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Sexually dimorphic activation of the accessory, but not the main, olfactory bulb in mice by urinary volatiles

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Abstract

Previous research suggests that volatile body odourants detected by the main olfactory epithelium (MOE) are processed mainly by the main olfactory bulb (MOB) whereas nonvolatile body odourants detected by the vomeronasal organ (VNO) are processed via the accessory olfactory bulb (AOB). We asked whether urinary volatiles from males and females differentially activate the AOB in addition to the MOB in gonadectomized mice of either sex. Exposure to urinary volatiles from opposite-sex but not same-sex conspecifics augmented the number of Fos-immunoreactive mitral and granule cells in the AOB. Volatile urinary odours from male as well as female mice also stimulated Fos expression in distinct clusters of MOB glomeruli in both sexes. Intranasal administration of ZnSO₄, intended to disrupt MOE function, eliminated the ability of volatile urinary odours to stimulate Fos in both the MOB and AOB. In ovariectomized, ZnSO₄-treated females a significant, though attenuated, AOB Fos response occurred after direct nasal exposure to male urine plus soiled bedding, suggesting that VNO signaling remained partially functional in these mice. Future studies will determine whether MOE or VNO signaling, or both types of input, drive the sexually dimorphic response of the AOB to volatile opposite-sex odours and whether this AOB response contributes to reproductive success.

Keywords

amygdala; castration; hypothalamus; ovariectomy; pheromone

Introduction

Volatile odourants bind to specific olfactory receptor neurons (ORNs) in the main olfactory epithelium (MOE), and ORNs expressing the same receptor protein project to the same one or two glomeruli in the ipsilateral main olfactory bulb (MOB; Buck & Axel, 1991; Chess *et al.*, 1992). The glomerular layer of the MOB is the first site at which discrimination of volatile social odours takes place, thereby allowing female mice to identify males by major histocompatibility complex (MHC) haplotype (Schaefer *et al.*, 2001, 2002) and ferrets of either sex to discriminate conspecifics' sex (Kelliher & Baum, 2001; Woodley & Baum, 2004). Several behavioural studies in mice (Pankevich *et al.*, 2004; Pierman *et al.*, 2006; Keller *et al.*, 2006a; Wesson *et al.*, 2006) have also implicated the main olfactory system in the ability to discriminate between male and oestrous female conspecific odours. We first asked whether differential patterns of MOB glomerular activation occur in female mice in response to male vs. oestrous female urinary volatiles which may contribute to sex discrimination.

Much evidence suggests that nonvolatile body odours, including components of urine (Moss *et al.*, 1997) and secretions from the extraorbital lacrimal gland (Kimoto *et al.*, 2005), are

detected by receptor neurons in the vomeronasal organ (VNO) which project to the accessory olfactory bulb (AOB) and subsequently to downstream medial amygdaloid and hypothalamic targets (Halpern & Martinez-Marcos, 2003). While conducting our MOB glomerular activation experiment in which female mice were exposed to volatile components of urine, we noticed robust Fos activation in the mitral and granule cell layers of the AOB in response to opposite-but not same-sex odours. These data led us more systematically to monitor Fos immunoreactivity (IR) in the mitral and granule cells of the AOB and in forebrain targets of the vomeronasal projection pathway (Halem *et al.*, 1999) in female (Experiment 1) and male (Experiment 2) mice treated intranasally with saline (control) or ZnSO₄, which functionally inactivates the MOE. Opposite- but not same-sex urinary volatiles augmented Fos expression in the AOB, and this response was eliminated by prior intranasal ZnSO₄ treatment.

Clearly the MOE is rendered nonfunctional by intranasal ZnSO₄ (McBride *et al.*, 2003); however, it was important to determine whether this treatment may have eliminated odour-induced AOB Fos expression by inadvertently damaging the VNO. In addition to using lectin staining to verify that VNO projections to the AOB glomerular layer were intact, we asked whether ovariectomized ZnSO₄-treated females would show an AOB Fos response to direct nasal contact with male body odours. The AOB Fos response in an initial cohort of ZnSO₄-treated females was intermediate between that observed in ZnSO₄-treated subjects exposed to clean bedding and saline-treated subjects exposed to soiled male bedding. Previous studies (Halem *et al.*, 1999, 2001a; Keller *et al.*, 2006b) showed that circulating sex hormones up-regulate murine VNO and forebrain Fos responses to both same- and opposite-sex urinary odourants. This led us to monitor AOB Fos responses to soiled male bedding in a second cohort of saline- or ZnSO₄-treated ovariectomized females that received daily injections of estradiol benzoate (EB).

Materials and methods

Subjects

Seventy-four female and 45 male sexually naïve Swiss Webster mice (Charles River Laboratories, Wilmington, MA, USA) were purchased at 5–6 weeks of age and housed in same-sex groups under a 12 h light–dark photoperiod with food and water provided *ad libitum*. All of the procedures were approved by the Boston University Animal Care and Use Committee and were in accordance with NIH guidelines. All behavioural testing and terminal odour exposure sessions were conducted during the light phase of the photoperiod. Both male and female subjects underwent bilateral gonadectomy using 2% isoflurane anaesthesia, and were allowed 1 week to recover. One cohort of ovariectomized female subjects used in Experiment 3 received injections of EB (details below), otherwise no replacement sex hormones were administered. Subjects were subsequently anaesthetized with ketamine (120 mg/kg) and xylazine (12 mg/kg), whereupon the nasal cavities were irrigated with either 7 µL of a 10% ZnSO₄ solution (0.56M) to damage the MOE or with 7 µL of sterile saline (control), via an Eppendorf capillary pipette tip (Fisher Scientific, Pittsburgh, PA, USA) attached to a Hamilton syringe. To ensure that subjects did not aspirate any of the ZnSO₄ solution, animals were held with their nose down for 2 min following nasal irrigation and then placed belly-down until they recovered from anaesthesia. Subjects were allowed 3 days to recover prior to administration of behavioural tests to assess olfactory function.

Behavioural tests

Subjects were housed individually 48 h prior to the onset of behavioural testing. In all testing paradigms, the experimenter was blind to the treatment group of each subject. Urine was obtained by holding the animal by the scruff of the neck over a plastic funnel and gently applying pressure to the abdomen. Urine was collected from three gonadally intact adult Swiss

Webster male mice and three ovariectomized adult Swiss Webster female mice, and was pooled according to sex and endocrine state and stored in 150- μ L aliquots at -80°C . In addition, oestrous female urine was collected following a series of subcutaneous injections of EB (20 μ g, 24 and 48 h prior to collection) and progesterone (500 μ g, 4 h prior to collection) dissolved in sesame oil. Ovariectomized female urine used for behavioural testing was collected from stimulus females that did not receive sex hormones.

The efficacy of ZnSO_4 lesions was assessed behaviourally using both a habituation–dishabituation testing paradigm (Baum & Keverne, 2002) and a hidden cookie test (Wersinger *et al.*, 2004) in which subjects were tested for their ability to detect volatile urinary odourants and a Nutter Butter[®] cookie, respectively, while housed singly in their home cage (29 $\times$ 18 $\times$ 13 cm). Habituation–dishabituation testing was conducted by pipetting 10 μ L of distilled water or ovariectomized female urine onto filter paper secured to a plastic weigh boat, and placing the weigh boat in a clean food hopper outside of the home cage. A wire mesh was placed between the filter paper and the cage top to prevent direct nasal access to the stimulus. In this way, only volatile urinary odourants were presented at body level. Subjects were first presented with three consecutive 2-min presentations of distilled water, followed by three consecutive presentations of ovariectomized female urine using 1-min intertrial intervals. Using an iPAQ Pocket PC (Hewlett-Packard, Houston, TX, USA) and Noldus Observer Software (Wageningen, The Netherlands), the experimenter recorded the amount of time that the subject spent with its nose at the wire mesh sniffing the stimulus during each 2-min interval. Subjects were tested 3 and 4 days after irrigation of the nasal cavities, and the data were averaged based on intranasal treatment solution (saline- vs. ZnSO_4 -treated) and terminal odour exposure treatments (male urinary volatiles, oestrous female urinary volatiles, or clean air). Wilcoxon signed-rank tests (two-tailed) were used to assess within-group differences in investigation times between the third presentation of water and the first presentation of ovariectomized female urine.

Immediately after habituation–dishabituation tests for urinary odourant detection, subjects were given a test in which a small piece of cookie was randomly hidden in the bedding of the home cage, whereupon the subject was placed in a separate random location. All subjects had been food-deprived for 48 h prior to testing. Each animal was given 5 min to locate the food, ‘find’ being defined as holding the cookie in both paws. Subjects’ latency to find the cookie was recorded by the investigator using an iPAQ Pocket PC and Noldus Observer Software. ZnSO_4 -treated subjects that showed evidence of olfactory function in either behavioural test were re-treated with ZnSO_4 irrigation of the nasal cavities 1 week later and retested for the efficacy of the treatment. Subjects were tested on days 3 and 4 after irrigation of the nasal cavities, and trials were averaged based on intranasal treatment solution and odour exposure group. Differences between groups were assessed using two-way ANOVAs followed by Student–Newman–Keuls *post hoc* tests.

General histological methods

Fos immunocytochemistry (ICC) as well as horseradish peroxidase-conjugated soybean agglutinin staining (SBA-HRP) were performed on coronal or sagittal olfactory bulb sections from each subject (details below). Olfactory bulb slices were preincubated with 7.5% normal goat serum in 0.1% Triton X-100 in PBS for 3 h, and then placed in primary rabbit polyclonal cFos antiserum (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) diluted 1:1000 in 2% normal goat serum and 0.1% Triton X-100 in PBS for 16 h at 4°C . Tissue was rinsed three times for 10 min in 1.5% normal goat serum in 0.1% Triton X-100 in PBS, and then incubated for 1 h with biotinylated goat antirabbit immunoglobulin G (Vector Laboratories, Burlington, CA, USA) diluted 1:200 with 2% normal goat serum in 0.1% Triton X-100 in PBS. Sections were then rinsed in 0.1 MPBS for 15 min before being placed in 3% hydrogen peroxide in 0.1

MPBS for 30 min. This incubation was followed by three 10-min rinses in 0.1 MPBS, and a 45-min incubation in avidin–biotin–peroxidase complex solution (ABC Kit; Vector Laboratories Elite Kit). Sections were rinsed three times for 10 min in 0.1 MPBS, and reacted with 0.025% 3,3'-diaminobenzidine (DAB) with nickel intensification for 5 min, followed by another series of three 10-min rinses in 0.1 MPBS. Olfactory bulb sections were mounted in sequence on gelatin-coated slides and coverslipped with Permount.

A second set of olfactory bulb sections was stained using SBA-HRP to determine whether the VNO axonal inputs to the AOB had been damaged by prior ZnSO₄ irrigation of the nasal cavities (Key & Giorgi, 1986; Wysocki & Wysocki, 1995). Sections were preincubated for 2 h with 3% normal goat serum in 1% hydrogen peroxide in 0.1 MPBS. The tissue was then rinsed four times for 10 min in 0.1 MPBS, and incubated for 40 min with 15 µg/mL SBA-HRP in 0.1 MPBS. Sections were once again rinsed four times for 10 min in 0.1 MPBS, reacted with DAB for 5 min, and finally rinsed with 0.1 MPBS. Sections were mounted on gelatin-coated slides and coverslipped with Permount.

Forebrain sections ipsilateral to the MOB subjected to glomerular analysis were cut coronally at 30 µm on a sledge microtome (Leica Microsystems Inc., Bannockburn, IL, USA) and stored as free-floating sections in 0.1 MPBS. Fos ICC was run on every other section as described above. Forebrain sections were mounted in sequence (rostral–caudal) on gelatin-coated slides and coverslipped with Permount. Each slide was coded prior to analysis in order to conceal subjects' treatment conditions. Forebrain Fos IR was quantified by counting cells in two anatomically matched sections using a camera lucida attachment to an Olympus microscope to trace individual Fos-IR cells onto paper, as previously described (Halem *et al.*, 2001b; Pankevich *et al.*, 2006a). Mean numbers of Fos-IR cells per circular standard counting area (0.1 mm²) were calculated for each animal in each forebrain region, and two-way ANOVAs (Student–Newman–Keuls *post hoc* tests) were used to determine significant differences between treatment (ZnSO₄ vs. saline) as well as odour exposure (clean air, oestrous female urinary volatiles and male urinary volatiles) groups.

Specific methods of procedure for individual experiments

Experiment 1—Forty-four ovariectomized female subjects given intranasal saline or ZnSO₄ (details above) underwent behavioural tests for anosmia prior to terminal odour exposure. Initially, two of the 18 ZnSO₄-treated subjects responded to volatile urinary odours in the habituation–dishabituation test and were able to locate a hidden cookie inside of the home cage. These subjects were determined to have ineffective lesions of the MOE and were retreated with intranasal ZnSO₄ 1 week later. Subsequent behavioural tests on days 3 and 4 following ZnSO₄ application showed that the re-treated subjects, like the remaining ZnSO₄-treated subjects, did not respond to ovariectomized female urinary volatiles following three presentations of distilled water in the habituation–dishabituation testing paradigm. Saline-treated subjects, however, all successfully dishabituated when first presented with the female urinary stimulus. Two-tailed Wilcoxon signed-rank tests indicated that time spent investigating the first presentation of female urine was significantly longer than time spent investigating the third presentation of water ($P < 0.05$) in each saline-treated group (data not shown). In addition, none of the ZnSO₄-treated subjects was able to locate a cookie hidden in the bedding of the home cage within 5 min following 48 h of food deprivation. The latency to find the cookie (163 ± 28 , 106 ± 40 and 74 ± 22 s for subjects exposed to clean air, male odours and female odours, respectively) was significantly lower in saline-treated than in ZnSO₄-treated subjects ($F_{5,44} = 70.5$, $P < 0.001$; data not shown). These behavioural results collectively indicate that ZnSO₄ irrigation of the nasal cavities functionally inactivated the MOE, rendering these subjects anosmic.

Female subjects were exposed to either clean air, male urinary volatiles or oestrous female urinary volatile odours, beginning at least 4 h after the completion of behavioural tests given 4 days after intranasal ZnSO₄ application. All volatile odour exposures took place in a clean, odour-free fume hood. Subjects were placed in a clean empty cage (20 × 20 × 20 cm), and air was passed via odour-free tubing through activated charcoal for purification, followed by drierite and fresh distilled water for rehydration. This purified air was then blown over a 20% solution of urine and distilled water or distilled water alone in the case of clean air exposure. Solutions were gently agitated using an orbital shaker during exposure, and air flow was adjusted to 1520 mL/min using a flow meter. Finally, urinary volatiles or clean air were blown into the cage via odourless tubing attached to a small funnel affixed to an opening in the side of the cage. All odour exposures began with 40 min of clean air pulsed at 3:1-min on:off intervals, followed by 30 min of 3:2-min on:off intervals of either clean air, oestrous female urinary volatiles or male urinary volatiles (Schaefer *et al.*, 2001). Subjects were anaesthetized with sodium pentobarbital and killed 90 min after the onset of odour exposure via transcardiac perfusion with 0.1 M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 MPBS. All brains were removed and postfixed in 4% paraformaldehyde for 4 h and then cryoprotected in 30% sucrose for 48 h. Forebrain hemispheres and olfactory bulbs were blocked and stored in OCT (Tissue-Tek, Elkhart, IN, USA) at −80 °C.

Olfactory bulb sections were cut coronally at 35 µm on a cryostat (Leica Microsystems Inc.) at −18 °C, whereupon free-floating sections were stored in 0.1 MPBS at 4 °C. Every other section was used for Fos ICC, and remaining sections were stained using SBA-HRP as previously described. Sections were then mounted sequentially (rostral–caudal) on gelatin-coated slides and coverslipped with Permount. Fos induction was quantified by counting Fos-IR cells per circular standard counting area (0.1 mm²) in rostral and caudal sections of the mitral and granule cell layers of the AOB in two anatomically matched sections (Halem *et al.*, 2001a). The number of Fos-IR cells counted in the rostral and caudal sections within each treatment and odour-exposure group did not differ, and thus were added and averaged within groups. Forebrain sections ipsilateral to the olfactory bulb used for Fos ICC were cut and stained as already described in *General histological methods*. Each slide was coded prior to analysis in order to conceal the treatment conditions for each subject.

The mapping technique used to generate contour plots depicting areas of odour-induced MOB glomerular activation was developed by Salcedo *et al.* (2005). Using Matlab software in conjunction with the GLOM-MAP program, we recorded the location of activated glomeruli based on rostral–caudal distance through the bulb as well as radial angle from a central point of origin. Activated glomeruli were defined as having 180° of continuous Fos expression or two 90° arcs of Fos expression in the periglomerular cells surrounding any given glomerulus (Schaefer *et al.*, 2001, 2002). To ensure that sections were orientated properly, olfactory bulbs were sectioned perpendicular to the lateral olfactory tract and analysed in sequence (70 µm between sections). Anatomical landmarks were used to determine the rostral and caudal limits for analysis, with the rostralmost section being the first to contain clear mitral cell and external plexiform layers, and the caudalmost section lying just posterior to the AOB. Using the thickness of each section (35 µm) and its sequence within the sections to be analysed, we were able to estimate the rostral–caudal location of each activated glomerulus. A central axis (0–180°) was also established for each section, which extended from the dorsal mitral cell layer to the ventral mitral cell layer. A central point of origin was then determined to be 1/3 the distance from the dorsal portion of the central axis in sections not containing the AOB, and 1/3 the distance from the granular cusp of the AOB in sections containing the AOB. This point of origin was used to determine the radial angle of an activated glomerulus relative to the central axis. For example, an activated glomerulus located at 0° would fall on the dorsal portion of the central axis, while a glomerulus located at 90° would lie on the lateral portion of the olfactory bulb (Fig. 1).

Using rostral–caudal distance and radial angle, GLOM-MAP allowed us to create two-dimensional colour contour plots that show the density of activated glomeruli throughout the MOB. Mapping was accomplished by capturing images of each section using a Nikon digital camera attached to an Olympus microscope. Activated glomeruli were identified using the microscope by an investigator who was blind to the treatment group of each subject, and annotated using the ACT1 imaging program. Annotated images were then imported into the GLOM-MAP OBS program, in which the central axis was established, and the positions of annotated glomeruli were recorded. Using the statistical toolbox, GLOM-MAP GDB, these data were compiled and smoothed, generating individual contour plots, group-averaged contour plots and statistical comparisons between groups. Mann–Whitney *U*-tests were used to determine significantly different regions of the maps, which are outlined in blue (Salcedo *et al.*, 2005). Significant *P*-values were determined using a false-discovery rate (FDR) critical value that was adjusted for multiple Mann–Whitney *U*-test comparisons. The use of an FDR reduced the occurrence of Type I statistical errors (Schaefer *et al.*, 2002).

Experiment 2—Thirty-nine castrated male subjects given intranasal saline or ZnSO₄ (details above) underwent behavioural tests for anosmia on days 3 and 4 following ZnSO₄ application prior to odour exposure. None of the ZnSO₄-treated subjects responded to ovariectomized female urinary volatiles following three presentations of distilled water in the habituation–dishabituation testing paradigm. Saline-treated subjects, however, all successfully dishabituated when first presented with the female urinary stimulus. Two-tailed Wilcoxon signed-rank tests indicated that time spent investigating the first presentation of female urine was significantly longer than time spent investigating the third presentation of water ($P < 0.05$) in each saline-treated group (data not shown). In addition, none of the ZnSO₄-treated subjects were able to locate a cookie hidden in the bedding of the home cage within 5 min following 48 h of food deprivation. The latency to find the cookie (42 ± 11 , 25 ± 2 and 44 ± 9 s, for groups that were later exposed to clean air, male odours and female odours, respectively) was significantly lower in saline-treated than in ZnSO₄-treated subjects ($F_{5,38} = 1267$, $P < 0.001$; data not shown). These behavioural results collectively indicate that ZnSO₄ irrigation of the nasal cavities functionally inactivated the MOE, rendering these subjects anosmic. Male subjects were subsequently exposed to either clean air, male urinary volatiles or oestrous female urinary volatile odours beginning at least 4 h after the completion of behavioural tests given 4 days after ZnSO₄ treatment. Histological analyses were carried out using the same methods described above for female subjects.

Experiment 3—In order to duplicate the endocrine state of the ovariectomized female subjects used in Experiment 1, an initial cohort of 13 subjects for Experiment 3 was comprised of ovariectomized females given daily s.c. injections of sesame oil vehicle for 7 days. Subjects were treated with either intranasal saline or ZnSO₄ and underwent behavioural testing for anosmia on days 3 and 4 after ZnSO₄ irrigation of the nasal cavities. ZnSO₄-treated subjects did not respond to ovariectomized female urinary volatiles in the habituation–dishabituation testing paradigm, while saline-treated subjects all successfully dishabituated when first presented with the female urinary stimulus. Two-tailed Wilcoxon signed-rank tests indicate that time spent investigating the first presentation of female urine was significantly longer than time spent investigating the third presentation of water ($P < 0.05$) in saline-treated subjects (data not shown). ZnSO₄-treated subjects were also unable to locate the hidden cookie in the bedding of the home cage within 5 min following 48 h of food deprivation. The latency to find the cookie was significantly lower in saline-treated subjects (32 ± 7 s) than in ZnSO₄-treated subjects ($P < 0.001$; data not shown).

Six gonadally intact male mice were individually housed in cages lined with Care Fresh bedding (Absorption Co., Bellingham, WA, USA) for 4 days prior to bedding collection. The soiled bedding was then pooled and placed into plastic cages (40 g/cage). Immediately after

behavioural testing on day 4 following intranasal saline or ZnSO₄ application, 20 µL of either saline or male urine was pipetted onto the oronasal groove of the female subjects, which were subsequently placed on either clean bedding or soiled male bedding, respectively. Subjects were killed 90 min later as described above.

Olfactory bulb sections were cut sagittally at 35 µm on a cryostat (Leica Microsystems Inc.) at -18 °C, whereupon free-floating sections were stored in 0.1 MPBS at 4 °C. Previous work suggests that odour-induced Fos expression in the AOB tends to be greater in the rostral portion of the mitral and granule cell layers than in the caudal portion of the AOB (Halem *et al.*, 2001a). Thus, we chose to use sagittal sections in this experiment in order to better quantify differences between the number of Fos-IR cells in the rostral and caudal portions of the mitral and granule cell layers of the AOB in response to clean vs. soiled male bedding. Every other section was used for Fos ICC, and remaining sections were stained using SBA-HRP as previously described. Sections were then mounted sequentially (lateral–medial) on gelatin-coated slides and coverslipped with Permount. All slides were coded in order to conceal the treatment group of each subject. Fos induction was quantified by counting Fos-IR cells per circular standard counting area (0.5 mm²) in the rostral and caudal portions of the mitral and granule cell layers of the AOB in three alternate sections closest to the midline (Halem *et al.*, 2001a; Keller *et al.*, 2006b). Upon further statistical analysis (one-way ANOVA), we discovered that significant odour-induced increases in Fos activation were similar in the rostral and caudal portions of the mitral and granule cell layers, and thus opted to combine the data from the rostral and caudal zones for ease of presentation.

As reported in the Results section, we found significant odour-induced Fos activation in the granule layer of the AOB in ZnSO₄-treated subjects given s.c. injections of oil vehicle; however, this effect was significantly reduced compared to that observed in saline-treated subjects exposed to the same stimulus. Previous work (Halem *et al.*, 1999) has suggested that the administration of EB to ovariectomized female mice up-regulates VNO neuronal responses to odours emitted from soiled male bedding. We wanted to know whether the lack of circulating sex hormone in our ovariectomized subjects could be responsible for this apparent deficit in VNO signaling. Therefore, a second cohort of 14 ovariectomized females was given daily s.c. injections of EB (1 µg/day for 7 days). Subjects were treated with either intranasal saline or ZnSO₄ on the third day of EB treatment, and underwent behavioural testing for anosmia 3 and 4 days later. ZnSO₄-treated subjects did not respond to ovariectomized female urinary volatiles in the habituation–dishabituation testing paradigm, whereas saline-treated subjects all successfully dishabituated when first presented with the female urinary stimulus. Two-tailed Wilcoxon signed-rank tests indicate that time spent investigating the first presentation of female urine was significantly longer than time spent investigating the third presentation of water ($P < 0.05$) in saline-treated subjects (data not shown). ZnSO₄-treated subjects were also unable to locate the hidden cookie in the bedding of the home cage within 5 min following 48 h of food deprivation. The latency to find the cookie was significantly lower in saline-treated subjects (41 ± 8 s) than in ZnSO₄-treated subjects ($P < 0.001$; data not shown). All subsequent methods of odour exposure, histology, and Fos-IR cell counting were the same as described for the first cohort of females prepared in Experiment 3.

Results

Experiment 1: effects of male vs. female urinary volatiles in ovariectomized females

Cellular activation in the AOB—Saline-treated female subjects exposed to male urinary volatiles showed a significantly augmented Fos response in the mitral and granule cell layers of the AOB compared to saline-treated subjects exposed to clean air or oestrous female urinary volatiles (Fig. 2). The number of Fos-IR cells counted in the rostral and caudal sections of the AOB within each treatment or odour-exposure group did not differ; therefore, values from the

two zones were added and averaged within groups. None of the ZnSO₄-treated subjects showed odour-induced Fos expression in the AOB, and the basal level of Fos-IR found in the mitral and granule cell layers of the AOB was lower in each of the ZnSO₄-treated odour-exposure groups than in their saline-treated counterparts. Significant overall effects of odour exposure (mitral layer, $F_{5,43} = 5.1$, $P < 0.05$; granule layer, $F_{5,43} = 8.9$, $P < 0.001$) as well as intranasal treatment solution (mitral layer, $F_{5,43} = 32.9$, $P < 0.001$; granule layer, $F_{5,43} = 27.2$, $P < 0.001$) were observed in both layers of the AOB (Fig. 2). SBA-HRP staining showed robust dark staining in the glomerular layer of the AOB in all subjects (data not shown).

Cellular activation in the forebrain—Several forebrain regions along the vomeronasal projection pathway of saline-treated females also showed an augmented Fos response to male urinary odours that was larger than the responses observed in other saline-treated females exposed to clean air or same-sex urinary volatiles (Table 1; for specific counting areas, see Pankevich *et al.*, 2006a). The bed nucleus of the stria terminalis (BNST) and medial preoptic area (MPA) both showed significant responses to male odours, while the medial amygdala (MeA), posterodorsal medial amygdala (MePD) and ventrolateral nucleus of the ventromedial hypothalamus (VMHVL) all showed similar nonsignificant trends compared with subjects exposed to female odours or clean air. In addition, the nucleus accumbens shell (AcbSh) showed a significantly augmented Fos response to male odours that was absent in saline-treated subjects exposed to female odours. Basal levels of Fos were lower in each of the ZnSO₄-treated groups than in their saline-treated counterparts. Significant effects of odour exposure (BNST, $F_{5,83} = 5$, $P < 0.01$; MPA, $F_{5,83} = 12.5$, $P < 0.001$; AcbSh, $F_{5,83} = 7.4$, $P = 0.001$) as well as intranasal treatment solution (BNST, $F_{5,83} = 18.4$, $P < 0.001$; MPA, $F_{5,83} = 34.6$, $P < 0.001$; MeA, $F_{5,83} = 26.5$, $P < 0.001$; MePD, $F_{5,83} = 32$, $P < 0.001$; AcbSh, $F_{5,83} = 13.1$, $P < 0.001$) indicate that activation of the accessory olfactory system by urinary volatiles was reflected further down the circuit of the vomeronasal projection pathway. Finally, amygdaloid targets of the main olfactory system including the posterolateral cortical nucleus of the amygdala (PLCo) as well as the anterior cortical nucleus of the amygdala (Aco) showed a significant effect of intranasal treatment solution (PLCo, $F_{5,83} = 17.1$, $P < 0.001$; Aco, $F_{5,83} = 26.8$, $P < 0.001$), but no effect of type of odour exposure.

Glomerular activation in the MOB—Exposure to male vs. female volatile urinary odours evoked spatially distinct patterns of glomerular activation in the MOB of ovariectomized saline-treated female mice (Fig. 3A–C). The ventral portion of the bulb showed the greatest number of activated glomeruli (outlined in black) in subjects exposed to either male or female urinary volatiles compared to subjects exposed to clean air. Despite the large area of overlap in the ventral portion of the bulb, as seen in the contour plots for saline-treated animals exposed to male or female odours, these maps show significant odour-induced differences in MOB activation, particularly in the dorsolateral portion of the bulb (rostral segments) and in the dorsomedial portion of the bulb (caudal segments). Point-by-point Mann–Whitney *U*-tests with FDR correction were performed, and regions of significant differences are shown in the bottom panels (G–I) of Fig. 3. In the comparisons between saline-treated subjects exposed to clean air vs. male or female urinary volatiles, the differences were widespread and largely significant. Mann–Whitney comparisons between saline-treated subjects exposed to male odours vs. female odours, however, showed more discrete differences. Nearly all glomerular activation in the MOB of ZnSO₄-treated animals was eliminated in all three odour-exposure groups (Fig. 3D–F). The greatly reduced MOB glomerular response to volatile urinary odours provides further evidence of the efficacy of the ZnSO₄ lesions of the MOE.

Experiment 2: effects of male vs. female urinary volatiles in castrated males

Cellular activation in the AOB—Castrated, saline-treated male subjects exposed to volatile oestrous female urinary odours had significantly more Fos-IR cells in the mitral and granule

cell layers of the AOB than saline-treated male subjects exposed to clean air or same-sex urinary volatiles (Fig. 4). These results resemble those obtained in females (Experiment 1) in that an augmented AOB Fos response was only observed in saline-treated subjects exposed to opposite-sex urinary volatiles. As in Experiment 1, Fos-IR cells in the rostral and caudal sections of the AOB were added and averaged within groups. None of the ZnSO₄-treated subjects showed odour-induced Fos expression in the AOB, and the basal level of Fos-IR found in the mitral and granule cell layers of the AOB was lower in each of the ZnSO₄-treated odour exposure groups than in their saline-treated counterparts. Significant effects of odour exposure (mitral layer: $F_{5,77} = 11.4$, $P < 0.001$, granule layer: $F_{5,77} = 35.4$, $P < 0.001$) as well as intranasal treatment solution (mitral layer, $F_{5,77} = 23.7$, $P < 0.001$; granule layer, $F_{5,77} = 82.2$, $P < 0.001$) were observed in both layers of the AOB (Fig. 4). Again, SBA-HRP staining showed robust dark staining in the glomerular layer of the AOB in all subjects (data not shown).

In saline-treated male subjects, augmented Fos responses in forebrain regions of the main olfactory as well as vomeronasal projection pathways were observed in subjects exposed to both oestrous female and male urinary volatiles compared to subjects exposed to clean air alone (Table 2; for specific counting areas, see Pankevich *et al.*, 2006a). As seen in female subjects in Experiment 1, intranasal ZnSO₄-treatment of males reduced Fos expression to or below the basal level of activated cells observed in saline-treated male subjects exposed to clean air. Significant effects of odour exposure were observed in forebrain targets of the accessory olfactory system including the BNST ($F_{5,38} = 12.3$, $P < 0.001$), MPA ($F_{5,38} = 16.3$, $P < 0.001$), MeA ($F_{5,38} = 3.4$, $P = 0.045$), VMHVL ($F_{5,38} = 5.1$, $P = 0.011$), nucleus accumbens core (AcbC; $F_{5,38} = 9.7$, $P < 0.001$) and AcbSh ($F_{5,38} = 6.8$, $P = 0.003$), and a significant interaction between odour exposure group and intranasal treatment solution was observed in the MePD ($F_{5,38} = 14.3$, $P < 0.001$). Main olfactory forebrain targets including the ACo ($F_{5,38} = 5.9$, $P = 0.006$) and the PLCo ($F_{5,38} = 6.6$, $P = 0.004$) also showed significant interaction between odour exposure group and intranasal treatment solution. Forebrain regions of the main (Aco, $F_{5,38} = 57.2$, $P < 0.001$; PLCo, $F_{5,38} = 37.4$, $P < 0.001$) and accessory (BNST, $F_{5,38} = 17.2$, $P < 0.001$; MPA, $F_{5,38} = 51.7$, $P < 0.001$; MeA, $F_{5,38} = 26.7$, $P < 0.001$; MePD, $F_{5,38} = 54.6$, $P < 0.001$; VMHVL, $F_{5,38} = 14.3$, $P < 0.001$; AcbC, $F_{5,38} = 4.2$, $P < 0.047$; AcbSh, $F_{5,38} = 13.7$, $P < 0.001$) olfactory pathways showed significant effects of intranasal treatment solution.

Glomerular activation in the MOB—Exposure to male and female volatile urinary odours evoked spatially distinct patterns of glomerular activation in the MOB of castrated, saline-treated male mice (Fig. 5A–C) just as female subjects had shown differential MOB glomerular activation after exposure to these same volatile urinary stimuli (Experiment 1). The ventral portion of the bulb showed the greatest number of activated glomeruli (outlined in black in Fig. 5) in subjects exposed to male or female urinary volatiles as opposed to clean air. Despite this overlap, contour plots for saline-treated animals exposed to male or female odours showed significant differences in MOB glomerular activation, particularly in the dorsolateral MOB (rostral sections) and in the dorsomedial MOB (caudal sections). Point-by-point Mann–Whitney *U*-tests with FDR correction were performed, and regions of significant differences are shown in the bottom panels of Fig. 5G–I. In the comparisons between saline-treated subjects exposed to clean air and male or female urinary volatiles, the differences were widespread and largely significant. Mann–Whitney comparisons between saline-treated subjects exposed to male odours vs. female odours, however, showed more discrete differences. Nearly all activation in the glomerular layer of ZnSO₄-treated animals was eliminated in all three odour-exposure groups (Fig. 5D–F), providing further evidence of the efficacy of the ZnSO₄ lesions of the MOE.

Experiment 3: induction of Fos in the AOB of ZnSO₄-treated female mice by male bedding

Ovariectomized ZnSO₄-treated female subjects that were given daily s.c. injections of sesame oil vehicle for 7 days prior to being exposed to nonvolatile plus volatile body odours via direct application of male urine onto the oronasal groove followed by placement on soiled male bedding for 90 min showed an intermediate amount of AOB Fos activation compared with saline-treated subjects exposed to these same male odour stimuli and ZnSO₄-treated subjects given nasal application of saline followed by exposure to clean bedding (Figs 6A and 7A–C; mitral layer, $F_{2,11} = 12.1$, $P = 0.002$; granule layer, $F_{2,11} = 33.2$, $P < 0.001$). *Post hoc* tests showed significant differences between each of these groups in AOB granule cell Fos expression. There was a nonsignificant trend for ZnSO₄-treated females to have more AOB mitral cell layer Fos-IR cells following application of male urine to the nose plus exposure to male bedding as opposed to application of saline to the nose plus exposure to clean bedding; however, both of these groups had significantly fewer Fos-IR cells in the mitral cell layer than saline-treated females that received male urine on the nose and exposure to male bedding.

The number of Fos-IR cells found in the AOB of ZnSO₄-treated subjects exposed to nonvolatile male body odours was lower than previously reported (Keller *et al.*, 2006b). In that study, ovariectomized, EB-treated female mice that had previously received nasal irrigation with ZnSO₄ or saline showed equivalent AOB Fos responses to nasal application of male urine followed by exposure to male bedding. We asked whether VNO signaling in ovariectomized ZnSO₄-treated females might be enhanced by EB treatment. A second cohort of female subjects given daily s.c. injections of EB (1 µg/day for 7 days) showed a robust response to nonvolatile plus volatile body odours which was more similar to those observed in saline-treated females exposed to these same male odours (Figs 6B and 7D–F; mitral layer, $F_{2,12} = 34.4$, $P < 0.001$; granule layer, $F_{2,12} = 83.7$, $P < 0.001$). The number of Fos-IR mitral cells in ZnSO₄- and saline-treated females exposed to male urine on the nose plus male bedding did not differ by *post hoc* tests. Among ZnSO₄-treated females, significantly greater numbers of Fos-IR granule cells were seen after exposure to male volatile and nonvolatile body odours as opposed to saline on the nose and clean bedding; however, this odour-induced increase was significantly lower in ZnSO₄- than in saline-treated females. An additional control group of ovariectomized, EB-treated female mice that received intranasal saline followed 4 days later by saline on the nose plus exposure to clean bedding would have helped us determine a basal level of AOB Fos expression in EB-treated subjects.

In order to further assess the possibility that the VNO was damaged by intranasal ZnSO₄ application, SBA-HRP staining was performed on all subjects used in each of the three experiments described in this study. All saline and ZnSO₄-treated subjects showed robust staining in the glomerular layer of the AOB (Fig. 7G–I), providing further evidence that inputs to the AOB from the VNO were intact even after the MOE was rendered nonfunctional by intranasal treatment with ZnSO₄. The possibility remains, however, that ZnSO₄ damaged VNO sensory neurons without causing observable loss of SBA staining in the AOB at the time subjects were killed 4 days after intranasal treatment.

Discussion

Results of Experiments 1 and 2 revealed a robust activation of the mitral and granule cell layers of the AOB in response to opposite-sex volatile odours in gonadectomized male and female mice. Ovariectomized female subjects showed an augmented number of Fos-IR mitral and granule cells when exposed to male, but not to female, urinary volatiles. Castrated male subjects, on the other hand, showed an augmented AOB Fos response to female, but not to male, urinary volatiles. The expression of the sex difference in the AOB response to male and female urinary volatiles did not depend on circulating sex steroids. Presumably, this sex difference in AOB responsiveness reflects a hard-wired difference in neural circuitry that is

organized perinatally by the actions of sex hormones in the male brain. A recent study (Muroi *et al.*, 2006) corroborating our results reported that sexually naïve, gonadally intact male mice showed significantly more Fos activation in the rostral and caudal portions of the granule cell layer of the AOB in response to volatile odours present in air drawn over an oestrous female mouse as opposed to volatiles emitted from a gonadally intact male mouse, soiled oestrous female bedding, or an ovariectomized female mouse. Whereas our data show that MOB glomeruli respond to both same- and opposite-sex volatile urinary odours in overlapping but spatially distinct patterns, these odours elicit a sexually dimorphic AOB response that may motivate male and female mice to seek out and maintain proximity to opposite-sex conspecifics.

Several behavioural studies had previously suggested that the main olfactory system plays a central role in murine sex discrimination. Thus, home cage habituation–dishabituation tests of urinary volatiles showed that male and female mice were able to detect differences in conspecifics' sex and endocrine status (Baum & Keverne, 2002; Pankevich *et al.*, 2004; Pierman *et al.*, 2006), and that this ability was not disrupted by removing the VNO (Pankevich *et al.*, 2004). Operant, hunger-motivated tests in an olfactometer showed that gonadectomized, EB-treated male and female mice could discriminate between male and female volatile urinary odours (Wesson *et al.*, 2006). In addition, Ma *et al.* (2002) showed that OMP-ntr transgenic mice lacking functional ORNs due to selective ablation using the pro-drug CB1954 were unable to discriminate between urinary volatiles from different male hybrid strains and showed a profound deficit in olfactory investigation of these stimuli. Our present results extend these behavioural findings by mapping MOB glomerular activation patterns in male and female subjects following exposure to volatile male vs. female urinary odourants. While activation patterns in the two sexes evoked by male and female odours were overlapping and concentrated primarily in the ventral portion of the MOB, the patterns were spatially distinct. In related studies (Schaefer *et al.*, 2001, 2002), female mice exposed to urinary odours from male conspecifics differing at a single gene locus of the MHC haplotype showed distinguishable activation of glomeruli located in the ventral portion of the MOB. Collectively, these data suggest that the ability to discriminate between male and female urinary volatiles begins at the level of the glomerular layer of the MOB, and that the main olfactory system plays a central role in identifying the sex and MHC status of conspecifics.

MOE vs. VNO contributions to AOB activation by urinary volatiles

ZnSO₄ irrigation of the nasal cavities eliminated subsequent MOB Fos activation in male and female subjects exposed to same- or opposite-sex urinary volatiles, as well as AOB Fos activation in response to opposite-sex urinary volatiles. One interpretation of the latter effect is that centrifugal inputs to the AOB, which originate from unspecified segments of the main olfactory pathway, normally convey selective information to the AOB about opposite-sex odours. Retrograde tract-tracing experiments showed that the locus coeruleus (Shipley *et al.*, 1985; McLean *et al.*, 1989) as well as the bed nucleus of the accessory olfactory tract, the MeA and the posteromedial cortical nucleus of the amygdala (Barber, 1982) provide input to the AOB. More research is needed to determine whether any of these brain regions play a role in the accessory olfactory response to opposite-sex volatile urinary odours detected by the MOE.

In both Experiments 1 and 2, volatiles emitted from dilute (20%) urine were presented to subjects. We cannot rule out the possibility that intranasal ZnSO₄ inadvertently damaged VNO sensory neurons so that any residual VNO signaling was not sufficient to activate the AOB after exposure to this relatively weak volatile odour stimulus. There is some controversy in the literature as to whether volatile body odourants can activate VNO sensory neurons. Electrophysiological evidence (Luo *et al.*, 2003) suggests that mice must have direct nasal contact with a conspecifics' nonvolatile body odours to activate AOB mitral cells in response

to VNO signaling. Other *in vitro* imaging (Sam *et al.*, 2001) and *in vivo* behavioural (Trinh & Storm, 2003) experiments suggest, however, that VNO sensory neurons can also detect urinary volatiles, raising the possibility that our observed sexually dimorphic Fos response to opposite-sex urinary volatiles in the AOB mitral and granule cell layers of saline-treated mice resulted, at least partially, from VNO signaling. Some support for this view was provided by Muroi *et al.* (2006) who reported that VNO removal in male mice eliminated Fos activation in the granule cell layer of the AOB otherwise shown in response to volatile estrous female odours. Likewise, Pankevich *et al.* (2006a) found that VNO removal in male mice eliminated Fos activation in the AOB that was otherwise shown after nasal application of oestrous female urine. In both of these studies, sensory stimuli were presented to males at least 2 weeks after VNO removal, raising the question of whether the degeneration of VNO axonal inputs into AOB glomeruli may have compromised the ability of centrifugal odour inputs originating in the MOB to activate denervated mitral cells. By contrast, stimuli associated with mating were still able to induce Fos expression in the AOB mitral cell layer of male rats studied 1 week after VNO removal (Kondo *et al.*, 2003). It is unclear, however, whether this activation occurred in response to genital somatosensory or main olfactory inputs to the AOB. In all three of our experiments, saline and ZnSO₄-treated subjects showed robust SBA-HRP staining in the glomerular layer of the AOB, suggesting that axons of VNO neurons had not degenerated at the time animals were killed 4 days following intranasal treatment with ZnSO₄. It is possible, however, that more subtle damage to VNO receptor neurons went undetected in mice given ZnSO₄ intranasally.

In Experiment 3, ovariectomized females previously given intranasal saline or ZnSO₄ received nasal application of saline or male urine followed by exposure to clean bedding or soiled male bedding, respectively. A significant increase in Fos-IR granule cells was observed in the AOB of ZnSO₄-treated subjects; however, this response was significantly lower than that observed in saline-treated subjects, and there was no significant odour-induced stimulation of Fos in the mitral cell layer of ZnSO₄-treated subjects. It is noteworthy that this apparent defect in VNO signaling was seen even after direct nasal application of undiluted urine, which was presumably a stronger stimulus to both MOE and VNO receptors than the volatiles emitted from the 20% urine solutions presented in Experiments 1 and 2. Several previous studies (Kelliher *et al.*, 1998; Paredes *et al.*, 1998; Halem *et al.*, 1999; Keller *et al.*, 2006b) suggested that circulating sex hormones up-regulate odour-induced Fos expression in the vomeronasal projection pathway in several mammalian species including mice. These results prompted us further to investigate AOB responses to nonvolatile body odours in ovariectomized, EB-treated females. Our data from EB-treated subjects that previously received intranasal saline or ZnSO₄ indicate that Fos activation in the mitral cell layer of the AOB was very similar following nasal application of undiluted male urine followed exposure to soiled male bedding. Significant odour-induced Fos activation was also observed in the AOB granule cell layer of ZnSO₄-treated subjects; however, once again this response was significantly lower than the one observed in saline-treated females. This outcome may reflect unwanted damage to VNO sensory neurons in mice treated intranasally with ZnSO₄. Additional studies in which VNO-lesioned subjects are exposed to volatile urinary stimuli at different intervals after surgery will be needed to assess the contribution of VNO signaling to the sexually dimorphic activation of the AOB by urinary volatiles.

Forebrain Fos responses to volatile urinary odours

Two segments (BNST and MPA) of the vomeronasal projection pathway as well as the AcbSh of the mesolimbic dopamine pathway of female subjects showed significantly augmented Fos expression in response to opposite-sex urinary volatiles (Experiment 1), which paralleled the response observed in the mitral and granule cell layers of the AOB. Other regions in this pathway, including the MeA, MePD and VMHVL also showed nonsignificant trends toward

an augmented response to opposite-sex odours. Direct projections from the AOB to the BNST and subsequent projections to the MPA (Halpern & Martinez-Marcos, 2003) may be responsible for the stronger activation observed in these regions. Male subjects, however, showed augmented Fos responses in several areas of the vomeronasal projection pathway (BNST, MPA, MeA, MePD and VMHVL), the main olfactory projection pathway (Aco and PLCo) and the mesolimbic dopamine pathway (AcbC and AcbSh) in response to both same- and opposite-sex urinary volatiles. These data do not correspond to the stimulus-specific Fos response observed in the mitral and granule cell layers of the AOB in male subjects (i.e. AOB Fos responses occurred only in response to volatile female urinary odours). It is possible that same-sex odours are more salient to male as opposed to female mice, and that direct main olfactory inputs of same-sex body odour information to the vomeronasal amygdala stimulated Fos expression in several forebrain sites of males. In addition, previous research in gonadally intact male hamsters (Fewell & Meredith, 2002) showed that mating experience augmented the ability of females' vaginal odours to induce Fos responses in several forebrain segments of the vomeronasal projection pathway. We used sexually naïve, castrated male mice in Experiment 2. Perhaps forebrain Fos responses to volatile urinary odours from oestrous females, as opposed to males, would have been stronger had we used testosterone-primed males with prior mating experience.

The present results partly corroborate several previous studies suggesting that sex discrimination is mediated by the main olfactory system whereas the reward salience of opposite-sex odours is selectively encoded by the accessory olfactory system. Thus, Pankevich *et al.* (2004 (2006a) found that male mice that underwent surgical removal of the VNO could distinguish the origin of urinary odours on the basis of sex or endocrine status (presumably via the main olfactory system). However, the absence of VNO inputs to the AOB and downstream forebrain targets reduced males' motivation to remain in nasal contact with opposite-sex as opposed to same-sex urinary odours. Similar effects of VNO removal have been reported in female mice (Keller *et al.*, 2006a), male guinea pigs (Beauchamp *et al.*, 1982), female hamsters (Petrulis *et al.*, 1999), and female ferrets (Woodley & Baum, 2004). Recent results (Pankevich *et al.*, 2006a) suggest, however, that main olfactory inputs may also contribute to the motivation of mice to seek out opposite-sex conspecifics. Thus male mice from which the VNO had been removed resembled sham-operated controls in their preference for approaching volatile urinary odours from oestrous females vs. water in Y-maze preference tests. The present results raise the possibility that volatile opposite-sex body odours detected by the MOE gain access to the AOB in addition to the medial amygdala, hypothalamus and mesolimbic dopamine system. Additional studies are needed to assess the possible contribution of main olfactory system–AOB signaling to the ability of opposite-sex social odours to motivate approach behaviours that lead to successful reproduction.

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Abbreviations

AcbC	nucleus accumbens core
AcbSh	nucleus accumbens shell
ACo	anterior cortical nucleus of the amygdala

AOB	accessory olfactory bulb
BNST	bed nucleus of the stria terminalis
EB	estradiol benzoate
FDR	false discovery rate
ICC	immunocytochemistry
IR	immunoreactivity or immunoreactive
MeA	medial amygdala
MePD	posterodorsal medial amygdala
MHC	major histocompatibility complex
MOB	main olfactory bulb
MOE	main olfactory epithelium
MPA	medial preoptic area
ORN	olfactory receptor neuron
PBS	phosphate-buffered saline
PLCo	posterolateral cortical nucleus of the amygdala
SBA-HRP	horseradish peroxidase-conjugated soybean agglutinin
VMHVL	ventrolateral nucleus of the ventromedial hypothalamus
VNO	vomeronasal organ

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Fig. 1. Identifying and mapping activated main olfactory bulb glomeruli using the presence of Fos-IR in periglomerular cells following exposure of female and male mice to volatile urinary odourants. (A) Activated glomeruli (GI) in the main olfactory bulb were identified as having a 180° arc or two 90° arcs of continuous Fos-IR in the periglomerular cells (PG) surrounding the glomerulus (Epl, external plexiform layer). Panel A shows a representative coronal section of the olfactory bulb located at point b in the pictures seen in panel B. The central axis (0–180°) extends from the dorsalmost portion to the ventralmost portion of the mitral cell layer (the arrow extends beyond these boundaries in this picture). The lateral portion of the bulb is located at a radial angle of 90°, the ventral portion at 180°, the medial portion at 270° and finally the dorsal portion at 0° as well as 360°. (B) The radial angle and rostral–caudal location in the bulb were used to map the location of each activated glomerulus. The section depicted in panel A is located at point b in the drawing of the olfactory bulb, and the activated glomerulus shown in the inset is located where the black box is drawn on the two-dimensional map. This

particular glomerulus is found in the ventromedial portion of the bulb, ~210 from the central axis.

Fig. 2.

Effects of volatile urinary odour exposure on Fos expression in the mitral (Mi) and granule (Gr) cell layers of the AOB in ovariectomized female mice previously given intranasal saline or ZnSO₄. (A–D) Fos expression in representative coronal AOB sections from saline-treated females exposed to (A) clean air, (B) male urinary volatiles or (C) female urinary volatiles, and (D) ZnSO₄-treated females exposed to male urinary volatiles. (E) The group data are expressed as Fos-IR cells per standard counting area (mean ± SEM); the number of subjects in each group is given in parentheses. Means with different superscript letters were significantly different from each other based on Student–Newman–Keuls *post hoc* tests after a significant overall ANOVA.

Fig. 3.

Contour plots showing glomerular activation in the main olfactory bulb of saline-treated female mice exposed to (A) clean air ($n = 8$), (B) male urinary volatiles ($n = 9$), or (C) female urinary volatiles ($n = 9$), and of ZnSO₄-treated female subjects exposed to (D) clean air ($n = 6$), (E) male urinary volatiles ($n = 5$) or (F) female urinary volatiles ($n = 7$). Regions of greatest activation are outlined in black. The scale for the number of activated glomeruli per bin is located to the right of the top two rows of panels. (G–I) Point-by-point Mann–Whitney *U*-tests showed significant differences ($P < \text{FDR cutoff value}$ indicated by the vertical black bar on the color scale located below each of plots G–I) between plots of data for saline-treated female subjects (outlined with blue lines) for (G) clean air versus male urinary volatiles, (H) clean air versus female urinary volatiles and (I) male versus female urinary volatiles. Outlined regions of greatest glomerular activation are superimposed on these plots to show overlap in highly activated regions of the MOB.

Fig. 4.

Effects of volatile urinary odour exposure on Fos expression in the mitral (Mi) and granule (Gr) cell layers of the AOB in castrated male mice previously given intranasal saline or ZnSO₄. (A–D) Fos expression in representative coronal AOB sections from saline-treated males exposed to (A) clean air, (B) male urinary volatiles or (C) female urinary volatiles, and ZnSO₄-treated males exposed to (D) female urinary volatiles. (E) Data are expressed as Fos-IR cells per standard counting area (mean $\pm$ SEM); the number of subjects in each group is given in parentheses. Means with different superscript letters were significantly different from each other based on Student–Newman–Keuls *post hoc* tests after a significant overall ANOVA.

Fig. 5.

Contour plots showing glomerular activation in the main olfactory bulb of saline-treated male mice exposed to (A) clean air ($n = 7$), (B) male urinary volatiles ($n = 6$) or (C) female urinary volatiles ($n = 6$), and of ZnSO_4 -treated female subjects exposed to (D) clean air ($n = 7$), (E) male urinary volatiles ($n = 7$) or (F) female urinary volatiles ($n = 6$). Regions of greatest activation are outlined in black. The scale for the number of activated glomeruli per bin is located to the right of the top two rows of panels. (G–I) Point-by-point Mann–Whitney U -tests showed significant differences ($P < \text{FDR cutoff value}$ indicated by the vertical black bar on the color scale located below each of plots G–I) between plots of data for saline-treated female subjects (outlined with blue lines) for (G) clean air versus male urinary volatiles, (H) clean air versus female urinary volatiles and (I) male versus female urinary volatiles. Outlined regions of greatest glomerular activation are superimposed on these plots to show overlap in highly activated regions of the main olfactory bulb.

Fig. 6.

Effects of 90-min exposure to clean or male soiled bedding on Fos expression in the mitral and granule cell layers of the AOB in ovariectomized female mice previously given intranasal saline or ZnSO₄. Prior to being placed on clean bedding, subjects received nasal application of saline; prior to being placed on soiled male bedding, subjects received nasal application of male urine. Subjects in panel A received seven daily s.c. injections of sesame oil vehicle whereas subjects in panel B received estradiol benzoate (1 g/day for 7 days) prior to odour exposure. Data are expressed as the number of Fos-IR cells per standard counting area (mean $\pm$ SEM); the number of subjects in each group is given in parentheses. Means with different superscript letters were significantly different from each other based on Student–Newman–Keuls *post hoc* tests following a significant ANOVA.

Fig. 7.

Examples of Fos and soybean agglutinin immunostaining in sagittal sections through the AOB of ovariectomized female mice previously treated intranasally with either saline or ZnSO_4 . (A–C) Fos immunostaining in the mitral (Mi) and granule (Gr) cell layers of the AOB for subjects injected daily s.c. with sesame oil vehicle; (D–F) Data for subjects injected daily s.c. with estradiol benzoate (1 g/day). Subjects shown in panels A and D received intranasal saline whereas subjects in panels B and E received intranasal ZnSO_4 4 days prior to being exposed for 90 min to soiled male bedding. Subjects shown in panels C and F received intranasal ZnSO_4 4 days prior to being exposed to clean bedding. Prior to being placed on clean bedding, subjects received nasal application of saline; prior to being placed on soiled male bedding, subjects received nasal application of male urine. (G–I) Soybean agglutinin immunostaining in the AOB glomerular layer (Gl) of subjects that had received either (G) intranasal saline or (H and I) ZnSO_4 4 days prior to being exposed for 90 min to either (G and H) soiled male bedding or (I) clean bedding.

Effects of volatile urinary odours on forebrain Fos responses in ovariectomized female mice previously treated intranasally with saline or ZnSO4

Brain region	Saline-treated			ZnSO ₄ -treated		
	Clean air (n = 8)	Male urine (n = 9)	Female urine (n = 8)	Clean air (n = 8)	Male urine (n = 5)	Female urine (n = 7)
Vomeranosal projection pathway						
MeA	14 ± 2 ^b	19 ± 2 ^b	13 ± 2 ^b	5 ± 2 ^a	9 ± 3 ^a	6 ± 2 ^a
MePD	13 ± 2 ^b	17 ± 2 ^b	12 ± 2 ^b	3 ± 1 ^a	7 ± 1 ^a	2 ± 2 ^a
BNST	8 ± 2 ^b	15 ± 2 ^c	11 ± 1 ^b	5 ± 1 ^a	8 ± 1 ^a	5 ± 1 ^a
MPA	20 ± 2 ^b	38 ± 3 ^c	28 ± 2 ^b	13 ± 3 ^a	22 ± 2 ^a	15 ± 2 ^a
VMHVL	8 ± 2	14 ± 4	10 ± 3	9 ± 3	7 ± 1	7 ± 4
Amygdaloid targets of main olfactory input						
ACo	18 ± 3 ^b	21 ± 3 ^b	19 ± 3 ^b	6 ± 2 ^a	8 ± 1 ^a	6 ± 2 ^a
PLCO	12 ± 2 ^b	14 ± 2 ^b	13 ± 3 ^b	5 ± 1 ^a	5 ± 1 ^a	5 ± 2 ^a
Mesolimbic dopamine system						
AcBc	1 ± 0.4	2 ± 1	1 ± 1	1 ± 1	2 ± 1	1 ± 0.4
AcbSh	14 ± 2 ^b	24 ± 2 ^c	16 ± 2 ^b	8 ± 2 ^a	15 ± 2 ^a	9 ± 3 ^a

Data are expressed as the number of Fos-IR cells per standard counting area (mean ± SEM); number of subjects in each group is given in parentheses. In any row, means with different superscript letters are significantly different from each other (*P* < 0.05) based on Student–Newman–Keuls *post hoc* tests following a significant ANOVA.

Table 2 Effects of volatile urinary odours on forebrain Fos responses in castrated male mice previously treated intranasally with saline or ZnSO₄

Brain region	Saline-treated			ZnSO ₄ -treated		
	Clean air (n = 7)	Male urine (n = 6)	Female urine (n = 6)	Clean air (n = 7)	Male urine (n = 7)	Female urine (n = 6)
Vomeronasal projection pathway						
MeA	12 ± 1 ^a	24 ± 2 ^b	25 ± 3 ^b	12 ± 2 ^a	10 ± 2 ^a	10 ± 2 ^a
MePD	8 ± 1 ^a	17 ± 2 ^b	16 ± 1 ^b	8 ± 1 ^a	4 ± 1 ^a	6 ± 1 ^a
BNST	6 ± 1 ^a	18 ± 1 ^b	17 ± 1 ^b	10 ± 1 ^a	10 ± 1 ^a	8 ± 1 ^a
MPA	17 ± 1 ^a	41 ± 4 ^b	48 ± 4 ^b	17 ± 3 ^a	20 ± 2 ^a	17 ± 3 ^a
VMHVL	4 ± 0.3 ^a	7 ± 1 ^{ab}	8 ± 1 ^b	4 ± 1 ^a	3 ± 1 ^a	4 ± 1
Amygdaloid targets of main olfactory input						
ACo	16 ± 3 ^a	24 ± 1 ^b	27 ± 2 ^b	12 ± 1 ^a	8 ± 1 ^a	10 ± 1 ^a
PLCO	9 ± 1 ^a	16 ± 1 ^b	16 ± 2 ^b	8 ± 1 ^a	4 ± 1 ^a	8 ± 1 ^a
Mesolimbic dopamine system						
AcbC	3 ± 0.1 ^a	7 ± 1 ^b	10 ± 1 ^b	3 ± 1 ^a	4 ± 1 ^a	6 ± 1 ^a
AcbSh	11 ± 2 ^a	27 ± 3 ^b	31 ± 4 ^b	14 ± 2 ^a	12 ± 2 ^a	16 ± 2 ^a

Data are expressed as the number of Fos-IR cells per standard counting area (mean ± SEM); number of subjects in each group is given in parentheses. In any row, means with different superscript letters are significantly different from each other ($P < 0.05$) based on Student–Newman–Keuls *post hoc* tests following a significant ANOVA.