Sexual Experience Does Not Compensate for the Disruptive Effects of Zinc Sulfate—Lesioning of the Main Olfactory Epithelium on Sexual Behavior in Male Mice

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Abstract

Recent studies point to an important role for the main olfactory epithelium (MOE) in regulating sexual behavior in male mice. We asked whether sexual experience could compensate for the disruptive effects of lesioning the MOE on sexual behavior in male mice. Male mice, which were either sexually naive or experienced, received an intranasal irrigation of either a zinc sulfate solution to destroy the MOE or saline. Sexual behavior in mating tests with an estrous female was completely abolished in zinc sulfate–treated male mice regardless of whether subjects were sexually experienced or not before the treatment. Furthermore, zinc sulfate treatment clearly disrupted olfactory investigation of both volatile and nonvolatile odors. Destruction of the MOE by zinc sulfate treatment was confirmed by a significant reduction in the expression of Fos protein in the main olfactory bulb following exposure to estrous female urine. By contrast, vomeronasal function did not seem to be affected by zinc sulfate treatment: nasal application of estrous female urine induced similar levels of Fos protein in the mitral and granule cells of the accessory olfactory bulb (AOB) of zinc sulfate– and saline-treated males. Likewise, the expression of soybean agglutinin, which stains the axons of vomeronasal organ neurons projecting to the glomerular layer of the AOB, was similar in zinc sulfate– and saline-treated male mice. These results show that the main olfactory system is essential for the expression of sexual behavior in male mice and that sexual experience does not overcome the disruptive effects of MOE lesioning on this behavior.

Key words: main olfactory system, male mice, sexual behavior, sexual experience, zinc sulfate

Introduction

In mice, olfaction plays a crucial role in mate recognition and subsequently sexual behavior. Two distinct sets of chemosensory receptors, the main olfactory epithelium (MOE) and the vomeronasal organ (VNO), exist in rodent species. Both systems play an important role in the detection of biologically relevant odors (reviewed in Restrepo et al. 2004). Even though controversy exists over whether the VNO is critical for mate recognition, there is more agreement about the role of the VNO in controlling sexual behavior (e.g., Stowers et al. 2002; Pankevich et al. 2004). Male mice in which the VNO was removed surgically showed normal male sexual behavior when paired with a sexually receptive female (Pankevich et al. 2004), although in an early study (Clancy et al. 1984) using shorter, daytime testing, VNO lesions reportedly disrupted mating in male mice. Again, in a recent study (Stowers et al. 2002) targeted deletion of transient receptor potential cation channel 2, a gene expressed in the VNO did not affect mating behavior. The VNO thus does not seem to be critical for the expression of male sexual behavior. A role for the MOE in regulating sexual behavior has been suggested by the observation that olfactory bulbectomy eliminated sexual behavior completely in male mice (Rowe and Edwards 1972). Indeed, recent studies showed that lesioning the MOE with dichlobenil completely disrupted male sexual behavior (Yoon et al. 2005). In addition, male mice carrying a targeted disruption of the Cyclic nucleotide-gated channel subunit A2 (CNGA2) channel, which is only expressed in the MOE, showed strong deficits in sexual behavior (Mandiyan et al. 2005). These results contrast, however, with an early report by Edwards and Burge (1973) who showed that lesioning the MOE with zinc sulfate solution did not affect sexual behavior in male mice. Perhaps these discrepancies are due to differences in

sexual experience of the subjects. The mice used in the Edwards and Burge study (1973) were sexually experienced, whereas Mandiyan et al. (2005) and Yoon et al. (2005) used sexually naive mice. Studies performed in hamsters have clearly demonstrated that sexual experience can compensate for the loss of information processed by one of the olfactory systems. Indeed, the attraction of male hamsters to vaginal fluids as well as sexual behavior are both usually regulated by the accessory olfactory system (e.g., Powers and Winans 1973, 1975; Winans and Powers 1977; Powers et al. 1979; Meredith 1986; Fernandez-Fewell and Meredith 1995; Westberry and Meredith 2003). Thus, lesioning the VNO induced a loss of sexual responses in sexually naive males, whereas lesioning the MOE was only slightly disruptive (Winans and Powers 1977). However, once males gain sexual experience, the main olfactory system becomes able to support sexual behavior in a way similar to that of the accessory olfactory system (Powers et al. 1979; Pfeiffer and Johnston 1994), and only lesioning both systems disrupts sexual behavior (Murphy and Schneider 1970; Winans and Powers 1977; Pfeiffer and Johnston 1994). Therefore, in the present study, we asked whether sexual experience might mitigate the disruptive effects of MOE lesioning on sexual behavior in male mice. Thus, the effect of zinc sulfate lesioning of the MOE on sexual behavior was compared in sexually naive and sexually experienced male mice.

We recently demonstrated that female mice in which the MOE was lesioned by intranasal application of zinc sulfate showed reduced olfactory investigation of both volatile and nonvolatile opposite sex odors (Keller, Douhard, et al. 2006). These results suggest that the MOE plays an important role in mate recognition. Therefore, in the present study, we assessed odor preferences in male mice in which the MOE was lesioned by zinc sulfate to determine whether the MOE plays a similar role in male mice.

Materials and methods

Subjects

Adult (10–12 weeks) male mice (n = 44) of the C57Bl6/j inbred strain were obtained from a local breeding colony at the University of Liège. Subjects were housed alone in macrolon cages and were placed in a climate-controlled (light, temperature, ventilation) animal housing unit (Iffa-Credo, L'Arbresle, France) on a reversed 12/12 h light–dark cycle. The air pressure in the housing units was higher than in the animal room, thereby avoiding the inflow of any odors from the room in which the housing unit was placed. Thus, care was taken that males were not exposed to any female-derived odors except when tested. Sawdust served as bedding and was not changed for at least 48 h before any behavioral test. Food and water were always available ad libitum.

All the procedures were conducted in accordance with the guidelines set forth by the National Institutes of Health Guiding Principles for the Care and Use of Research Animals and were approved by the Ethical Committee for Animal Use of the University of Liège.

Zinc sulfate treatment

Before the onset of behavioral testing, mice received an intranasal application of 10% zinc sulfate or saline solution under general anesthesia using a mixture of ketamine (80 mg/kg per mouse) and medetomidine (Domitor, Pfizer, Groton, CN; 1 mg/kg per mouse). Mice received atipamezole (Antisedan, Pfizer, 4 mg/kg per mouse) subcutaneously (s.c.) at the end of the treatment in order to antagonize medetomidine-induced effects, thereby accelerating their recovery. Subjects were placed on their back, and each naris was injected with 10 μl of a sterile 10% zinc sulfate solution or saline. Immediately after zinc sulfate irrigation, the mice were held with their head down for several seconds to minimize spread of the solution to the oral cavity.

Because regeneration of olfactory receptor cells in the MOE typically occurs within 7 days after zinc sulfate treatment (for a review, see McBride et al. 2003), all behavioral tests were completed within 1 week after intranasal application of zinc sulfate. Peripheral anosmia was first assessed using habituation/dishabituation tests following zinc sulfate treatment. Undiluted urinary odors from estrous females were used as odor stimulus. Only mice that no longer responded to the odor stimulus following zinc sulfate treatment were subsequently tested for odor preferences or sexual behavior. In order to obtain sufficient data, all males were retreated with zinc sulfate or saline approximately 1 week following the first treatment and retested again for odor preferences or sexual behavior.

Experimental procedures

Role of the MOE in sexual behavior

Male mice were divided into 4 groups: 2 groups of sexually naive males, consisting of 7 zinc sulfate– and 6 saline-treated males, and 2 groups of sexually experienced males, consisting of 8 zinc sulfate– and 8 saline-treated males. To gain sexual experience, these males were placed with a sexually receptive female at least 3 times on separate days for 2 h before being treated with zinc sulfate or saline. No behavior was scored during these pretests. However, all males showed mounting behavior. Then the sexual behavior of these males was tested during 1 pretest session of 30 min to ensure that these animals continued to show high levels of mating behavior. Following treatment with either zinc sulfate or saline, males were tested for mating behavior with a sexually receptive female a total of 4 times.

Mating behavior of zinc sulfate– and saline-treated males, which were either sexually naive or experienced before the onset of the experiment, was quantified by recording the number of mounts with or without intromission. All mating

tests were conducted in a plexiglas aquarium (35 cm long × 25 cm high × 19 cm wide) whose floor was covered with fresh sawdust. At the beginning of each test, males were placed alone in the aquarium and allowed to adapt for 15 min. By placing an estrous female in the aquarium, the test began and lasted 30 min or until the male had ejaculated. Behavioral estrus was induced in female mice by ovariectomy and subsequent treatment with estradiol by silastic capsule and a s.c. injection of 500 µg progesterone 2–3 h before each behavioral test (Bakker et al. 2002). Behavioral tests were always performed on days 2 and 5 following zinc sulfate treatment, and anosmia was reassessed by habituation/dishabituation tests on day 6 following zinc sulfate treatment. All behavioral tests were performed between 11:00 AM and 3:00 PM, during the dark phase of the light/dark cycle.

Role of the MOE in mate recognition

All olfactory discrimination tests were conducted in an enclosed plexiglas Y-maze (for a full description of the maze, see Bakker et al. 2002). When subjects were tested for mate recognition using volatile body odors as odor stimuli, removable opaque plexiglas doors were placed at the distal end of each arm to separate the goal boxes from the rest of the maze. Volatile body odors were derived from anesthetized stimulus animals placed behind these doors. It should be noted that the top of each opaque door was perforated to allow air to flow from the goal boxes into the maze. However, these holes were placed at the upper part of the door so that subjects could not see the stimulus animals. The level of anesthesia was checked regularly and adjusted—if necessary—between each trial. Also, stimulus animals were placed on a heating pad to prevent hypothermia. The time was recorded that the subject spent poking his nose in the holes of the door or actively sniffing the door. When subjects were tested for mate recognition using nonvolatile odors, the doors were removed to allow direct access to the odor stimuli that were placed in the back of each goal box. Soiled bedding was placed in bowls, and the time that the mouse spent in direct physical contact with the stimulus was recorded.

At the beginning of each test, the subject was placed in the start box with the door closed to adapt for 1 min. The test began when the door was removed, and the subject could freely move around in the Y-maze. The time the subject spent investigating each odor stimulus was recorded with a stopwatch. Subjects were first tested for 5 min in the Y-maze with no stimulus animals in the goal boxes to adapt to the testing apparatus and to determine whether they would develop any side preferences. This test was conducted before animals were treated with either zinc sulfate or saline. The maze was cleaned with 70% ethanol between trials. All Y-maze tests lasted 5 min and were separated by at least 2 days. For each test, cages were taken randomly out of the housing unit to prevent the same animals always being tested first or last.

Preparation of odor stimuli. To obtain soiled bedding, groups of females that were ovariectomized (OVX) under general anesthesia at least 2 weeks prior to testing and implanted with (*n* = 5) or without (*n* = 5) a silastic capsule containing estradiol (diluted 1:1 with cholesterol; for more details, see Bakker et al. 2002) were placed in clean cages containing fresh sawdust. Females that were implanted with a capsule containing estradiol were injected with 500 µg of progesterone 2–3 h before being placed in a clean cage. At the same time, OVX females not treated with estradiol or progesterone were placed in a clean cage. Bedding was collected 10 h later. All bedding was stored in plastic freezer bags at −80 °C prior to being used in the experiment. When anesthetized animals were used to provide volatile body odors, females that were implanted with a capsule containing estradiol were also injected with progesterone at least 2 h before being used in the experiment.

Determine whether lesioning the MOE affects anxiety

Because olfactory bulbectomy is commonly used as a model of depression in rodents (Kelly et al. 1997; Song and Leonard 2005), we determined whether the behavioral changes observed during the sexual interactions with the female might be due to any changes in subjects' state of anxiety. Therefore, zinc sulfate– and saline-treated animals were tested for their behavior in the elevated plus maze. Male mice were brought into the test room at least 1 h before the onset of behavioral testing and remained in the same room throughout the test. The maze consisted of 4 arms (each arm 30 cm long × 15 cm high × 8 cm wide); 2 open and 2 closed arms formed a cross, which was raised 80 cm above the floor. At the beginning of the test, each mouse was placed in the center area, and subsequently, the time spent in the center, open, and closed arms was recorded for 5 min. In addition, the number of entries into either the open or closed arms was registered. Behavioral variables were recorded using a stopwatch. It was considered that the mouse was in the open (or closed) arm when its 4 legs were no longer in the center area. Males were tested individually under normal white lighting in a random order. The maze was cleaned with 70% ethanol to eliminate odors after each test.

Assessment of the specificity of the destruction of the MOE

Habituation/dishabituation tests. To assess whether animals that were treated with zinc sulfate were anosmic, habituation/dishabituation tests were conducted using volatile urinary odors derived from estrous females as odor stimulus on days 1 and 6 following zinc sulfate treatment. Animals were tested in their home cages as described by Baum and Keverne (2002) and Pierman et al. (2006). The stainless steel cage top containing their food and water was removed and replaced with a clean top. Odor stimuli were presented by pipetting 30 µl of estrous female urine onto a piece of filter paper that was glued to a plastic weighing boat (4.3 × 4.3 cm), which was then placed in the food hopper so that volatile odors from the

stimulus were available at body level. Subjects were unable to make physical contact with the filter paper using either their snout or paws. Each test was constituted of a sequence of 3 water presentations followed by 3 odor presentations. The duration of investigation of the odor stimuli was recorded using a stopwatch; any significant increase of olfactory investigation (dishabituation) when being exposed to the odorant stimulus was considered as the subject detecting the odor.

Histological assessment of the specificity of MOE destruction. When behavioral testing was completed, the specificity of the zinc sulfate lesioning was assessed histologically. Thus, we determined whether infusions with zinc sulfate had damaged the functioning of VNO sensory neurons and thus disrupted the functioning of the accessory olfactory system. Brains of several zinc sulfate– or saline-treated males were processed for soybean agglutinin (SBA) and Fos immunocytochemistry. Therefore, zinc sulfate– and saline-treated males were stimulated by direct application of 30 μl of either estrous female urine (saline: $n = 5$, zinc sulfate: $n = 7$) or water (saline: $n = 5$) onto the oronasal groove. Ninety minutes after stimulation, animals were anesthetized with ketamine/domitor and perfused transcardially with saline followed immediately by 4% cold paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) (pH = 7.4). Brains were removed and postfixed in 4% paraformaldehyde for 2 h. Then brains were cryoprotected in 30% sucrose/PBS solution and when sunken, frozen on dry ice, and kept at −80 °C. Sagittal sections (30 μm thick) of the olfactory bulbs were cut on a Leica cryostat, and alternate sections were stained for SBA conjugated with horseradish peroxidase (SBA-HRP) or Fos. Sections were saved in antifreeze solution and maintained at −20 °C for later immunocytochemistry.

Accessory olfactory bulb morphology using SBA. SBA-HRP stains the axons of VNO neurons that project to the glomerular layer of the accessory olfactory bulb (AOB) and serves as a useful marker for the presence of intact VNO neurons in mice and rats (Key and Giorgi 1986; C.J. Wysocki and L.M. Wysocki 1995). After surgical removal of the VNO, the lack of SBA staining in the AOB provides evidence that the VNO was successfully removed. In this study, SBA-HRP staining was performed to ensure that zinc sulfate treatment did not affect the integrity of the VNO. Sagittal sections of the olfactory bulbs were first incubated in 3% normal goat serum (NGS)/1% H_2O_2/PBS for 2 h, followed by washes in 0.1 M PBS. Sections were then incubated in SBA-HRP (15 μg/ml; Sigma Chemicals, St Louis, MO) for 40 min at room temperature, followed by washes in PBS. Sections were reacted with nickel-3,3′-diaminobenzidine (DAB) for 5 min and then mounted onto gelatin-coated slides and coverslipped using permount.

Functional assessment of the main and AOB using the expression of c-fos. Every fourth section was processed for Fos immunoreactivity as previously described (Keller, Douhard, et al. 2006; Keller, Pierman, et al. 2006). All incu-

bations were done at room temperature and all washes in PBS. Briefly, sections were preincubated for 3 h in 7.5% NGS in Tris-buffered saline containing 0.1% Triton X-100 (TBST). Then sections were incubated overnight with a rabbit polyclonal anti c-fos antibody (Santa Cruz, CA; SC-52; 1:3000 in TBST/2% NGS) and then incubated for 1 h in a goat anti-rabbit biotinylated antibody (Dako Cytomation, Carpinteria, CA; 1:200 in TBST/2% NGS). To eliminate endogenous peroxidase activity, sections were incubated for 30 min in PBS containing H_2O_2 at a final concentration of 3%. Sections were then incubated for 45 min in avidin–biotin complex (Vector Lab, Burlingam, CA) and reacted for 5 min with DAB containing nickel chloride (Vector Lab, prepared according to the manufacturer's recommendations). Then sections were washed, mounted onto gelatin-coated slices, dried, dehydrated through graded alcohol, cleared in toluene, and coverslipped using permount. Numbers of Fos-immunoreactive cells were quantified throughout the mitral and granular cell layers of the AOB and main olfactory bulb (MOB), using a microscope with a camera lucida attachment. Slides were coded so that the investigator did not know the treatment of the subject. For both the mitral and granular cell layers of the AOB and MOB, respectively, 3 sagittal sections (from lateral to medial) from both bulbs were analyzed for numbers of Fos-immunoreactive neurons. For all 3 sections, all Fos-immunoreactive neurons visible in a field of view under the 40× objective were traced on a piece of paper using the camera lucida attachment and subsequently counted. For the AOB, the number of Fos-immunoreactive cells visible in one single field of the 40× objective were traced on a piece of paper and subsequently counted. For the MOB, even though differences in Fos immunoreactivity between zinc sulfate– and saline-treated animals were consistent throughout the olfactory bulb, we analyzed Fos immunoreactivity at 3 different locations in the MOB in order to avoid possible confounding effects of regional differences in Fos activation. For instance, the ventral part of the MOB has been demonstrated to be particularly sensitive to urinary stimulation (Schaefer et al. 2001; Lin et al. 2005). Therefore, we analyzed Fos immunoreactivity at the dorsal and ventral parts separately. Because no regional differences were found, we later combined these data. Thus, in Results, Fos immunoreactivity is expressed as the number of cells per standard counting area. Thus, only relative differences between saline- and zinc sulfate–treated animals are reported and not total numbers of Fos-immunoreactive cells.

Statistical analysis

All data were analyzed using repeated measures analysis of variance (ANOVA). When appropriate, all ANOVAs were followed by Tukey highest signification difference (HSD) post hoc comparisons adapted for repeated measures ANOVA. Only significant ($P < 0.05$) effects detected by the ANOVAs are mentioned in detail in Results.

Results

Role of MOE in sexual behavior

Sexually experienced males showed high levels of sexual behavior during the pretest (Figure 1). Furthermore, no differences were observed between males that were destined to receive zinc sulfate or saline. Because the achievement of an ejaculation might be more relevant to getting sexually experienced than mounting behavior by itself, we recorded whether the males ejaculated. Ten out of 15 males ejaculated during this test (5/8 in the group destined to receive saline, and 5/7 in the group destined to receive zinc sulfate). Thus, an equal proportion of males ejaculated in both groups.

Zinc sulfate lesions completely abolished sexual behavior in both sexually experienced and sexually naive males, whereas saline infusions had no effect on sexual behavior (Figure 1). This was confirmed by 2-way ANOVA with treatment and sexual experience as independent factors and tests as repeated factor showing a significant effect of zinc sulfate treatment on mount frequency ($F_{(1,25)} = 19.11$, $P < 0.001$) and intromission frequency ($F_{(1,25)} = 14.3$, $P < 0.001$) but no significant effect of sexual experience, repeated testing, or a significant interaction between these factors. Latencies

A Mount frequency

B Intromission frequency

Figure 1   Mean (±standard error of mean) mount **(A)** and intromission frequencies **(B)** of male mice that had received either an intranasal irrigation with zinc sulfate (ZnSO₄) to destroy the MOE or saline. *$P < 0.05$, significant effect of zinc sulfate treatment.

to show first mount, intromission, or ejaculation were also strongly affected in zinc sulfate–treated males (Table 1). Two-way ANOVA showed a significant effect of zinc sulfate treatment on mount latency ($F_{(1,25)} = 28.84$, $P < 0.001$), intromission latency ($F_{(1,25)} = 23.1$, $P < 0.001$), and ejaculation latency ($F_{(1,25)} = 5.0$, $P = 0.034$) but no significant effect of sexual experience, of repeated testing, or a significant interaction between any of the factors. It should be noted that 1 zinc sulfate–treated male started to copulate in test 4 (Table 1), even though he did not respond to the presentation of estrous female odors in habituation/dishabituation tests conducted the next day, suggesting that he was still anosmic.

Role of the MOE in detecting volatile odors

Zinc sulfate–treated males failed to show an odor preference, whereas saline-treated males clearly preferred to investigate estrous over OVX female volatile body odors (Figure 2A). Overall, zinc sulfate–treated males investigated the odor stimuli for significantly less time, suggesting that they could not detect the odors. Two-way ANOVA with treatment as independent factor and odor stimulus (estrous vs. anestrous female) showed a significant effect of zinc sulfate treatment ($F_{(1,13)} = 25.4$, $P < 0.001$), of odor stimulus ($F_{(1,13)} = 6.85$, $P = 0.021$), and a significant interaction between these 2 factors ($F_{(1,13)} = 10.36$, $P = 0.007$). Post hoc analysis showed that saline-treated males spent more time investigating estrous over OVX female odors as well as spending more time investigating either odor compared with zinc sulfate–treated males. The lower investigation time of zinc sulfate–treated males was probably not due to the males not exploring the Y-maze and thus the odor stimuli. Two-way ANOVA on the number of visits to each arm showed a significant effect of zinc sulfate treatment ($F_{(1,13)} = 7.3$, $P = 0.018$; Table 2); however, zinc sulfate–treated males did explore the Y-maze, although not to the same levels as saline-treated males.

Role of the MOE in detecting nonvolatile odors

As was observed when volatile body odors were used as odor stimuli, zinc sulfate–treated males failed to show an odor preference when provided with the choice between soiled bedding from estrous females and that of OVX females (Figure 2B). In addition, zinc sulfate–treated males spent less time investigating either type of bedding. Saline-treated males, however, showed a clear preference to investigate estrous female bedding. Two-way ANOVA revealed a significant effect of the zinc sulfate treatment ($F_{(1,13)} = 18.4$, $P < 0.001$), of the odor stimulus ($F_{(1,13)} = 5.7$, $P = 0.032$), and a significant interaction between these 2 factors ($F_{(1,13)} = 9.7$, $P = 0.008$). Zinc sulfate–treated males continued to visit both arms of the Y-maze as indicated by the number of arm visits (Table 2), but this number was significantly lower than what was observed in saline-treated males (effect of treatment: $F_{(1,13)} = 4.9$, $P = 0.045$).
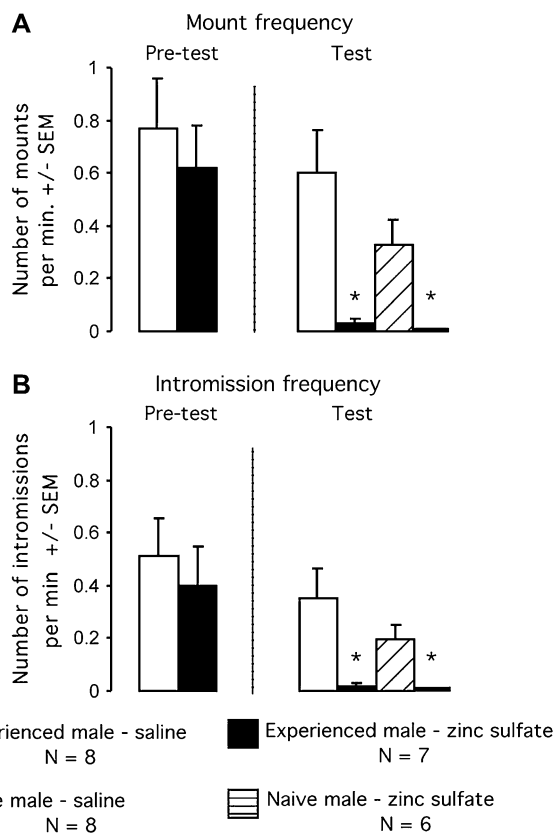
Table 1 Mount and intromission latencies in minutes during pair tests with an estrous female

	First mount latency (min)				First intromission latency (min)				Ejaculation latency (min)			
	Exp-saline	Exp-ZnSO₄	Naïve-saline	Naïve-ZnSO₄	Exp-saline	Exp-ZnSO₄	Naïve-saline	Naïve-ZnSO₄	Exp-saline	Exp-ZnSO₄	Naïve-saline	Inexp-ZnSO₄
Pretest	6.1 ± 1.5	5.6 ± 1.8			8.7 ± 3.0	10.3 ± 3.3			23.3 ± 3.0	25.3 ± 1.7		
Test nr 1	13.4 ± 4.0	26.0 ± 3.9	21.5 ± 3.7	/	16.9 ± 5.0	/	23.6 ± 2.8	/	22.0 ± 4.0	/	28.8 ± 0.9	/
Test nr 2	9.8 ± 3.8	/	11.9 ± 4.3	/	18.0 ± 4.0	/	15.5 ± 3.9	/	24.5 ± 3.7	/	24.5 ± 2.6	/
Test nr 3	16.9 ± 4.8	/	18.0 ± 4.9	/	14.5 ± 4.8	/	25.9 ± 3.4	/	23.7 ± 3.2	/	29.8 ± 0.2	/
Test nr 4	18.2 ± 4.5	23.4 ± 4.5	25.0 ± 2.5	/	21.8 ± 4.6	23.5 ± 4.4	27.3 ± 1.6	/	27.8 ± 2.2	27.3 ± 0.4	29.5 ± 0.4	/

Male mice had received either an intranasal infusion with zinc sulfate to destroy the MOE or saline to serve as control. Furthermore, males were either sexually naive (naive) or sexually experienced (Exp) prior to zinc sulfate or saline treatment. The "/" indicates that the mean of the group is 30 min, so no behavior was observed during the test; no pretest for the 2 groups without sexual experience.
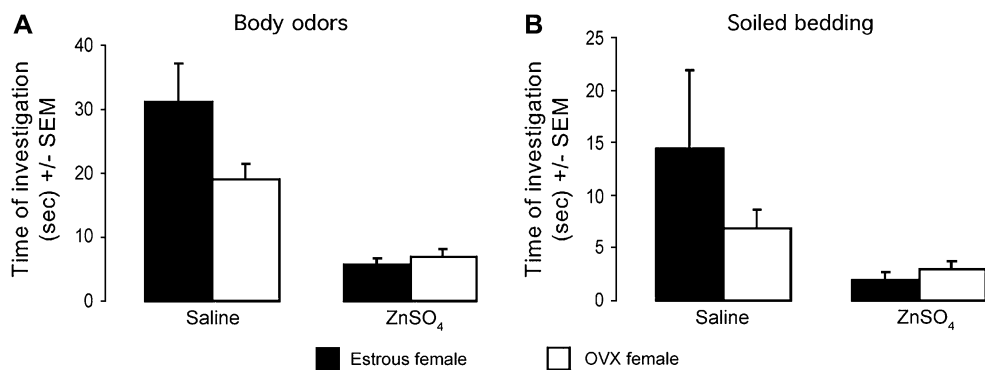
Figure 2 The mean (±standard error of mean) amount of time that male mice spent investigating volatile **(A)** and nonvolatile **(B)** odors derived from estrous females or OVX females in a Y-maze. Males had received either a intranasal irrigation with zinc sulfate to destroy the MOE (ZnSO₄) or saline.

Determine whether MOE lesioning affects anxiety

No significant differences were observed between ZnSO₄- and saline-treated males in their behavior in the elevated plus maze, suggesting that the zinc sulfate lesions did not affect their state of anxiety (saline-treated males—mean [±standard error of mean] time spent in open arm: 54 ± 10 s, closed arm: 197 ± 16 s, center of the maze: 50 ± 9 s; zinc sulfate–treated males—open arm: 69 ± 9 s, closed arm: 163 ± 7 s, center of the maze: 88 ± 19 s). This was confirmed by 2-way ANOVA with treatment as independent factor and arm of the maze (open or closed) as repeated factor showing a significant effect of arm of the maze ($F_{(2,32)}$ = 13.2, $P < 0.001$; Figure 3) but no effect of zinc sulfate treatment. Post hoc analysis revealed that all males spent more time in the closed than in the open arm or in the center of the maze.

Assessment of the specificity of the destruction of the MOE

Habituation/dishabituation tests

For all tests performed, none of the zinc sulfate–treated animals showed dishabituation responses when provided with volatile urinary odors from estrous females. By contrast, all saline-treated males consistently showed dishabituation responses when provided with estrous female urine (data not shown).

AOB morphology using SBA

Both zinc sulfate– and saline-treated males showed robust SBA-HRP labeling in every section containing the AOB (Figure 3). Gross anatomical differences could be observed neither in the morphology of the glomerular layer of the AOB nor in the intensity of the labeling between zinc sulfate– and saline-treated males.

Functional assessment of the MOB and AOB using the expression of c-fos

Exposure to estrous female urine significantly induced Fos protein in the mitral cell layer of the AOB of both zinc sulfate– and saline-treated males (Figure 4). This effect was also noticeable, although not significantly, in the granular cell layer of the AOB. By contrast, only a significant induction of c-fos was observed in the MOB of saline-treated females but not of zinc sulfate–treated males following exposure to estrous female urine, thereby confirming a substantial destruction of the MOE. One-way ANOVA on the number

Table 2 Mean number of visits to each arm of the Y-maze when given the choice between volatile odors derived from anesthetized conspecifics or nonvolatile odors derived from soiled bedding

	Saline		Zinc sulfate	
	OVX	Estrous	OVX	Estrous
Anesthetized conspecifics	4.6 ± 0.4	4.7 ± 0.7	3.0 ± 0.5*	2.7 ± 0.4*
Soiled bedding	3.6 ± 0.4	4.0 ± 0.5	2.7 ± 0.4*	2.6 ± 0.5*

*$P < 0.05$ post hoc comparisons between saline- and zinc sulfate–infused males.
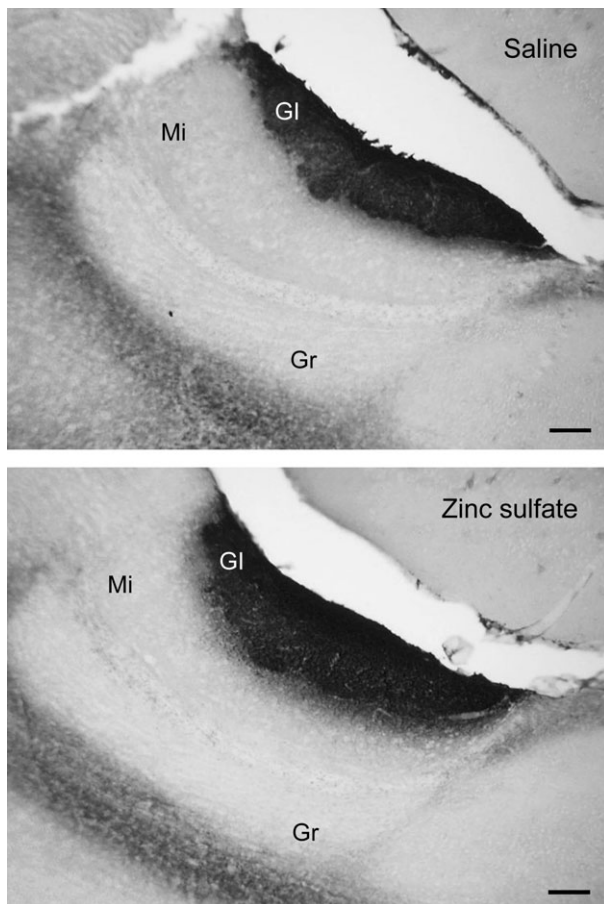
Figure 3 Representative photomicrographs showing sagittal sections stained with SBA-HRP of the AOB of male mice that had either undergone zinc sulfate or saline treatment. The presence of SBA-HRP staining in the glomerular layer of both groups of animals was taken as evidence of intact VNO function in zinc sulfate–treated males. Gl, glomerular cell layer; Mi, mitral cell layer; Gr, granular cell layer. Scale bar: 100 μm.

of Fos-immunoreactive cells in the AOB revealed a significant effect of odor exposure in the mitral cell layer ($F_{(2,14)} = 6.2$, $P = 0.012$) but no significant differences between estrous female urine-exposed zinc sulfate– and saline-treated males (Figure 5). By contrast, 1-way ANOVA on the number of Fos-immunoreactive cells in the MOB
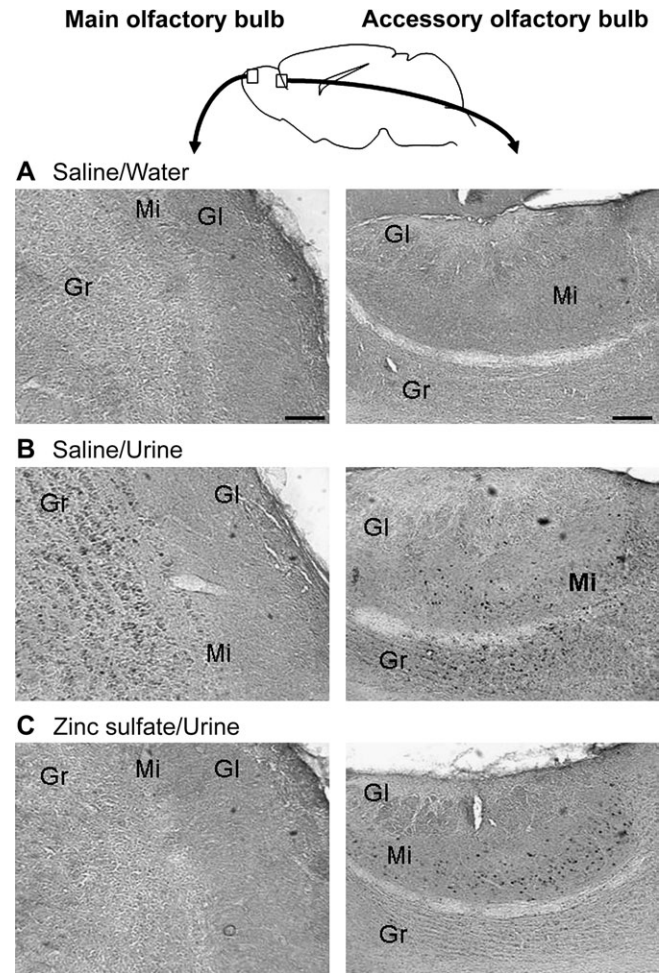

Figure 4 Representative sagittal sections showing Fos-immunoreactive cells in the mitral and granular cell layers of the main and AOB. Male mice in which the MOE was destroyed by intranasal application of zinc sulfate did not show a significant induction of c-fos in both cell layers of the MOB after exposure to estrous female urine. By contrast, they showed a similar Fos induction in both cell layers of the AOB. **(A)** Saline-treated male exposed to water, **(B)** saline-treated males exposed to estrous female urine, and **(C)** zinc sulfate–treated male exposed to estrous female urine. Scale bar: 100 μm.

revealed significant differences between urine-exposed zinc sulfate– and saline-treated males in the mitral cell layer ($F_{(2,14)} = 7.8$, $P = 0.005$) and granular cell layer ($F_{(2,14)} = 4.8$, $P = 0.026$). In fact, zinc sulfate–treated males exposed to estrous female urine showed the same level of Fos activation as saline-treated males exposed to water.

Discussion

The present study clearly shows that destruction of the MOE by intranasal application of zinc sulfate completely disrupts sexual behavior, indicating that the MOE plays a central role in regulating mating behavior in male mice. Our results thus corroborate recent observations that also showed strong deficits in sexual behavior in CNGA2 mutant mice (Mandiyan et al. 2005) and following treatment with dichlobenil (Yoon
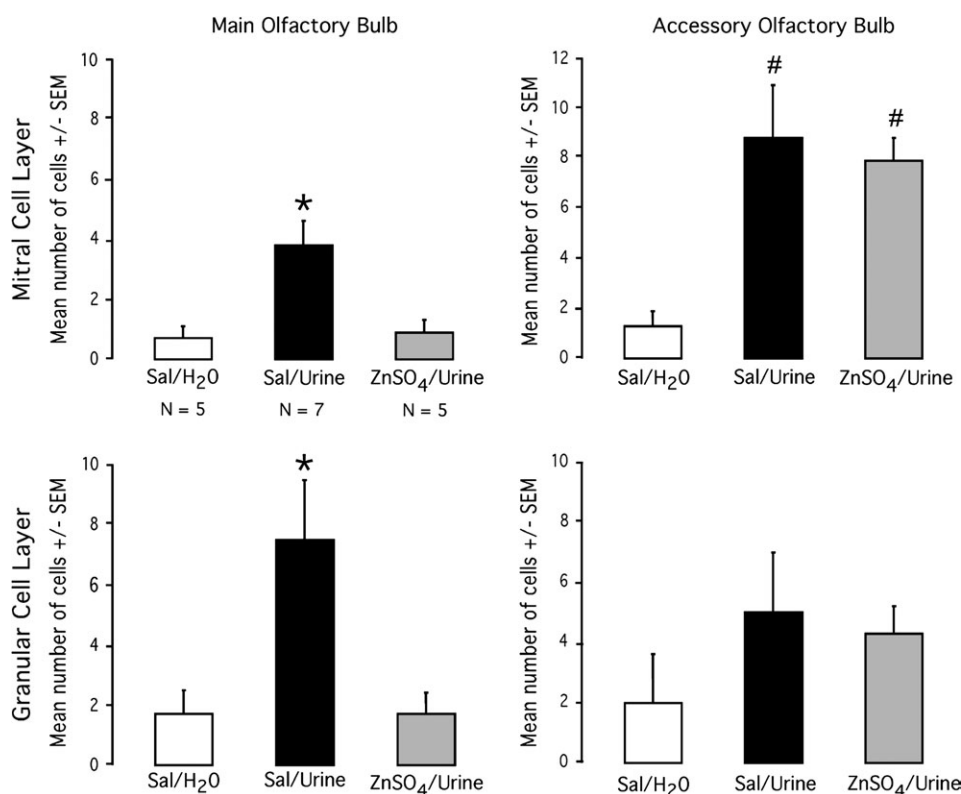
Figure 5 Mean (±standard error of mean) numbers of Fos-immunoreactive cells in the mitral and granular cell layers of both the MOB and AOB. Male mice which were either treated with zinc sulfate to destroy the MOE (ZnSO₄) or saline (Sal) were either exposed to estrous female urine or water placed directly on the nose. *$P < 0.05$, significantly higher in Sal-treated males exposed to female urine compared with Sal-treated males exposed to water or zinc sulfate–treated males exposed to female urine. #$P < 0.05$, significantly different between Sal-treated males exposed to water and Sal- and zinc sulfate–treated males exposed to female urine.

et al. 2005). In contrast with what has been observed in male hamsters (Pfeiffer and Johnston 1994), sexual experience did not compensate for the loss of input from the main olfactory system in male mice. Zinc sulfate lesions had very similar disruptive effects on sexual behavior in sexually naive and sexually experienced males. One possibility to explain this discrepancy may be that the amount of sexual experience given to the animals before being treated with zinc sulfate was not sufficient. Perhaps longer periods of exposure with various females would allow compensation processes to occur, through accessory olfactory or other sensory cues, for the loss of the main olfactory system. However, according to the level of sexual experience of our animals, our study does not explain the discrepancy between the early study by Edwards and Burge (1973) and those by Mandiyan et al (2005) or Yoon et al. (2005). Perhaps the zinc sulfate treatment in the study of Edwards and Burge was less effective in lesioning the MOE. The authors did not provide any histological evidence that they successfully lesioned the MOE. They assumed that their males were anosmic based on their inability to find a piece of cookie buried in the sawdust; this test may not be the most sensitive method to assess MOE function in mice. However, there was some

indication of a recovery in one zinc sulfate–treated male. He started to copulate until ejaculation in the final test even though he did not show any dishabituation responses to the presentation of estrous female odors the next day, suggesting that he was still anosmic. Perhaps, if we had continued to test zinc sulfate–treated males for a more prolonged period of time, all of them would eventually have recovered, suggesting that some compensatory mechanism may exist.

In the present study, we used different behavioral and histological methods to assess independently the loss of olfactory function in zinc sulfate–treated males. Thus, zinc sulfate–treated animals failed to respond to volatile female urinary odors in habituation/dishabituation tests, and when tested in the Y-maze, these animals showed a dramatic reduction in their time of investigation toward volatile body odors derived from either an estrous or OVX female in comparison with saline-infused controls. In addition, zinc sulfate–treated males showed a significant lack of induction of c-fos, in both the mitral and granular cell layers of the MOB, after being exposed to female urine. These data demonstrate that the destruction of the MOE through zinc sulfate application was efficient and rendered male mice anosmic for the whole duration of the experiment. In addition,

the normal induction of c-fos in the AOB following urinary stimulation as well as the morphological analysis of the glomerular layer of the AOB by means of SBA-HRP histochemistry made us confident that our lesion was restricted to the MOE and that the behavioral effects observed in this study were not due to any collateral VNO damage and disruption of the accessory olfactory system.

The dramatic reduction observed in olfactory investigation of both volatile and nonvolatile odor stimuli in a Y-maze in zinc sulfate–treated males is very similar to what we observed in our previous study using zinc sulfate–treated females (Keller, Pierman, et al. 2006). As observed in our previous study, the lower level of olfactory investigation shown by zinc sulfate–treated males was not due to any side effects of the zinc sulfate treatment on locomotor activity or general state of anxiety. Indeed, zinc sulfate–treated males continued to explore the Y-maze, albeit significantly less compared with saline-treated males, and when tested in the elevated plus maze were not found to be more anxious than saline-treated controls. Thus, the absence of any odor preferences for estrous female odors indicates that zinc sulfate–treated males were not able to detect estradiol-dependent female chemosignals just as zinc sulfate–treated females were unable to detect to testosterone-dependent male chemosignals. These results support the view that both male and female mice use volatile odors for mate recognition (Powers et al. 1979; O'Connell and Meredith 1984; Bakker et al. 2002; Pankevich et al. 2004; Keller, Douhard, et al. 2006; Keller, Pierman, et al. 2006). A similar conclusion was reached in a study (Kelliher and Baum 2001) showing that peripheral anosmia disrupted mate recognition in both male and female ferrets.

When comparing the results of several studies with the role of the main versus the accessory olfactory system in regulating sexual behavior (e.g., Stowers et al. 2002; Pankevich et al. 2004; Keller, Douhard, et al. 2006; Keller, Pierman, et al. 2006), clear sex differences emerge with regard to which olfactory system plays a more predominant role in which sex. For instance, VNO lesions completely abolished lordosis behavior in female mice (Keller, Douhard, et al. 2006), whereas it had no effect on mounting and intromissive behaviors in male mice (Pankevich et al. 2004). Likewise, MOE lesions completely abolished sexual behavior in male mice (Yoon et al. 2005; present study), whereas these lesions only had moderate effects on lordosis behavior in female mice (Edwards and Burge 1973; Keller, Pierman, et al. 2006). Thus, in male mice, the main and not the accessory olfactory system seems to be more important in regulating sexual behavior, whereas in female mice, it seems to be the accessory and to a lesser extent the main olfactory system.

Acknowledgements

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