Published in final edited form as:

Physiol Behav. 2007 February 28; 90(2-3): 438–449.

Bilateral damage to the sexually dimorphic medial preoptic area/ anterior hypothalamus of male ferrets causes a female-typical preference for and a hypothalamic Fos response to male body odors

Olga V. Alekseyenko, Patricia Waters, Huiquan Zhou, and Michael J. Baum*

Department of Biology, 5 Cummington St., Boston, MA 02215, USA

Abstract

Previous studies showed that bilateral lesions of the male ferret's preoptic area/anterior hypothalamus (POA/AH), centered in the sexually dimorphic nuclei present in this region, caused subjects to seek out a same-sex male, as opposed to a female conspecific. Male subjects with POA/AH lesions (which were also castrated and given estradiol) displayed female-typical receptive behavior in response to neck gripping by a stimulus male, implying that subjects' approaches to a same-sex conspecific were sexually motivated. We asked whether the effect of POA/AH lesions on males' partner preference reflects a shift in the central processing of body odorant cues so that males come to display a female-typical preference to approach male body odorants. Sexually experienced male ferrets in which electrolytic lesions of the POA/AH caused bilateral damage to the sexually dimorphic male nucleus (MN) resembled sham-operated females by preferring to approach body odors emitted from anesthetized male as opposed to female stimulus ferrets confined in the goal boxes of a Y-maze. This lesion-induced shift in odor preference was correlated with a significant increase in the ability of soiled male bedding to induce a Fos response in the medial POA of males with bilateral damage to the MN-POA/AH. No such partner preference or neural Fos responses were seen in sham-operated males or in other groups of males with POA/AH lesions that either caused unilateral damage or no damage to the MN-POA/AH. Male-typical hypothalamic processing of conspecifics' body odorants may determine males' normal preference to seek out odors emitted by female conspecifics, leading to mating and successful reproduction.

Keywords

Pheromone; Sexual behavior; Estradiol; Brain sexual differentiation

1. Introduction

In mammals exposure to testosterone derived from the testes during species-specific perinatal periods is responsible for male-typical differentiation of the capacity to show mating behavior later in life and for the motivation to seek out and approach opposite-sex (female) as opposed

* Corresponding author. Tel.: +1 617 353 3009, fax: +1 617 353 0996. E-mail address: baum@bu.edu (M.J. Baum).

Publisher's Disclaimer: This article was originally published in a journal published by Elsevier, and the attached copy is provided by Elsevier for the author's benefit and for the benefit of the author's institution, for non-commercial research and educational use including without limitation use in instruction at your institution, sending it to specific colleagues that you know, and providing a copy to your institution's administrator. All other uses, reproduction and distribution, including without limitation commercial reprints, selling or licensing copies or access, or posting on open internet sites, your personal or institution's website or repository, are prohibited. For exceptions, permission may be sought for such use through Elsevier's permissions site at: <http://www.elsevier.com/locate/permissionusematerial>

to same-sex (male) conspecifics for the purpose of mating and reproduction [33]. In the male ferret the differentiation of male-typical partner preference depends on the action of testosterone beginning over the last quarter of the 41-day gestation and extending out to ~ postnatal day 20. Thus administration of testosterone to female ferrets over this entire perinatal period masculinized their partner preference profile in T-maze tests given later in life (following ovariectomy and treatment with estradiol) and caused these female subjects to display male-typical neck grip behavior towards the stimulus female that they approached [3]. In the absence of perinatal testosterone treatment, control females later displayed a female-typical partner preference (for a male) in T-maze tests and showed sexually receptive behavior in response to neck gripping by a stimulus male. The fact that subjects in these T-maze tests engaged in mating behavior after approaching a particular stimulus animal suggests their approach behaviors were sexually motivated.

Fetal exposure over the last quarter of the 41-day gestation of the male ferret's brain to estradiol, which is derived from the neural aromatization of testosterone, organizes the differentiation of a sexually dimorphic cluster of neurons in the dorsal preoptic area/anterior hypothalamus (POA/AH), which we have called the male nucleus (MN) of the POA/AH because it is present only in the male [9,30]. Several previous studies suggest that the integrity of the male ferret's MN-POA/AH is required for the male-typical preference to seek out and mate with a female as opposed to a male conspecific. In an initial study [10] discrete, bilateral electrolytic lesions of the MN-POA/AH caused castrated, adult male ferrets to display a female-like motivation to approach a stud male in an L-maze in response to increasing doses of estradiol benzoate (EB). These lesioned males also displayed receptive behavior in response to the neck grips of a stimulus male, suggesting that their approach behaviors were sexually motivated. In a subsequent study [22] bilateral excitotoxic lesions centered in the MN-POA/AH caused male ferrets to display a female-like preference to approach and interact sexually (display receptive behavior) with a stud male as opposed to an estrous female in T-maze tests. In another study [19] bilateral electrolytic lesions centered in the MN-POA/AH caused male ferrets to switch their preference to approach distal cues (odor, sight, and sound) from a stud male as opposed to an estrous female in T-maze tests with subjects restrained in the goal boxes behind a wire mesh. In both of these latter studies castrated male subjects, like ovariectomized female controls, all received EB at the time a female-typical preference to approach the male goal box was observed in males with bilateral damage to the MN-POA/AH.

The observation [19] that males with bilateral lesion damage to the MN-POA/AH, like sham-operated females, showed a strong preference to approach distal cues from a stud male as opposed to an estrous female raised the possibility this female-typical partner preference reflected subjects' motivation to approach body odorants from the stud male. Subsequent experiments established an obligatory role of olfactory cues in ferrets' heterosexual partner preference. Thus inducing peripheral anosmia disrupted the preference of ferrets of both sexes to approach an opposite-sex conspecific in Y-maze tests [17]. Surgical destruction of the vomeronasal organ (VNO) failed to disrupt female ferrets' motivation to approach volatile odors emitted from a stud male as opposed to an estrous female [36]. This implies that the detection of volatile body odors by the main olfactory epithelium, and not the VNO/accessory olfactory system, is responsible for odor-based mate recognition in the ferret. Indeed, ferrets of both sexes showed differential patterns of glomerular Fos expression in the main olfactory bulb following exposure to male vs. female anal scent gland odorants [35]. In other studies gonadectomized male and female ferrets that were primed with testosterone and estradiol, respectively, showed a strong preference to approach volatile body odors emitted from anesthetized opposite-sex ferrets [16] as well as volatile anal scent gland odorants from opposite-sex ferrets [11] in Y-maze tests. Female subjects also showed a preference to approach urinary odors from males, although male subjects failed to show a strong preference to approach female urinary odors in Y-maze tests [11].

Early studies in female rats [2,5], hamsters [29] and mice [13] showed that exposure to body odors emitted from soiled male bedding in mice and rats and from vaginal secretion in hamsters stimulated Fos expression in a VNO projection circuit including the accessory olfactory bulb, the medial amygdala, bed nucleus of the stria terminalis, medial preoptic area, and ventromedial hypothalamic nucleus. In male rodents exposure to soiled bedding from other males typically activated Fos expression in the distal portions of the VNO circuit (AOB; medial amygdala), but not in the above-listed hypothalamic targets of VNO inputs. In ferrets of both sexes exposure to soiled male bedding stimulated Fos expression to an equivalent degree in the main olfactory bulb whereas no Fos activation was seen in the AOB [18]. Likewise, odors emitted from soiled male bedding stimulated equivalent Fos in the medial amygdala and BNST of male and female ferrets; however, only females showed Fos responses to male odors in the mPOA and VMH [18].

In the present study we hypothesized that the placement of electrolytic lesions of the medial POA/AH of male ferrets which damaged the MN-POA/AH would cause them to display a female-typical profile of preference for body odors, including anal scents and urinary odors. In addition, we predicted that adult male ferrets with bilateral lesion damage to the MN-POA/AH would show a female-typical profile of hypothalamic Fos responses in response to odors emitted from soiled male bedding.

2. Methods

2.1. Subjects

Retired breeder male (castrated) and female (ovo-hysterectomized) European Fitch ferrets were purchased from Marshall Breeding farms (North Rose, NY, USA). Males were housed individually and females in pairs in modified rabbit cages under controlled illumination (lights on from 6:00 am to 10:00 pm). Ferrets were fed moistened Purina ferret chow once daily, and water was available ad libitum. Animal housing and all experiments were conducted in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Boston University IACUC.

2.2. Placement of brain lesions

Male ferrets received either bilateral electrolytic lesions targeting the MH-POA/AH or sham lesions whereas females only received sham lesions using sterile technique under combined xylazine (4 mg/kg)/ketamine (35 mg/kg) anesthesia. Following induction of anesthesia, ferrets' heads were fixed in Kopf stereotaxic apparatus equipped with a squirrel monkey head adapter and ferret ear bars. A tungsten lesioning electrode (0.5 mm diameter) insulated with epoxy except for 1 mm at the tip was lowered using the following coordinates which were determined empirically: 5.5 mm anterior to the intra-aural line, 0.8 mm lateral to the midline sinus, and 12.4 mm below dura. The anode of a Grass Lesion Maker was connected to the lesioning electrode while the cathode was inserted into the anus. Lesions were made by passing 2.5 mA of direct current for 60 s. Sham lesions were performed using identical surgical procedure without passing any electrical current. The actual lesion sites were verified in each subject by visual inspection of Cresyl Violet stained brain sections after the animals were sacrificed (see below).

2.3. Experimental design

2.3.1. Hormone treatments—Beginning 3 days after arrival in the colony, castrated male began receiving daily s.c. injections of testosterone propionate (TP; 5 mg/kg in sesame oil) whereas ovo-hysterectomized females received daily s.c. injections of estradiol benzoate (EB; 15 µg/kg in sesame oil). Beginning 7 days later male and female subjects received a preliminary series of Y-maze odor preference tests (details below) over a 9-day period while these hormone

treatments continued. Males and females received TP and EB, respectively, in order to duplicate the profile of major circulating sex steroids in adult male and female ferrets, thereby giving subjects of both sexes an optimal circumstance in which to display preferences for different social body odors in pre-operative Y-maze tests. After completion of these preliminary tests, hormone treatments were stopped whereupon subjects received brain lesions or sham operations. Beginning 7 days later, daily s.c. injections of EB (15 µg/kg) were given to both male and female subjects. We adopted this procedure of giving EB to both sexes from our previous experiments [19,22] so that any differences in odor preferences between the sexes would reflect sex differences in brain mechanisms processing odors as opposed to a sex difference in the steroid hormones that circulated at the time of testing. Beginning 7 days after the onset of EB injections, subjects received a series of post-operative Y-maze tests of odor preferences over a 9-day period. Subjects continued to receive EB injections over the next 1–2 weeks until they were exposed to selected odors and sacrificed so that odor-induced Fos responses could be studied in the amygdala and hypothalamus.

2.3.2. Y-maze tests—Ferrets were tested in a Y-maze [17] that allowed the presentation of various types of odors from male and female ferrets in breeding condition. The maze consisted of a “start” box (18×12×12 in.), two “goal” boxes and a stainless-steel box (48×36×12 in.) divided by stainless-steel triangle into a Y-shaped maze connecting the start box with goal boxes. The opaque Plexiglas doors of the goal boxes were constructed so that they prevented the sight of a stimulus behind the doors while allowing the passage of air from the goal box into the maze. The entire maze was covered with Plexiglas panels that created an airtight seal. Air was pulled into the maze through the goal boxes using an exhaust fan, which was attached to the start box with PVC tubing. This air was vented from the test room. The maze was washed with a diluted bleach solution followed by 70% ethanol before the onset of tests for each subject. During each subject’s repetitive trials the maze was cleaned with 70% ethanol whenever the subject soiled the apparatus.

All subjects received Y-maze tests to assess their preference for different olfactory stimuli emitted by a breeding male vs. an estrous female ferret. Animals received three separate series of tests for their preferences to approach the goal boxes containing the following olfactory stimuli: body odors emitted from anesthetized male vs. female stimulus ferrets; odors emitted from male vs. female anal scent gland secretions; and odors emitted from male vs. female urine, exactly as described previously [11]. Breeding male and female stimulus ferrets were anesthetized with a combination of xylazine (4 mg/kg) and ketamine (35 mg/kg) prior to being placed into separate goal boxes. Frozen anal scent glands from two breeding males and two estrous females were purchased from Marshall Farm, thawed, the contents were removed and sonicated. The secretions from two individuals of the same sex were combined and diluted 1:100 in mineral oil, aliquotted and frozen until use. Each subject was presented with 1 ml aliquot of the solution from each sex, pipetted into weigh boats (4.5×4.5 cm) and placed approximately 3 cm behind the closed opaque goal box door. Urine was collected from breeding males and estrous females placed in a clean cage alone for 4 hours over a stainless steel collecting tray. Undiluted urine that had not been contaminated with fecal matter was combined from the same-sex stimulus ferrets, aliquotted and frozen until use. 1 ml of undiluted urine of each sex was presented to the subject in the same way as the anal scent secretions were presented.

Each of the 3 types of olfactory stimuli was presented for three consecutive days, comprising a total of 9 days of odor preference testing. Each day’s testing began by placing a subject in the start box for 30 s, whereupon the door was raised and the subject was allowed to approach (free trial) stimuli emanating from either of the two goal boxes (one emitting odorant stimuli from a breeding male and the other emitting odors from an estrous female). A choice for male or female stimuli was recorded when the subject made nasal contact with one of the goal box

doors. The latency to approach the preferred goal box was recorded during each trial. Subjects were given up to 120 s to make a choice, whereupon they were returned to the start box. If the subject failed to make a choice within 120 s, the trial was counted as void, and the animal was returned to the start box. After each free trial (tests with anesthetized stimulus ferrets only), a barrier was placed in the Y-maze to block access to the goal box chosen by a particular subject on the previous free trial. The start box door was then raised so that the subject could only approach (guided trial) the goal box not chosen on the previous trial. In this way, subjects were frequently reminded of the location of the two alternative social stimuli available in the two goal boxes. Daily test sessions were comprised of eight free trials and seven guided trials (a total of 24 free trials and 21 guided trials over the three days per stimulus condition). After subjects made a choice to run to particular anesthetized stimulus ferrets in each trial, the goal box door was lifted and the subject was allowed to make nasal contact with the stimulus for 5 s, as in our previous study [11]. We reasoned that such direct contact would lead to an activation of VNO inputs, which would enhance the reward salience of the particular suite of odor stimuli emitted from male vs. female stimulus ferrets. Tests of animals' preferences to approach anal scent and urinary stimuli only included 'free trials' (8 per day for 3 days) in so far as a previous study [11] suggested that ferrets of both sexes showed a sex-typical preference for these odorants in Y-maze tests in the absence of any 'guided trials'. Also, in the present study subjects were never allowed to gain direct physical access to anal scent gland and urinary stimuli after each trial. This was done because in a pilot study we found that ferrets destroyed these stimuli whenever they were allowed physical access to them. Since these anal scent gland and urinary odorants were in scarce supply, we decided to avoid this problem by restricting ferrets' physical access to them. Every subject was presented with fresh anal scent gland and urinary stimuli during daily test sessions. Male and female olfactory stimuli were placed in the same respective goal boxes on each test day; however, the location of the goal boxes was alternated each day. The mean percentage of free trials (out of 24) on which each subject approached the male and female stimuli were computed for each pair of stimulus conditions.

2.3.3. Terminal odor exposure—Soiled male bedding was collected from a breeding male ferret that was placed on fresh wood-chip bedding for 2 days. The bedding was collected and used the same day as a source of male body odorants [18]. Prior to sacrifice, half of all sham-operated male and female subjects were exposed to clean bedding; the other half received the exposure to freshly soiled male bedding. All lesioned males were exposed only to soiled male bedding. Subjects were food and water deprived 24 hours prior to odor exposure in order to reduce the chance of self-soiling during the terminal odor exposure session. Prior to the final odor presentation session, individual subjects were placed in a clean test chamber (26×26×20 cm) for 90 min of habituation. If the chamber was soiled during the habituation time, the session was interrupted, and the chamber was replaced by a new clean chamber for another 90 min. Then, subjects were placed in a same size chamber with either clean wood chip bedding or soiled male bedding for another 90 min. At the end of this period ferrets were given a lethal i.p. injection of sodium pentobarbital (100 mg/kg; Butler Co., Columbus, OH) where-upon they were perfused via the heart with PBS followed by 4% paraformaldehyde.

2.4. Tissue preparation and Fos — immunohistochemistry on brain sections

After transcardial perfusion, the brains were removed and further fixed in 4% paraformaldehyde for 2 hours followed by cryoprotection in 30% sucrose for 48 hours at 4 °C. Brains were sectioned coronally at 30 µm using a freezing sledge microtome. Every fourth section (120 µm intervals) was collected as a set (4 sets total). One set was mounted onto gelatin-coated slides in exact sequential order and stained with Cresyl Violet to assess any lesion damage. Each individual subject's lesion was mapped on a series of coronal templates of the ferret forebrain [19]. After group membership was determined, individual maps were superimposed in order to create the summary diagrams shown in Fig. 1.

Another set of free-floating sections was processed for Fos protein immunohistochemistry (Fos-IR). A primary rabbit polyclonal anti-*Fos* antibody (1:5000; Santa Cruz Biotechnology, CA) was used according to the protocol described elsewhere [18]. After incubation in the primary Fos antiserum, sections were incubated in biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA; 1:400) followed by ABC kit/nickel-diaminobenzidine visualization (Vector Laboratories). Sections were mounted on slides; washed in dionized water, and cover-slipped using Permount (Fisher Scientific Co., Pittsburgh, PA).

2.5. Data analysis

All statistical analyses were carried out using SPSS for Windows software (Version 10.0; SPSS, Inc., Chicago, IL, USA). Preference score data were analyzed across all groups using non-parametric Kruskal–Wallis 1-way ANOVAs. Provided a significant H value was obtained, 2-tailed Mann–Whitney U tests were used for post-hoc comparisons of pairs of means.

For neuronal *Fos* activation analysis slides were coded so that the investigator had no knowledge of the sex and treatment of the individual subjects. *Fos*-positive cells were counted for mPOA, BNST, MA and VMH brain regions. One section was picked and analyzed for each brain region (Fig. 5, left panel), with the exception of the VMH where five adjacent sections were used to calculate the mean value for each animal. mPOA and MA brain sections were viewed under $\times 40$ whereas BNST and VMH under $\times 20$ objective. All *Fos*-positive cells present in the microscope field of view were traced onto a blank sheet of paper with the aid of a camera lucida microscope attachment and counted for both left and right hemispheres separately, and then averaged. In mPOA/AH lesioned animals *Fos*-positive cells in mPOA region were counted rostral to the lesion. In one animal with extensive bilateral lesion damage of the mPOA *Fos*—positive cells were left uncounted for these brain regions. Another animal from bilateral MN-POA/AH damage group had a poor morphology of VMH sections resulting in 5 instead of 6 data points for *Fos*-counts in VMH for this experimental group. *Fos* data were analyzed using parametric one-way ANOVAs, with lesion as a main factor run for male subjects exposed to soiled bedding only and followed by a post-hoc LSD test to compare the *Fos* responses in different lesion groups. When needed, comparisons of pairs of means between clean bedding- and soiled bedding-induced *Fos*-activation data were made using independent two-tailed *t*-tests.

3. Results

3.1. Extent of electrolytic lesion damage

Based on an analysis of brain sections, male subjects that received brain lesions were divided into 3 groups according to lesion placement: bilateral lesion group with lesions centered in the dorsomedial POA/AH so as to cause bilateral damage to the sexually dimorphic MN-POA/AH ($n = 8$); unilateral lesion group when MN-POA/AH damage was observed in one hemisphere leaving the MN-POA/AH intact in the other hemisphere ($n = 5$, 4 males with lesions in the left hemisphere and 1 with a right hemisphere lesion); missed lesion group when no damage to the sexually dimorphic MN-POA/AH was found in either hemisphere despite the presence of extensive damage to the mPOA/AH ($n = 16$). Schematic maps showing the maximal extent as well as the common overlap of lesions given to ferrets in these three different groups are shown in Fig. 1. Two animals from “no MN-POA/AH damage” group had lesions extending beyond the final caudal section shown on the map.

No evidence of neural damage was found in sham-operated males ($n = 13$) or females ($n = 10$).

3.2. Odor preferences in Y-maze tests

Before surgery all 4 groups of male subjects preferred to approach an anesthetized female whereas females that later received sham operations preferred to approach an anesthetized male (Fig. 2). An overall ANOVA revealed a significant effect of group ($H = 11.3$, $df = 4$, $P = 0.023$), and *post-hoc* comparisons showed that all 4 groups of males approached the anesthetized male stimulus on significantly fewer trials than females that later received a sham operation. After brain surgery, sham-operated females, sham-operated males, and the groups of males in which there was no damage or only unilateral damage to the MH-POA/AH showed the same preferences to approach anesthetized stimulus animals that they had shown pre-operatively (Fig. 2). By contrast, males with bilateral lesion damage to the MN-POA/AH switched their preference so that they, like sham-operated females, preferred to approach odors emitted from an anesthetized stimulus male. An overall ANOVA revealed a significant effect of group ($H = 22.0$, $df = 4$, $P < 0.001$), and *post-hoc* comparisons showed that sham-operated males as well as males that sustained either no damage or unilateral damage to the MN-POA/AH (but not males that had bilateral damage to this nucleus) preferred to approach the anesthetized male on significantly fewer trials than sham-operated females. Furthermore, males with bilateral damage to the MN-POA/AH preferred to approach the anesthetized stimulus male on significantly more trials than sham-operated males.

When anal scent gland secretions were used as olfactory stimuli, all groups of males, when tested before brain surgery, showed a clear preference to approach the female odorants whereas females destined to receive sham operations preferred to approach the male odorants (Fig. 3). An overall ANOVA revealed a significant effect of group ($H = 14.4$, $df = 4$, $P = 0.006$), and *post-hoc* comparisons showed that males in all 4 treatment groups preferred to approach the male stimulus on significantly fewer trials than the females destined to receive a sham operation. However, after brain surgery, there were no significant differences among the 5 groups of ferrets in the preference to approach either male or female anal scent gland odorants ($H = 3.1$, $df = 4$, $P = 0.534$; Fig. 3).

When male vs. female urinary stimuli were presented before surgery, female subjects destined to receive sham operations preferred to approach the male urinary odorants whereas 3 out of 4 groups of males showed a significantly lower preference to approach these odorants (Fig. 4). An overall ANOVA revealed a significant effect of groups ($H = 12.6$, $df = 4$, $P = 0.013$), and *post-hoc* tests showed that males destined to receive sham lesions or lesions that caused either no or bilateral damage to the MN-POA/AH preferred to approach the male urinary odorants on significantly fewer trials than females destined to receive sham operations. As with anal scent gland odorants, postoperatively, there were no significant differences in urinary odor preferences among the 5 groups of ferrets ($H = 1.9$, $df = 4$, $P = 0.751$; Fig. 4).

The latency to approach either male or female odorants was not significantly different among experimental groups in all three olfactory stimuli testing series (data not shown).

3.3. Neuronal Fos responses to soiled male bedding

Sham-operated females exposed to soiled male bedding had significantly ($t = 2.7$, $df = 13$, $P = 0.019$) more Fos-IR cells in the MA than other sham-operated females that were exposed to clean bedding (Figs. 5 and 6A–B). A similar non-significant trend was seen in the MA between sham-operated males that were exposed to these same 2 types of bedding (Fig. 5). There was a non-significant trend for sham-operated females exposed to soiled male bedding to have more Fos-IR cells in the BNST, mPOA (Fig. 6C–D), and VMH (Fig. 5) than females exposed to clean bedding. Males with bilateral lesion damage to the MN-POA/AH had significantly more Fos-IR cells in the mPOA than any of the other 3 groups of male subjects following exposure to soiled male bedding (Figs. 5 and 6E–F). Among males exposed to male soiled bedding, there

was an overall effect of lesion location ($F_{(3,22)} = 3.7$, $P = 0.03$), and subsequent *post-hoc* tests showed that males with bilateral MN-POA/AH damage had significantly more Fos-IR cells in the mPOA than any other group of males. Further analysis (independent *t*-tests on pairs of means) revealed that following exposure to soiled male bedding, the number of Fos-IR cells in the mPOA of sham-operated females was similar to the number present in males with bilateral MN-POA/AH damage ($t = 0.5$, $df = 12$, $P = 0.656$), but significantly higher than the number of Fos-IR cells present in the mPOA of the 3 other groups of males ($t = 2.4$, $df = 15$, $P = 0.033$ for sham-operated males; $t = 2.8$, $df = 12$, $P = 0.019$ for lesioned males with no damage to the MH-POA/AH; and $t = 3.1$, $df = 12$, $P = 0.013$ for lesioned males with unilateral MN-POA/AH damage). In addition, the number of Fos-IR cells in the mPOA was significantly higher ($t = 2.4$, $df = 8$; $P = 0.046$) in males with bilateral MH-POA/AH damage that were exposed to soiled male bedding than in sham-operated males that were exposed to clean bedding (Fig. 5).

4. Discussion

The major finding of this study was that bilateral damage to the sexually dimorphic MN-POA/AH caused male ferrets to display a female-like preference to approach odors emitted from anesthetized male as opposed to female conspecifics. In addition, there was a suggestion that these same males showed a female-typical pattern of neural activation, as indexed by increased Fos-IR in the mPOA, in response to male odors. The profile of odor preference shown in the present study by males with bilateral MN-POA/AH damage when tested with anesthetized stimulus ferrets corroborates our previous observation [19] that such lesions caused males to prefer to approach awake, active males restrained behind a wire mesh barrier. The present findings strongly suggest that the motivation for this female-typical approach behavior reflected the central processing of odor cues, as opposed to visual or auditory signals. Indeed, in another previous study [17] anosmic male and female ferrets were unable to use visual or auditory cues from conspecifics to direct a heterosexual partner preference in Y-maze tests similar to those used in the present study. This was even true when anosmic subjects were allowed to interact sexually with a tethered stimulus female after each Y-maze trial. As explained in the Introduction, there is additional evidence from several of our previous studies [3,10,19,22] that ferrets' odor-based motivation to approach conspecifics in T-maze tests was also sexually motivated.

In a recent study [24] bilateral electrolytic lesions of the VMH eliminated the preference of ovariectomized, estradiol-treated female ferrets to approach an anesthetized male as opposed to a female ferret in Y-maze tests identical to those run in the present experiment. In contrast to the effects of bilateral MN-POA/AH damage in male ferrets (present study), placement of VMH lesions in females eliminated their preference to approach male odors but did not motivate them to seek out same-sex (female) odors. These results suggest that female-typical odor-based mate recognition depends critically on the integrity of the VMH. As explained in the Introduction, in male ferrets perinatal exposure to testosterone and/or to estradiol synthesized by the neural aromatization of this androgen promotes the male-typical development of a preference to seek out body odors emitted from females as well as the differentiation of a sexually dimorphic MN-POA/AH. Results of the present study raise the possibility that in male ferrets afferent inputs from the MN-POA/AH to the VMH may normally override the processing of body odors which would otherwise motivate a female-typical preference for male odorants. New studies are needed to test this hypothesis.

It is noteworthy that in the post-operative tests given in the present study male and female sham-operated subjects failed to show the sexually dimorphic preference to approach anal scents from opposite sex ferrets which they had displayed in pre-operative tests. Perhaps not surprisingly, there was also no statistically reliable shift in the preference for male vs. female

anal scents in males with bilateral damage to the MN-POA/AH, although there was a trend in this direction. It seems likely that one factor contributing to the preoperative preference of male subjects to approach female anal scent gland odors was that these castrated subjects received TP at the time of testing. By contrast, during post-operative testing male as well as female subjects received EB. This was done so that any female-typical preferences seen in lesioned male subjects would reflect the neural damage as opposed to a difference between the sexes in the sex hormone being administered at the time that partner preference was assessed. In a previous study [8] castrated male ferrets showed a significant preference to investigate wood blocks previously soiled by female vs. male conspecifics when they received TP but not EB. By contrast, in that study ovariectomized female ferrets preferred to investigate wood blocks soiled by male conspecifics, regardless of whether they received TP or EB at the time of testing. In the present study sham-operated males, like males with unilateral or no damage to the MN-POA/AH, continued to prefer to approach an anesthetized stimulus female even though all males were receiving EB during these tests. During this part of the study each subject was given a brief opportunity for nasal contact with the anesthetized stimulus ferret after each Y-maze trial. Previous studies using female ferrets [36] as well as male and female mice [15, 21] suggest that VNO inputs that occur when subjects have direct nasal contact with an opposite sex conspecific may be rewarding, and thus enhance subjects' motivation to approach this same stimulus on subsequent trials. For practical reasons explained in the Methods section, no opportunity was provided for direct nasal contact with either anal scents or urinary odors after each Y-maze trial in the present study. In males especially, the absence of VNO inputs in preference tests for these latter odorants, together with the fact that they were receiving EB instead of TP, may have attenuated their motivation to approach these stimuli.

In the present study, as in two previous studies [18,34] exposing female ferrets to soiled male bedding caused a significant increase in Fos-IR cells in the MA over the level seen in females exposed to clean bedding. In one of these previous studies [18] male ferrets also showed a significant increase in MA Fos after exposure to male bedding. By contrast in the present study there was only a non-significant trend of increased Fos in the MA of sham-operated males. As in the case of ferrets' Y-maze preference responses, the different outcomes between the present and past studies may reflect, in part, the fact that EB was administered to the castrated males in the present study whereas previously [18] TP was administered at the time subjects were exposed to odors and killed. Even so, castrated, EB-treated males that sustained bilateral damage to the MN-POA/AH did have significantly more Fos-IR cells in the mPOA than any other group of males, regardless of whether they were exposed to soiled male or clean bedding. This mPOA Fos response in these lesioned males resembled that seen in the mPOA of female ferrets exposed to male odors in previous studies [18,34] and the non-significant trend that was present in sham-operated females from the present study. Again, the absence of a significant difference in mPOA Fos in our ovariectomized female subjects may have reflected the fact that we administered EB to our present subjects whereas in previous studies we used either gonadally intact estrous females [34] or ovariectomized, TP-treated females [18]. The observation that males with bilateral damage to the MN-POA/AH showed a female-like profile of odor induced Fos expression in the mPOA correlates with the observation that these same males previously showed a female-like preference in Y-maze tests to approach odors emitted from an anesthetized stud male as opposed to an estrous female.

The present results, like those of our previous two experiments [19,22] establish a causal link between the integrity of the sexually dimorphic MN-POA/AH in male ferrets and the existence of a sexually differentiated, male-typical preference to seek out a female conspecific. A related phenomenon has been reported in rats. Thus in one study [23] bilateral lesions of the medial POA/AH, which included the sexually dimorphic sexually dimorphic nucleus (SDN) [12], caused male rats to switch their preference from approaching a female to another male stimulus rat. In a subsequent experiment [14], medial POA/AH lesions disrupted males' preference to

investigate soiled bedding from an estrous as opposed to an anestrous female, although these lesions failed to disrupt the ability of soiled estrous bedding to induced Fos responses throughout the VNO projection pathway of male rats.

Several studies carried out in other species suggest that the presence of a male-typical, sexually dimorphic sub-division of the POA/AH contributes to the male-typical preference of male mammals to seek out females, as opposed to other males, for the purpose of mating. The first example involves the report [26] that ~8% of rams prefer to approach and mount other rams as opposed to ewes. In sheep, as in rat and ferret, a sexually dimorphic group of cells exists in the POA/AH (ovine SDN) which is significantly larger in males than in females [25]. The volume of this ovine SDN was twice as great in female-oriented than in male-oriented rams [25]. These correlative results raise the question of whether variations in the dimensions of the male-typical ovine SDN control heterosexual vs. homosexual partner orientation in rams. More research is needed that directly links the differentiation of a male-typical ovine SDN and the development in later life of an orientation towards female as opposed to other male conspecifics. It will also be important to determine whether the ovine SDN plays any role in the processing of olfactory and/or visual information that conveys information about sex and endocrine status of other sheep.

Rhesus macaques, like rat, ferret and sheep, also possess a nucleus in the dorsocentral portion of the anterior hypothalamus (AHdc) whose volume is nearly 3 times greater in males than in females [6]. In a recent study [32] this same sex dimorphism in AHdc volume was also documented in Japanese macaques, a species in which during the breeding season ~50% of females actually prefer to interact sexually (mount) other females instead of displaying female-typical sexual behavior towards male conspecifics [31]. Vasey and Pfaus [32] argued that a male-typical AHdc is likely not required for female Japanese monkeys to display a male-typical profile of sexual orientation, although a direct correlation between individual females' female-oriented mounting behavior and AHdc volume was not made in that study.

Three independent groups of investigators [1,7,20] have published results showing that the volume of the third interstitial nucleus of the anterior hypothalamus (INAH3) is significantly greater in (presumptive) heterosexual men than women. LeVay reported that the volume of INAH3 was significantly greater in heterosexual than in homosexual men. The homosexual men whose brains were analyzed in this study had nearly all died of HIV/AIDS whereas the heterosexual subjects typically died of non-HIV related causes. This difference in cause of death led some critics to attribute the differences in INAH3 volume observed between homosexual vs. heterosexual men to the presence of HIV infection in the homosexual sample as opposed to a difference in sexual orientation. A more recent study [7] suggested, however, that there was no effect of HIV infection on the dimensions of INAH3 in either heterosexual or homosexual male subjects. These investigators also reported only a non-significant trend for INAH3 volume to be greater in heterosexual as compared with homosexual men.

There are several recent reports of a correlation between subjects' hypothalamic responses (indexed using PET activation) to a putative human pheromone, 4,16-androstadien-3-one (AND), which is excreted in male axillary sweat, and sexual orientation in humans. In an initial experiment [27], applying AND to the upper lip of women subjects activated subdivisions of hypothalamic regions including the ventromedial and preoptic nuclei whereas this compound had no such effect in men. Subsequently [28] this sexually dimorphic effect of AND on hypothalamic neuronal activity was replicated. In addition, application of AND to the upper lip of self-identified homosexual men evoked a female-typical profile of hypothalamic activity. Most recently [4], application of AND to the upper lip of self-identified homosexual women failed to induce the hypothalamic activation otherwise seen in heterosexual women. The results of these 3 studies are surprisingly congruent with the animal results, already reviewed, in which

exposure to male body odorants stimulated a sexually dimorphic profile of Fos responses in the olfactory projection pathway to the hypothalamus. Whereas in animals it is well established that body odorants are prime determinants of sex partner preference, no such role of AND or any other body odorants has yet been demonstrated in humans. Likewise, there are no established links between variations in the volume of the sexually dimorphic nucleus, INAH-3, and hypothalamic responses to AND. Thus the functional significance of the reported effects of sex and sexual orientation on hypothalamic responses to AND in humans remains to be determined.

In conclusion, the present results raise the possibility that in male ferrets inputs from the sexually dimorphic MN-POA/AH to the VMH somehow impose a male-typical profile of body odor processing which overrides the female-typical function of the latter structure thereby leading males to seek out females for the purpose of mating and reproduction.

Acknowledgements

This research was supported by NIH grant HD 21094 awarded to M.J. Baum. We thank Daniel Roberts for the technical assistance and the staff of the Boston University Animal Care Facility for the care of our ferrets.

References

1. Allen LS, Hines M, Shryne JE, Gorski RA. Two sexually dimorphic cell groups in the human brain. *J Neurosci* 1989;9:497–506. [PubMed: 2918374]
2. Bakker J, Baum MJ, Slob AK. Neonatal inhibition of brain estrogen synthesis alters adult neural Fos responses to mating and pheromonal stimulation in the male rat. *Neuroscience* 1996;74:251–60. [PubMed: 8843090]
3. Baum MJ, Erskine MS, Kornberg E, Weaver CE. Prenatal and neonatal testosterone exposure interact to affect differentiation of sexual behavior and partner preference in female ferrets. *Behav Neurosci* 1990;104:183–98. [PubMed: 2317276]
4. Berglund H, Lindstrom P, Savic I. Brain response to putative pheromones in lesbian women. *Proc Natl Acad Sci U S A* 2006;103:8269–74. [PubMed: 16705035]
5. Bressler SC, Baum MJ. Sex comparison of neuronal Fos immunoreactivity in the rat vomeronasal projection circuit after chemosensory stimulation. *Neuroscience* 1996;71:1063–72. [PubMed: 8684610]
6. Byne W. The medial preoptic and anterior hypothalamic regions of the rhesus monkey: cytoarchitectonic comparison with the human and evidence for sexual dimorphism. *Brain Res* 1998;793:346–50. [PubMed: 9630719]
7. Byne W, Tobet S, Mattiace LA, Lasco MS, Kemether E, Edgar MA, et al. The interstitial nuclei of the human anterior hypothalamus: an investigation of variation with sex, sexual orientation, and HIV status. *Horm Behav* 2001;40:86–92. [PubMed: 11534967]
8. Chang YM, Kelliher KR, Baum MJ. Steroidal modulation of scent investigation and marking behaviors in male and female ferrets (*Mustela putorius furo*). *J Comp Psychol* 2000;114:401–7. [PubMed: 11149544]
9. Cherry JA, Basham ME, Weaver CE, Krohmer RW, Baum MJ. Ontogeny of the sexually dimorphic male nucleus in the preoptic/anterior hypothalamus of ferrets and its manipulation by gonadal steroids. *J Neurobiol* 1990;21:844–57. [PubMed: 2077101]
10. Cherry JA, Baum MJ. Effects of lesions of a sexually dimorphic nucleus in the preoptic/anterior hypothalamic area on the expression of androgen- and estrogen-dependent sexual behaviors in male ferrets. *Brain Res* 1990;522:191–203. [PubMed: 2224522]
11. Cloe AL, Woodley SK, Waters P, Zhou H, Baum MJ. Contribution of anal scent gland and urinary odorants to mate recognition in the ferret. *Physiol Behav* 2004;82:871–5. [PubMed: 15451652]
12. Gorski RA, Gordon JH, Shryne JE, Southam AM. Evidence for a morphological sex difference within the medial preoptic area of the rat brain. *Brain Res* 1978;148:333–46. [PubMed: 656937]
13. Halem HA, Cherry JA, Baum MJ. Vomeronasal neuroepithelium and forebrain Fos responses to male pheromones in male and female mice. *J Neurobiol* 1999;39:249–63. [PubMed: 10235679]

14. Hurtazo HA, Paredes RG. Olfactory preference and Fos expression in the accessory olfactory system of male rats with bilateral lesions of the medial preoptic area/anterior hypothalamus. *Neuroscience* 2005;135:1035–44. [PubMed: 16165294]
15. Keller M, Douhard Q, Baum MJ, Bakker J. Destruction of the main olfactory epithelium reduces female sexual behavior and olfactory investigation in female mice. *Chem Senses* 2006;31:315–23. [PubMed: 16484502]
16. Kelliher K, Baum M. Effect of sex steroids and coital experience on ferrets' preference for the smell, sight and sound of conspecifics. *Physiol Behav* 2002;76:1–7. [PubMed: 12175583]
17. Kelliher KR, Baum MJ. Nares occlusion eliminates heterosexual partner selection without disrupting coitus in ferrets of both sexes. *J Neurosci* 2001;21:5832–40. [PubMed: 11466455]
18. Kelliher KR, Chang YM, Wersinger SR, Baum MJ. Sex difference and testosterone modulation of pheromone-induced Neuronal Fos in the ferret's main olfactory bulb and hypothalamus. *Biol Reprod* 1998;59:1454–63. [PubMed: 9828192]
19. Kindon HA, Baum MJ, Paredes RJ. Medial preoptic/anterior hypothalamic lesions induce a female-typical profile of sexual partner preference in male ferrets. *Horm Behav* 1996;30:514–27. [PubMed: 9047276]
20. LeVay S. A difference in hypothalamic structure between heterosexual and homosexual men. *Science* 1991;253:1034–7. [PubMed: 1887219]
21. Pankevich DE, Cherry JA, Baum MJ. Accessory olfactory neural Fos responses to a conditioned environment are blocked in male mice by vomeronasal organ removal. *Physiol Behav* 2006;87:781–8. [PubMed: 16516252]
22. Paredes RG, Baum MJ. Altered sexual partner preference in male ferrets given excitotoxic lesions of the preoptic area/anterior hypothalamus. *J Neurosci* 1995;15:6619–30. [PubMed: 7472423]
23. Paredes RG, Tzschenke T, Nakach N. Lesions of the medial preoptic area/anterior hypothalamus (MPOA/AH) modify partner preference in male rats. *Brain Res* 1998;813:1–8. [PubMed: 9824656]
24. Robarts DW, Baum MJ. Ventromedial hypothalamic nucleus lesions disrupt olfactory mate recognition and receptivity in female ferrets. *Horm Behav.* in press
25. Roselli CE, Larkin K, Resko JA, Stellflug JN, Stormshak F. The volume of a sexually dimorphic nucleus in the ovine medial preoptic area/anterior hypothalamus varies with sexual partner preference. *Endocrinology* 2004;145:478–83. [PubMed: 14525915]
26. Roselli CE, Larkin K, Schrank JM, Stormshak F. Sexual partner preference, hypothalamic morphology and aromatase in rams. *Physiol Behav* 2004;83:233–45. [PubMed: 15488542]
27. Savic I, Berglund H, Gulyas B, Roland P. Smelling of odorous sex hormone-like compounds causes sex-differentiated hypothalamic activations in humans. *Neuron* 2001;31:661–8. [PubMed: 11545724]
28. Savic I, Berglund H, Lindstrom P. Brain response to putative pheromones in homosexual men. *Proc Natl Acad Sci U S A* 2005;102:7356–61. [PubMed: 15883379]
29. Swann J, Fiber JM. Sex differences in function of a pheromonally stimulated pathway: role of steroids and the main olfactory system. *Brain Res Bull* 1997;44:409–13. [PubMed: 9370205]
30. Tobet SA, Zahniser DJ, Baum MJ. Differentiation in male ferrets of a sexually dimorphic nucleus of the preoptic/anterior hypothalamic area requires prenatal estrogen. *Neuroendocrinology* 1986;44:299–308. [PubMed: 3808217]
31. Vasey PL. Same-sex sexual partner preference in hormonally and neurologically unmanipulated animals. *Annu Rev Sex Res* 2002;13:141–79. [PubMed: 12836731]
32. Vasey PL, Pfaus JG. A sexually dimorphic hypothalamic nucleus in a macaque species with frequent female–female mounting and same-sex sexual partner preference. *Behav Brain Res* 2005;157:265–72. [PubMed: 15639177]
33. Wallen, K.; Baum, MJ. Masculinization and defeminization in altricial and precocial mammals: comparative aspects of steroid hormone action. In: Pfaff, DW.; Arnold, AP.; Etgen, AM.; Fahrback, SE.; Rubin, RT., editors. *Hormones, brain and behavior*. New York: Academic Press; 2002. p. 385–423.
34. Wersinger SR, Baum MJ. Sexually dimorphic processing of somatosensory and chemosensory inputs to forebrain luteinizing hormone-releasing hormone neurons in mated ferrets. *Endocrinology* 1997;138:1121–9. [PubMed: 9048618]

35. Woodley SK, Baum MJ. Differential activation of glomeruli in the ferret's main olfactory bulb by anal scent gland odours from males and females: an early step in mate identification. *Eur J Neurosci* 2004;20:1025–32. [PubMed: 15305871]
36. Woodley SK, Cloe AL, Waters P, Baum MJ. Effects of vomeronasal organ removal on olfactory sex discrimination and odor preferences of female ferrets. *Chem Senses* 2004;29:659–69. [PubMed: 15466811]

Fig. 1. Schematic representation (240 μ m intervals between coronal sections) of the extent of the damage caused by electrolytic lesions of the medial preoptic area/anterior hypothalamus (mPOA/AH) of male ferrets. Light-gray areas show the maximal extent of lesion damage for any of the individual subjects included in a particular group. Dark-gray areas show the overlapping lesion damage that was present in all members of a particular group. Left panel: subjects with extensive lesions of the mPOA/AH but no damage to the sexually dimorphic male nucleus of the POA/AH (MN-POA/AH) ($n=16$). Middle panel: subjects with unilateral damage to the MN-POA/AH ($n=5$; overlapping lesion damage is depicted for one hemisphere, although actually four males had MN-POA/AH damage on the left side and one male had MN-POA/AH damage in the right hemisphere). Right panel: subjects with extensive lesion of mPOA/AH that included overlapping bilateral MN-POA/AH damage ($n=8$).

Fig. 2.

Percentage of free trials in Y-maze tests during which ferrets chose to approach anesthetized male vs. female stimulus ferrets before (top panel) and after (bottom panel) the placement of electrolytic lesions in the medial preoptic area/anterior hypothalamus (POA/AH) of male subjects or of a sham operation in both sexes. Groups of males with brain lesions were distinguished by the presence of damage in the sexually dimorphic male nucleus of the POA/AH (MN-POA/AH); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. sham-operated females; ## $p < 0.01$ vs. sham-operated males by Mann-Whitney *post-hoc* tests. The data are expressed as means $\pm$ SEM. The number of subjects in each group is given in parentheses.

Fig. 3.

Percentage of trials in Y-maze tests during which ferrets chose to approach male vs. female anal scent gland odorants before (top panel) and after (bottom panel) the placement of electrolytic lesions in the medial preoptic area/anterior hypothalamus (POA/AH) of male subjects or of a sham operation in both sexes. Groups of males with brain lesions were distinguished by the presence of damage in the sexually dimorphic male nucleus of the POA/AH (MN-POA/AH). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. sham-operated females by Mann-Whitney *post-hoc* tests. The data are expressed as means $\pm$ SEM. The number of subjects in each group is given in parentheses.

Fig. 4.

Percentage of trials in Y-maze tests during which ferrets chose to approach male vs. female urinary odorants before (top panel) and after (bottom panel) the placement of electrolytic lesions in the medial preoptic area/anterior hypothalamus (POA/AH) of male subjects or of a sham operation in both sexes. Groups of males with brain lesions were distinguished by the presence of damage in the sexually dimorphic male nucleus of the POA/AH (MN-POA/AH). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. sham-operated females by Mann-Whitney *post-hoc* tests. The data are expressed as means $\pm$ SEM. The number of subjects in each group is given in parentheses.

Fig. 5.

Effect of exposure to either soiled male or clean bedding on the number of Fos-immunoreactive cells present in different brain regions of male ferrets which had previously received electrolytic lesions of the medial preoptic area/anterior hypothalamus (POA/AH) or sham operations in both sexes. Groups of males with brain lesions were distinguished by the presence of damage in the sexually dimorphic male nucleus of the POA/AH (MN-POA/AH). The locations of the Fos counting regions (shaded areas) in the medial amygdala (MA), bed nucleus of the stria terminalis (BNST), medial preoptic area (mPOA), and ventromedial hypothalamus (VMH) are shown in the left panels. * $p < 0.05$ soiled male vs. clean bedding effect in sham-operated females by an independent t -test. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ comparisons with Sham.

males that sustained bilateral MN-POA/AH damage by *post-hoc* LSD tests. The data are expressed as means $\pm$ SEM, and the number of subjects in each group is given in parentheses. Black dots represent the individual subjects' data points. Additional abbreviations: 3V — third ventricle, IC — internal capsule, OT — optic tract, LV — lateral ventricle, FX — fornix, AC — anterior commissure, OC — optic chiasm, ME — median eminence.

Fig. 6.

Representative bright-field photomicrographs showing coronal ferret forebrain sections that were immunostained for Fos protein. Top row: medial amygdala sections from sham-operated females exposed to either clean (A) or soiled male (B) bedding. Middle row: medial preoptic area (mPOA) sections from sham-operated females exposed to either clean (C) or soiled male (D) bedding. Bottom row: mPOA sections from males that had sustained either a bilateral damage to the sexually dimorphic male nucleus of the preoptic area/anterior hypothalamus (E) or a sham operation (F) prior to exposure to soiled male bedding.