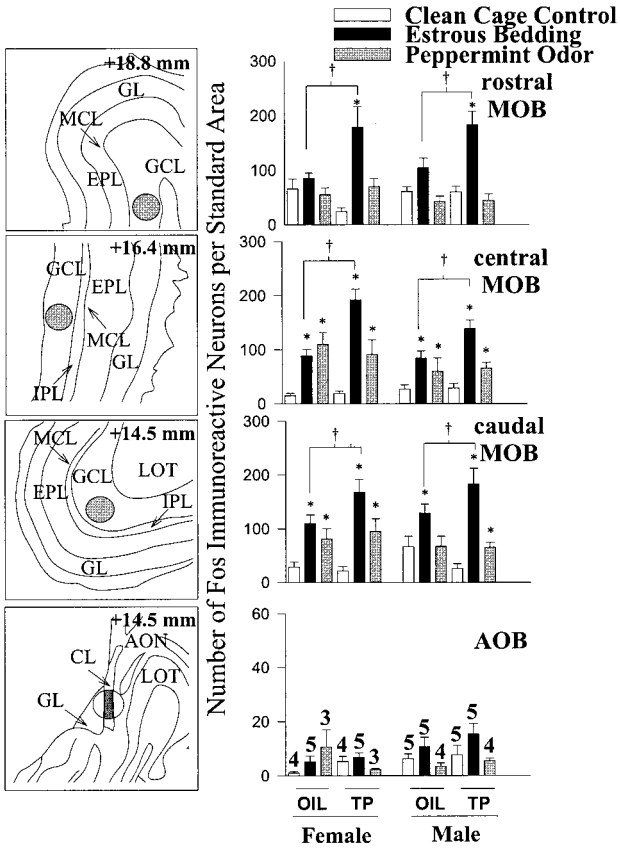

FIG. 3. **Right**) Mean number (± SEM) of Fos-IR cells in the granule cell layer of the rostral (+18.8 mm in front of the interaural line), central, and caudal MOB and in the AOB. *$p < 0.05$ compared to clean-cage control animals using post-hoc Student-Neuman-Keuls tests following a significant three-way ANOVA. †$p < 0.05$ compared to oil-treated, pheromone-exposed animals by post-hoc Student Neuman-Keuls tests following a significant three-way ANOVA. **Left**) Schematic drawings showing the location of the Fos-IR cell counting areas at three levels of the MOB (0.1 mm²) and in the AOB (0.03 mm²). GL, glomerular layer; IPL, internal plexiform layer; LOT, lateral olfactory tract; AON, anterior olfactory nucleus; and CL, AOB cell layer.

ule cell layer of the MOB because previous work in the rat [37] showed that Fos-IR was augmented in this layer after exposure to peppermint odor. Mitral cells were not counted because without Nissl counterstaining we found it impossible to distinguish them from other cell types within that layer. The cell layer of the AOB (Fig. 1) was chosen for analysis because it contains the granule cells associated with this structure.

Additional segments of the chemosensory projection pathway, including the MA, BNST, VLH, and mPOA, were studied because significant increments in neuronal Fos-IR had been seen previously in each of these regions in estrous female ferrets that received intromissive stimulation from a male [32, 28]. We also examined neuronal Fos responses to pheromones in the dorsal (d) POA/anterior hypothalamus (AH), which in male ferrets contains a sexually dimorphic male nucleus [38] and in the ventral (v) POA/AH, which contains a non-dimorphic nucleus. In experiment 1, the number of Fos-IR cells in different groups was analyzed using a three-way ANOVA (sex × steroid treatment × chemosensory stimulus). In experiment 2, Fos data were analyzed using a two-way ANOVA (sex × chemosensory stimulus). After a significant overall ANOVA result, post-hoc comparisons of pairs of group means were carried out using the Student-Neuman-Keuls procedure.

RESULTS

Experiment 1: Neuronal Fos-IR Responses in the Chemosensory Pathway after Exposure to Soiled Female Bedding or Peppermint Odors

Odors from estrous bedding significantly augmented neuronal Fos-IR in the granule cell layer of the central and caudal MOB of gonadectomized, oil-treated male and female subjects. In both sexes, TP treatment further enhanced neuronal Fos-IR responses to estrous female odors (Figs. 2 and 3). In addition, TP treatment was needed for a neuronal Fos-IR response to occur in the rostral MOB. In both sexes, peppermint odor also augmented neuronal Fos-IR in the central and caudal MOB regions studied; however, TP treatment failed to enhance the magnitude of this effect (Fig. 3). Statistical analysis showed a significant effect of odor stimulus on the number of Fos-IR cells in the most rostral counting area (+18.8 mm; Fig. 3) of the MOB ($F[2,51] = 20.642$, $p < 0.01$). In addition there was a significant steroid treatment × odor stimulus interaction ($F[2,51] = 6.055$, $p < 0.01$), reflecting the observation that estrous odors augmented neuronal Fos-IR in this site only in TP-treated females and males. There were also significant overall effects of odor stimulus in the central MOB ($F[2,51] = 40.336$, $p < 0.01$) and in the caudal MOB ($F[2,51] = 57.822$, $p < 0.01$), and there were significant steroid treatment × odor stimulus interactions in these two regions (central MOB: $F[2,51] = 5.117$, $p < 0.05$; caudal MOB: $F[2,51] = 12.679$, $p < 0.01$). In contrast to the MOB, there were no stimulatory effects in either sex of estrous odors

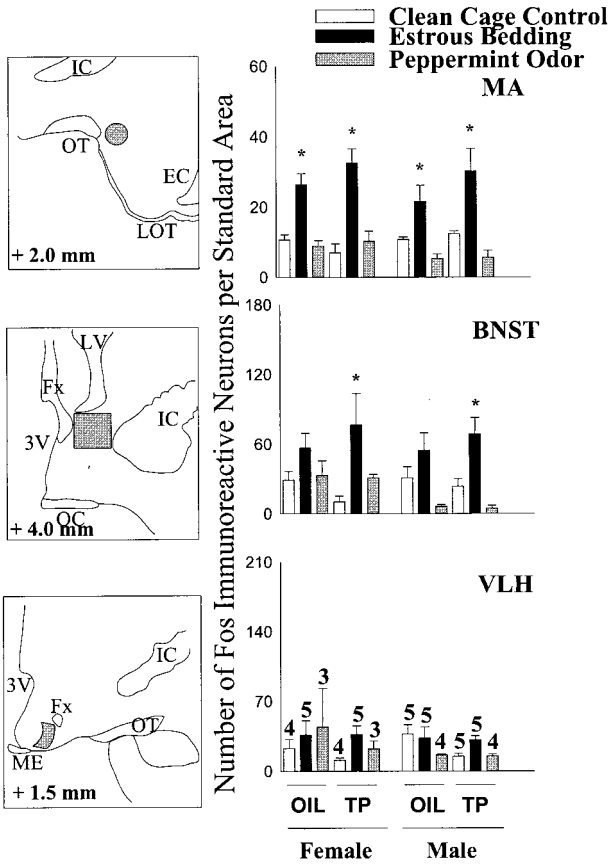

FIG. 4. **Right**) Mean number (± SEM) of Fos-IR cells in the MA (+ 2.0 mm in front of the interaural line), the BNST, and the VLH. *$p < 0.05$ compared to clean-cage control animals using post-hoc Student Neuman-Keuls tests following a significant three-way ANOVA. **Left**) Schematic drawings showing the location of the Fos-IR cell counting areas in the MA (0.1 mm^2), BNST (1.0 mm^2), and VLH (0.2 mm^2). IC, internal capsule; OT, optic tract; EC, external capsule; LV, lateral ventricle; 3V, third ventricle; Fx, fornix; ME, median eminence.

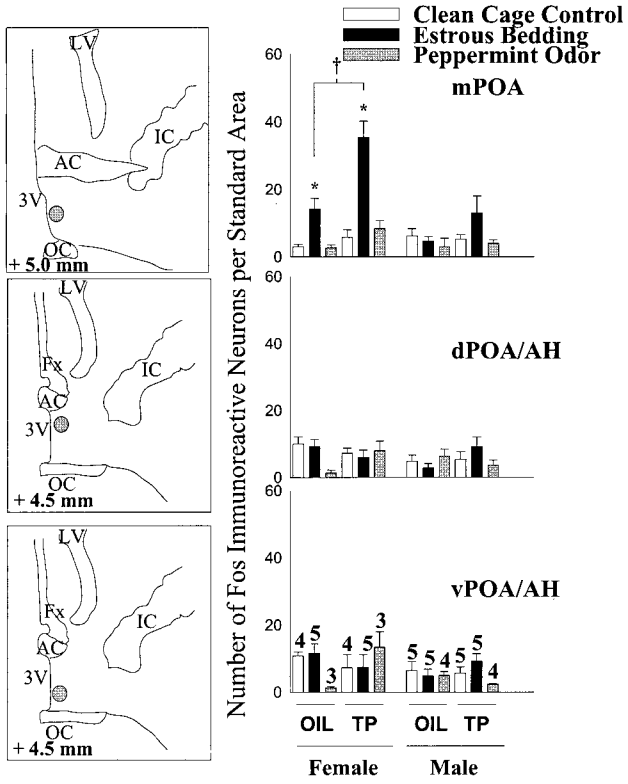

FIG. 5. **Right**) Mean number (± SEM) of Fos-IR cells in the mPOA (+ 5.0 mm in front of the interaural line), the dPOA/AH, and the vPOA/AH. * $p < 0.05$ compared to clean-cage control animals using post-hoc Student Neuman-Keuls tests following a significant three-way ANOVA. †$p < 0.05$ compared to oil-treated, pheromone-exposed females by post-hoc Student Neuman-Keuls tests following a significant three-way ANOVA. **Left**) Schematic drawings showing the location of respective Fos-IR cell-counting areas (0.1 mm^2). AC, anterior commissure; OC, optic chiasm.

or peppermint on Fos-IR in the cell layer of the AOB (Fig. 3, bottom panel).

Exposure to pheromones from estrous females induced equivalent increases in neuronal Fos-IR in the MA of gonadectomized male and female ferrets, regardless of whether they were treated with TP or oil vehicle (Fig. 4, top panel). In the BNST, estrous odors elicited significant increases in neuronal Fos-IR only in gonadectomized, TP-treated males and females (Fig. 4, middle panel). There was no effect of peppermint on neuronal Fos-IR in the MA, BNST, or VLH, nor was there any effect of estrous odors on neuronal Fos-IR in the VLH (Fig. 4, bottom panel). Statistical analyses revealed significant effects of odor stimuli on the number of Fos-IR cells in the MA ($F[2,49] = 38.399$, $p < 0.01$) and BNST ($F[2,49] = 13.363$, $p < 0.01$).

A sexually dimorphic pattern of neuronal Fos-IR was observed in the mPOA after exposure to odors from estrous females (Fig. 5, top panel). Gonadectomized, oil-treated females showed significant increments in neuronal Fos-IR, and the magnitude of this effect of estrous odors was further enhanced in females that received TP. There was no effect of estrous odors on the number of Fos-IR cells in the mPOA of males. Statistical analyses revealed a significant sex × odor stimulus interaction ($F[2,49] = 9.088$, $p < 0.01$) as well as a significant steroid treatment × odor stimulus interaction ($F[2,49] = 5.815$, $p < 0.01$). Peppermint failed to augment neuronal Fos-IR in any of the subregions of the POA, and estrous odors failed to affect neuronal Fos-IR in either the dPOA/AH or vPOA/AH (Fig. 5, middle and bottom panels).

Androgen receptor-IR was present in the MOB and AOB in both sexes. Immunopositive cells were primarily restricted to the granule and mitral cell layers of the MOB and to the cell layer of the AOB (Fig. 6) in all five animals studied. By contrast, ER-IR was not seen anywhere in the MOB or AOB of any of the five ferrets studied for this purpose (Fig. 6). Both AR-IR and ER-IR were present in the mPOA, as expected from previous reports in the ferret [33, 38].

Experiment 2: Neuronal Fos-IR Responses in the Chemosensory Pathway after Exposure to Soiled Bedding from a Male in Breeding Condition

Pheromones from soiled male bedding caused significant increases in neuronal Fos-IR in all three regions of the MOB in gonadectomized, TP-treated male and female ferrets (Table 1). As with estrous female odors (experiment 1), exposure to male odors failed to influence neuronal Fos-IR in the AOB of either sex. Exposure to male odors also caused significant increases in neuronal Fos-IR in the MA of both male and female subjects. Statistical analyses revealed significant effects of odor stimulus in the rostral MOB ($F[1,19] = 3.531$, $p < 0.05$), central MOB ($F[1,19] = 3.462$, $p < 0.05$), and caudal MOB ($F[1,19] = 8.993$, $p < 0.01$) in addition to the MA ($F[1,16] = 18.108$, $p < 0.01$).

TABLE 1. Total number of Fos-IR cells (mean ± SEM) per standard area in gonadectomized, TP-treated male and female ferrets after exposure to either a clean cage or to soiled bedding from a breeding male (experiment 2).

Brain region	Female subjects		Male subjects	
	Clean cage control (n=3)	Male bedding (n=4)	Clean cage control (n=5)	Male bedding (n=5)
MOBr[a]	48 ± 13	108 ± 34*	29 ± 9	84 ± 24*
MOBc[b]	104 ± 13	154 ± 38*	42 ± 10	112 ± 24*
MOBca[c]	72 ± 20	156 ± 18*	33 ± 13	99 ± 34*
AOB	5 ± 3	10 ± 2	6 ± 2	9 ± 3
MA	10 ± 3	30 ± 7*	8 ± 2	22 ± 3*
BNST	15 ± 8	83 ± 28*†	6 ± 1	21 ± 8*
VLH	17 ± 2	59 ± 19*†	10 ± 2	17 ± 3
mPOA	6 ± 0	36 ± 13*†	5 ± 1	6 ± 1
dPOA/AH	7 ± 2	16 ± 6	4 ± 1	7 ± 2
vPOA/AH	2 ± 1	10 ± 6	1 ± 1	3 ± 1

[a] MOBr, rostral (+18.8 mm).
[b] MOBc, central (+16.4 mm).
[c] MOBca, caudal (+14.5 mm).
* $p < 0.05$; Student Neuman-Keuls post-hoc comparisons with same-sex, clean-cage controls.
† $p < 0.05$; Student Neuman-Keuls post-hoc comparisons with males exposed to male bedding.

A sexual dimorphism was observed in the ability of male odors to elicit significant neuronal Fos-IR responses in more central segments of the chemosensory pathway. Thus male odors stimulated neuronal Fos-IR in the BNST of both sexes ($F[1,16] = 8.034$, $p < 0.05$; Table 1); however, this effect was significantly stronger in female than in male subjects ($F[1,16] = 5.904$, $p < 0.05$). Only females showed significant increases in neuronal Fos-IR in the mPOA ($F[1,16] = 5.528$, $p < 0.05$) and in the VLH ($F[1,16] = 6.516$, $p < 0.05$) after exposure to male bedding (Table 1).

Experiment 3: Effects of Chemosensory Cues and Testosterone Treatment on Scent-gathering Behavior

There was no indication that the ability of TP treatment to enhance ferrets' neuronal Fos-IR responses to estrous female odors (experiment 1) resulted from increased locomotor activity or scent-gathering behaviors in steroid-treated subjects. Treatment with TP actually reduced locomotor activity in males, but not in females, regardless of which olfactory stimulus was present at the time of assessment (Table 2). This was reflected in a significant sex × steroid treatment interaction ($F[1,56] = 15.413$, $p < 0.01$). Exposure to odors from estrous females or breeding males caused significant increases in sniffing behavior ($F[2,56] = 20.633$, $p < 0.01$) in both sexes; however, these increments were similar in gonadectomized subjects given TP as opposed to oil vehicle.

DISCUSSION

Pheromones Are Processed by the Main Olfactory System in Ferrets

In experiment 3, both male and female ferrets spent significantly more time investigating soiled than clean bedding. This finding suggests that pheromones emitted by urine, feces, and sebaceous glands may be used by ferrets to mark territory and to attract or repel conspecifics. The results of experiments 1 and 2 suggest that in ferrets of both sexes the pheromones derived from estrous females as well as from breeding males are detected by receptors in the MOE, whereupon signals are passed to the MOB. Information is then conveyed to the MA and then on to the BNST, mPOA, and/or VLH in a manner that varies with the source of the pheromones (male versus female) and the

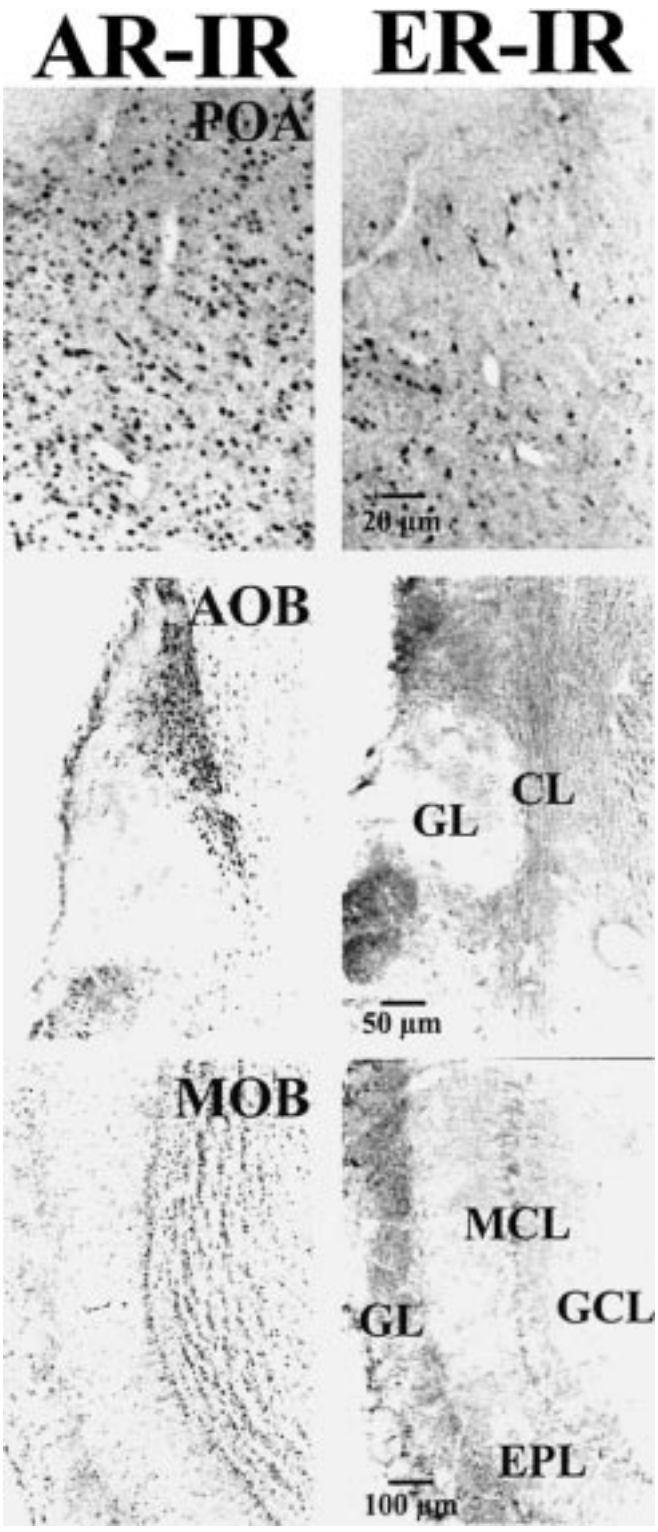

FIG. 6. Representative photomicrographs of AR-IR (**left**) in a gonadectomized, TP-treated female, and ER-IR (**right**) in a gonadectomized, oil-treated female ferret. The top two panels show examples of AR-IR and ER-IR, respectively, in the mPOA. The middle and bottom panels show AR-IR in the AOB (middle left) and MOB (bottom left), but no ER-IR in the AOB (middle right) or the MOB (bottom right). GL, glomerular layer; EPL, external plexiform layer; MCL, mitral cell layer; GCL, granule cell layer; CL, AOB cell layer.

TABLE 2. Effect of TP treatment on locomotor activity and scent-gathering behaviors displayed by gonadectomized female and male ferrets placed for 30 min either in a clean cage or in a cage containing pheromones from soiled bedding from either an estrous female or a breeding male ferret (experiment 3).

			Behavioral parameter	
Sex	Hormone treatment	Olfactory stimulus	Grid crossings[a] (total number)	Sniffing (total[a] duration in sec)
Female	Oil	Clean cage	371 ± 48	110 ± 48
		Estrous bedding	351 ± 9	289 ± 26*
		Male bedding	295 ± 27	288 ± 45*
	TP	Clean cage	422 ± 31	181 ± 31
		Estrous bedding	346 ± 34	340 ± 27*
		Male bedding	321 ± 18	336 ± 40*
Male	Oil	Clean cage	267 ± 33	104 ± 14
		Estrous bedding	347 ± 39	225 ± 33*
		Male bedding	335 ± 29	233 ± 62*
	TP	Clean cage	247 ± 28†	104 ± 28
		Estrous bedding	251 ± 28†	318 ± 53*
		Male bedding	230 ± 29†	190 ± 33*

[a] Data are expressed as mean ± SEM; n = 5.
* $p < 0.05$; Student Neuman-Keuls post-hoc comparisons with same-sex clean-cage controls.
† $p < 0.01$; represents a significant three-way ANOVA sex × steroid interaction.

sex of the subject. In contrast to their effects in the MOB, pheromones failed to stimulate neuronal Fos-IR in the ferret's AOB. This absence of response in ferrets contrasts with results from rats [39], hamsters [8], voles [40], and mice [11], in which pheromones derived from soiled bedding, urine, or vaginal secretions readily stimulated neuronal Fos-IR in the AOB granule and mitral cell layers. The absence of an AOB neuronal Fos response in the present studies using ferrets cannot, by itself, be taken as absolute proof that VNO receptors were not activated by the pheromones presented. All subjects were killed 90 min after the onset of odor presentation, and it is possible that the timing of neuronal Fos-IR induction in the AOB somehow differs from that seen in other brain regions. In a study using male hamsters [8], pheromones from female hamster vaginal secretions augmented neuronal Fos-IR simultaneously in the MOB and AOB 60 min later. It seems unlikely that the 30-min difference in the interval between the onset of odor presentation and death can account for the absence of any AOB neuronal Fos response in the present experiments or in our previous study [28] using breeding ferrets, although a time-course experiment is needed to definitively rule out this possibility. It is also possible that other biologically relevant odors, such as those derived from prey or from neonatal offspring, might activate the ferret's VNO-AOB system.

Pheromones as well as peppermint odor augmented neuronal Fos-IR in the ferret MOB; however, only pheromonal stimuli stimulated neuronal Fos-IR in the MA and in other limbic and hypothalamic segments of the olfactory pathway. Kevetter and Winans [41] showed that MOB mitral cells project to the anterior cortical and posterolateral cortical amygdala, which in turn provide input to the medial amygdaloid nucleus. Thus olfactory cues detected by the MOB potentially have access to the same limbic-hypothalamic regions that are activated by inputs from the VNO-AOB, although there is no evidence that the VNO-AOB system is connected to cortical olfactory areas. The present results suggest that pheromones detected by the MOE and initially processed by the MOB are able to augment neuronal activity in the MA, and depending on the sex of the subject and pheromonal stimulus presented, stimulate hypothalamic neurons as well. Several previous studies corroborate this conclusion. For example, Licht and Meredith [42] found that individual neurons in the hamster's MA could be activated by an electrical stimulus delivered either to receptor neurons in the MOE or in the VNO. Fiber and Swann [43] observed significant increments in neuronal Fos-IR in both the MOB and the AOB of male hamsters 1 h after exposure to female hamster vaginal secretions. Surgical removal of the VNO caused significant deficits in the sexual behavior of male hamsters, provided animals were not sexually experienced [44]. However, complete elimination of mating in sexually experienced hamsters was only achieved when chemosensory inputs from both the VNO and the MOE were eliminated [45]. This suggests that both the VNO-AOB and the MOE-MOB projections to the hypothalamus are required in order for pheromones to facilitate sexual arousal in this rodent species. Additional examples of the detection and processing of pheromones by the MOE-MOB system are found in the opossum and domestic pig. Male opossums, with their VNOs removed and their MOE intact, still discriminated between their own scent marks and those left by a conspecific [46]. Estrous sows showed proceptive and receptive sexual responses to the male's pheromone, androsterone, after the VNO was occluded [23]. Each of these results implies that pheromones affected behavior after detection and processing by the MOE-MOB olfactory pathway.

Facilitation by Testosterone of Pheromone-Induced Neuronal Fos

Three MOB sites were found at which estrous females' pheromones caused significant increases in neuronal Fos-IR. The magnitude of these effects was significantly greater in gonadectomized male and female ferrets that had received TP. Furthermore, this facilitation of neuronal responsiveness was never seen in ferrets exposed to peppermint odor. These results resemble those obtained previously by Pfaff and Pfaffman [47], who recorded extracellularly from presumed mitral cells in the MOB of castrated male rats exposed to different chemosensory stimuli. Administration of testosterone enhanced neuronal responsiveness to pheromones derived from estrous female urine but not to a nonreproductive odor, amyl acetate.

Several possible explanations for the facilitatory effect of TP on ferrets' neuronal responses to pheromones exist.

First, this androgen could act by increasing scent-gathering behaviors. Kauer [48] found that in the salamander, individual mitral cells responded to different concentrations of a particular odorant. In the present studies, a TP-induced increase in locomotion and scent gathering could have exposed ferrets to increased concentrations of particular pheromones, resulting in an activation of more mitral cells and their associated granule cells. Indeed, castrated male rats preferred to approach the pheromones in soiled estrous bedding only if they had received TP [14], and TP treatment was required in order for castrated male rats to show a significant increase in neuronal Fos-IR in the VNO-AOB olfactory pathway after exposure to these pheromones [16]. In experiment 3, we found that scent gathering (sniffing) was significantly increased in gonadectomized ferrets exposed to pheromones, regardless of whether they received TP or oil vehicle. In agreement with a previous study using rats [49], locomotor activity in male ferrets was actually reduced by TP treatment. It seems unlikely that the facilitatory effect of TP on ferrets' neuronal Fos-IR responses to pheromones can be explained by a hormone-induced increase in scent-gathering behavior.

A second alternative is that TP acted directly via ARs to augment pheromone-induced Fos-IR in the ferret's MOB. Our observation that AR-IR was present in the ferret's MOB and AOB corroborates a previous study [12] in which AR mRNA was found in the rat's MOB and AOB. Simerly et al. [12] also found low levels of ER mRNA in the MOB; however, we were unable to detect any ER-IR in either the MOB or AOB of gonadectomized ferrets. Our results suggest that if testosterone acts directly at the level of the MOB, it does so via AR and not after aromatization to estradiol and a subsequent activation of ER. Testosterone may facilitate pheromone-stimulated neuronal Fos-IR in the MOB by acting on a steroid response element in the c-*fos* gene promoter. Sex steroids have previously been shown to influence c-*fos* gene transcription (reviewed in [50]). The c-*fos* gene contains both estrogen and glucocorticoid response elements on its promoter [51], and these may also bind androgen and progesterone receptors [52]. While estrogens have been reported to increase c-*fos* gene expression [53–57], androgens have, if anything, been reported to inhibit c-*fos* gene transcription [58]. Interactions between steroid hormone receptors and their response elements on immediate early genes such as c-*fos* are sufficiently complex so as not to rule out a direct modulation of c-*fos* gene transcription by testosterone.

A third alternative is that testosterone, or estradiol formed in the brain via aromatization of testosterone, augmented activity in groups of forebrain neurons that send centrifugal projections to the MOB. For example, noradrenergic neurons in the rat's locus coeruleus project to the MOB and AOB [59] and contain AR mRNA [12]. The MOB also receives centrifugal inputs from other brain regions including the diagonal band of Broca, the Raphe nuclei, the MA, and the BNST [60], all of which contain AR as well as ER [33, 35]. Aromatization of androgen occurs in the adult ferret's MA, BNST, and mPOA [61]. Thus estradiol working via ER could mediate any effects of testosterone in these brain regions.

Sex Difference in Neuronal Fos Responses to Pheromones

Male and female ferrets showed similar neuronal Fos-IR responses to pheromones in the peripheral regions of the olfactory pathway (i.e., MOB, MA), whereas only females responded in more central regions of this circuit (i.e., BNST, VLH, mPOA). This sex difference is summarized diagrammatically in Figure 7 for gonadectomized, TP-treated ferrets exposed to pheromones derived from estrous females (experiment 1) and from breeding males (experiment 2). The present results corroborate previous studies in which similar sex differences were observed in the regional distribution of neuronal Fos-IR in gonadally intact breeding ferrets both after mating [32] and after exposure to pheromones from ferrets of the opposite sex [28]. The present experiments involved gonadectomized subjects in which the effects of the same pheromonal stimuli were systematically compared in males and females that had received either oil vehicle or TP. Our results show unambiguously that the functional responsiveness of neurons in the central segments of the olfactory pathway (i.e., BNST, mPOA, VLH) is sexually dimorphic, with pheromones derived either from estrous females or breeding males more effectively eliciting neuronal Fos responses in females.

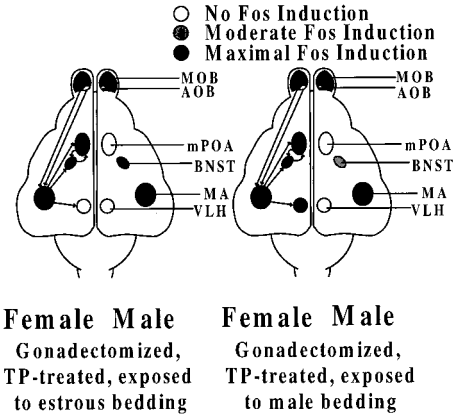

FIG. 7. Schematic representation of the observed sex differences (experiments 1 and 2) in the ability of the pheromones derived from soiled bedding of an estrous female or a breeding male to augment neuronal Fos-IR in different segments of the chemosensory projection pathway of gonadectomized, TP-treated male and female ferrets. Sexually dimorphic responses to both female and male pheromones occurred only in the central segments of the pathway (i.e., BNST, mPOA, and VLH), with females being more responsive than males. Black shading designates brain regions in which maximal neuronal Fos induction occurred. Gray shading designates a brain region (BNST) in which male subjects had significantly more neuronal Fos-IR than clean-cage controls but significantly less neuronal Fos-IR than female subjects. Unshaded regions designate brain regions in which pheromone-exposed ferrets had the same number of Fos-IR cells as clean-cage controls.

The volumes of the VNO neuroepithelium and the AOB are significantly greater in male than in female rats [62]. Recently, Herrada and Dulac [63] reported that the distribution across the apical-basal extent of the VNO neuroepithelium of mRNAs encoding putative pheromone receptor proteins is different in male and female rats. Also, the volume of more central segments of the VNO-AOB olfactory pathway, including the BNST [64] and mPOA [65] is significantly greater in males than in females. In the ferret, the volumes of the VNO neuroepithelium and the AOB [25] as well as the MA (unpublished observations) are equivalent in males and females. Obvious morphological sex differences do, however, exist in the ferret's dorsal POA at its caudal border with the AH. Males, but not females, possess a Nissl-stained cluster of large cells in this region [38], and many of these large neurons in males express the neuropeptide galanin [66]. Exposure to pheromones failed, how-

ever, to induce neuronal Fos-IR in the dorsal POA/AH of either sex. After males received bilateral excitotoxic lesions centered in this sexually dimorphic dorsal POA/AH region, they showed a female-typical preference to approach and interact sexually with other stimulus males in T-maze tests [67]. The present observation that pheromones augmented neuronal Fos-IR in the mPOA and VLH of female, but not male, ferrets raises the question of whether pheromones play a more central role in females' expression of heterosexual partner preference. Indeed, the VLH, which is activated in females after exposure to male pheromones, has been implicated in the steroidal control of feminine sexual behavior in female rodents [68] and in cats [69].

There are both similarities and differences between ferrets and rodents in their neuronal Fos responses to pheromones. For example, gonadectomized, estradiol-treated male and female rats showed equivalent increases in neuronal Fos-IR in the AOB and MA after exposure to soiled bedding from sexually active males [10]. However, only females showed significant neuronal Fos responses in the BNST and mPOA in response to male odors. These results in rodents are comparable to our results in the ferret (experiment 2). In contrast, gonadectomized, TP-treated male and female rats exposed to soiled estrous female bedding showed equivalent increments in neuronal Fos-IR throughout the VNO-AOB projection pathway, including the mPOA [9, 16]. This isomorphic profile of neuronal Fos-IR responses to female pheromones may reflect the extensive neural masculinization sustained by female as well as male rats in response to fetal testosterone exposure [70]. Independent confirmation of this hypothesis stems from reports [71, 72] that adult, gonadectomized, TP-treated female rats show nearly as much male-typical coital behavior as males. Interestingly, Fiber and Swann [43] reported that the ability of vaginal pheromones to augment neuronal Fos-IR in the magnocellular preoptic nucleus of gonadectomized, TP-treated hamsters is sexually dimorphic, with only males showing a response. The inability of female hamsters to show a neuronal Fos-IR response to vaginal secretions in this brain region is correlated with their lack of male-typical coital responsiveness to adult treatment with testosterone [73]. In experiment 1, female ferrets showed significant increases in neuronal Fos-IR in the BNST and mPOA after exposure to estrous female odors. This result is surprising, in so far as female ferrets are exposed to very low levels of testosterone perinatally and as a result are capable of showing only minimal levels of male-typical coital behavior when given TP and tested in adulthood with estrous females (reviewed in [29]). Thus female pheromones would not be expected to activate hypothalamic neurons in female conspecifics. These odors may affect other, yet-to-be-identified aspects of social interaction among female ferrets. The absence of neuronal Fos-IR responses to female pheromones in the BNST and mPOA of male ferrets (experiment 1 and ref. [28]) is even more surprising, in light of several reports (reviewed in [74]) of such effects in male rodents. The absence of a neuronal Fos response in a particular brain region does not necessarily mean that neurons in that region are totally unresponsive to a particular stimulus. The different effects of estrous female pheromones on hypothalamic Fos responses in male rodents and ferrets suggests, however, that significant differences exist in the roles that pheromones play in aspects of social interaction and neuroendocrine function among males of different mammalian orders.

ACKNOWLEDGMENTS

We thank Drs. Gerard Evan and David Hancock for generously providing the DCH-1, and Dr. Geoffrey Greene for the PG-21 and H222 antibodies. We also thank the staff of the Boston University Laboratory Animal Care Facility for caring for our ferrets.

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