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Effect of bilateral accessory olfactory bulb lesions on volatile urinary odor discrimination and investigation as well as mating behavior in male mice

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Abstract

Previous research raises the possibility that urinary volatiles from estrous female mice activate mitral cells in the accessory olfactory bulb (AOB) of male mice following detection via the main olfactory epithelium as opposed to the vomeronasal organ. We asked whether bilateral lesions of the AOB would disrupt the ability of male mice to discriminate between urinary volatiles from mice of different sexes or endocrine states, or affect their interest in investigating these odors when they were presented sequentially in home-cage habituation/dishabituation tests. Males with either partial or complete bilateral lesions of the AOB resembled sham-operated control males in their ability to discriminate between ovariectomized and estrous female urinary volatiles as well as between male and estrous female urinary volatiles. However, males with either complete or partial AOB lesions spent significantly less time than sham-operated control males investigating urinary volatiles from estrous females, especially during tests when the alternative stimulus presented was male urine. Placement of AOB lesions failed to disrupt males' mating performance. Our results suggest that the incentive value of opposite-sex (female) volatile urinary odors which are initially detected by the main olfactory system is enhanced when they are further processed by the male's AOB.

Keywords

Pheromone; Testosterone; Main olfactory system; Accessory olfactory system; Vomeronasal organ

1. Introduction

Rodents use body odors for social communication [4]. Current dogma holds that volatile components of urine are detected by the main olfactory epithelium (MOE) and processed in the main olfactory bulb (MOB) [25], whereas non-volatile components of urine [14], as well as extraorbital lacrimal gland secretions [12], are detected by receptor neurons in the vomeronasal organ (VNO) and processed in the accessory olfactory bulb (AOB). Although details remain to be worked out, several studies [3,6,9,10,11,13] suggest that socially relevant olfactory cues from both the MOB and AOB are integrated in the medial amygdala prior to being conveyed to different hypothalamic regions. In previous studies [21,22], we found that male mice retained the ability to discriminate between volatile urinary odors from male vs. estrous female conspecifics after surgical removal of the VNO, implying that odor-based sex discrimination depends on the detection and initial processing of these odors via the MOE and MOB. Whereas VNO removal failed to disrupt odor-based sex discrimination, it did significantly reduce the time that male mice spent in nasal contact with non-volatile

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components of urine spots from estrous females, suggesting that VNO inputs in the male may activate forebrain mechanisms that increase the reward salience of female odor cues. Likewise, recent evidence [18] suggests that VNO inputs mediate the reward salience in female mice of non-volatile odors present in soiled male bedding.

Results of another recent study [17] raise the possibility that both the ability of mice to identify and the subsequent motivation to approach opposite-sex urinary volatiles is facilitated by the processing of these cues via the AOB. Exposure of either male or female gonadectomized mice to volatile urinary odors from the opposite, but not the same, sex stimulated Fos expression in the mitral and granule cell layers of the AOB, in addition to the medial amygdala, sub-regions of the hypothalamus, and shell region of the nucleus accumbens. Surprisingly, disruption of MOE function by intranasal zinc sulfate treatment blocked the subsequent ability of opposite-sex urinary volatiles to stimulate Fos expression in the AOB and downstream forebrain target sites. Additional studies suggested that VNO signaling remained viable in mice even after intranasal zinc sulfate treatment. We cannot rule out the possibility that destruction of the MOE reduced activity of the VNO 'pump' [20], thereby reducing the ability of urinary volatiles to gain access to VNO sensory neurons. Although there is evidence that volatile constituents of urine can activate VNO neurons *in vitro* [23], the best available evidence suggests that *in vivo* activation of the AOB occurs mainly in response to non-volatile body odorants [14]. Taken together, our recent results [17] raise the possibility that volatile opposite-sex urinary odors detected by the MOE subsequently gain access to the AOB via a pathway that has yet to be specified. In a previous study [21] we found that removal of the VNO failed to disrupt the ability of male mice to discriminate between volatile urinary odors emitted either from estrous vs ovariectomized females or from males vs estrous females in home-cage habituation/dishabituation tests. In the present study we hypothesized that disrupting AOB processing of volatile urinary odor inputs following bilateral lesions of this structure would also fail to affect the ability of male mice to discriminate between these same pairs of volatile urinary odors. In another previous study [22] we found that VNO removal attenuated the motivation of male mice to remain in direct nasal contact with urine from estrous females following their detection in home-cage habituation/dishabituation tests. In the present study, we hypothesized that AOB lesions would reduce males' motivation to investigate opposite-sex (estrous female) urinary volatiles without affecting their motivation to investigate other types of urinary odors. Finally, in our previous study [21] we found the VNO removal failed to affect males' mating behavior in tests with estrous females, and in the present study we predicted that a similar persistence of mating would be seen in male mice following bilateral lesions of the AOB.

2. Materials and methods

2.1. Subjects

All procedures were approved by the Boston University Animal Care and Use Committee. Twenty sexually naïve male Swiss Webster mice were purchased from Charles River Breeding Facility (Wilmington, MA) at 10–14 weeks of age. They were singly housed on a reversed 12 h light/dark cycle with food and water provided *ad libitum*. During the first week all subjects underwent bilateral castration (under 2% Isofluorane anesthesia) and at the same time had Silastic capsules (length 1.5 cm; inner diameter 0.10 cm, outer diameter 0.22 cm) with crystalline testosterone implanted under the skin on the back of the neck. One week later, 12 subjects underwent bilateral AOB lesions and 8 underwent sham lesions. Subjects were anesthetized with ketamine (100 mg/kg) and xylazine (20 mg/kg). The head was secured in a stereotaxic instrument whereupon the AOB was lesioned bilaterally with a tungsten electrode, fully insulated except for a 1 mm bare tip. Skull holes were drilled over the MOB of each hemisphere. The electrode was lowered at a 40° angle to a spot on the dura located 1 mm anterior and 0.8 mm lateral to the point at which the skull midline and the inferior cerebral vein

intersect. 300 μA of current was passed for 30 s at three different depths: −2.2 mm, −1.8 mm, and −1.4 mm from the surface of the MOB dura in each hemisphere. The indifferent electrode was attached to the subject's tail, which was covered with saline-soaked gauze and aluminum foil. For sham lesions, the electrode was lowered until it touched the surface of the MOB dura, and no current was passed. Behavioral testing commenced a week after brain surgery.

2.2. Habituation/dishabituation tests

2.2.1. Urine collection—The urine stimuli used in habituation/dishabituation tests were collected from 12 gonadally intact adult males, from 20 ovariectomized adult females (at least 2 weeks post-surgery), and from 15 estrous females (these animals were ovariectomized and treated with 20 μg of estradiol benzoate 48 and 24 h followed by 500 μg of progesterone 4 h prior to urine collection). Urine was collected while holding mice by the scruff of the neck. Urine from 3–5 mice of the same sex and endocrine state was mixed, alliquoted, and frozen at −80 °C prior to later thawing and presentation in home cage habituation/dishabituation tests.

2.2.2. Home-cage test procedure—Subjects received habituation/dishabituation tests [2] to assess their ability to discriminate between different volatile urinary odors. The experimenter was blind during testing to the particular surgical treatment received by individual subjects. The testing was carried out in the subject's home cage, in which the bedding had not been changed for at least 48 hr. At the beginning of each test, the cage top was replaced with a clean one that had a wire mesh placed against the food hopper. This wire mesh prevented any direct nasal contact with the stimuli placed on top of it, thus ensuring that the animal was responding only to volatile urinary odors. Habituation/dishabituation tests were carried out on four consecutive days. The following procedure was followed in each daily test: The subject was given three 2-minute presentations of distilled water, followed by three two-minute presentation of one urine stimulus, followed by three two minute presentations of another urine stimulus, with all stimulus presentations separated by a one-minute interval. Distilled water, estrous female urine, gonadally intact male urine, and ovariectomized female urine were used as stimuli. In any given stimulus presentation, 10 μL of urine or of water was pipetted onto a filter paper fastened to a small plastic weigh boat, which was then placed, facing downward, on top of the wire mesh. The time the subject spent with its nose against the wire mesh directly underneath the weigh boat containing water or a urine spot was recorded using an HP iPAQ Pocket PC (Hewlett-Packard, Houston, TX, USA) and Noldus Observer Software (Wageningen, Netherlands). On the first test day, three presentations of distilled water were followed by three presentations of ovariectomized female urine which were, in turn, followed by three presentations of estrous female urine. On the second test day, the urinary odor sequence was reversed so that after three presentations of distilled water subjects were presented three times with estrous female urine followed by three presentations of ovariectomized female urine. On the third test day, three presentations of distilled water were followed by three presentations of gonadally intact male urine which, in turn, were then followed by three presentations of estrous female urine. Finally, on the fourth test day, the urinary odor sequence was reversed so that after three presentations of distilled water subjects were presented three times with estrous female urine followed by three presentations of gonadally intact male urine.

2.3. Mating

Several weeks after the completion of home-cage habituation/dishabituation tests, a single four-hour long mating test was conducted to assess the effect of AOB lesions on males' mating performance. The test was conducted in the subjects' home cages which had not been cleaned at least 48 h prior to testing. At the beginning of the test, an estrous female was placed into the cage and interactions between the pair were recorded for 4 h. The observer recorded the latency and number of anogenital investigations, mounts with and without intromission, and the occurrence of ejaculation. After the test the female was left in the cage with the male for an

additional 16 hours, and the presence or absence of a vaginal plug was recorded at the end of that interval. The presence of a vaginal plug was taken as evidence that the male subject had ejaculated during the period when animals' behavior was not directly observed by the experimenter. We acknowledge, however, that we may have under estimated the occurrence of ejaculation in male subjects during this latter period by relying only on the presence of a vaginal plug in the female partner as evidence of its occurrence.

2.4. Histology

At the conclusion of behavioral testing, subjects were injected with sodium pentobarbital (100 mg/kg), and perfused with saline followed by 4% paraformaldehyde in 0.1 M PBS, pH 7.4. Brains were removed, placed into 4% paraformaledehyde and stored at 4 °C overnight. The following day they were placed into 30% sucrose solution and stored at 4 °C until they sank to the bottom of the vials. The right and left olfactory bulbs (including both the MOB and AOB) were separated from the rest of the brain and cut sagittally on a sledge microtome (Leica Microsystems Inc., Bannockburn, IL). Fifty- μm thick sections were collected sequentially from the lateral to the medial aspect of the olfactory bulb and stored in 0.1 M PBS pH 7.4. The following day, they were mounted on gelatin-coated slides in the sequence that they were collected. Brain sections were then stained with Cresyl Violet to differentiate the cell layers. An independent observer, unaware of the animal's intended lesion treatment, examined the slides to determine the extent of the lesions. Three groups (sham-operated controls n=8; partial bilateral AOB lesions n=5; complete bilateral AOB lesions n=5) of male subjects were formed, based on the results of this histological analysis. The extent of the AOB lesion damage in the partial and complete lesion groups is shown in Fig. 1.

3. Results

3.1. Habituation/dishabituation tests

Males with partial or complete AOB lesions, like sham-operated controls, usually distinguished successfully between estrous and ovariectomized female urinary volatiles (Fig. 2A and B) and between male and estrous female urinary volatiles (Fig. 3A and B), regardless of the order in which either of these pairs of odor stimuli were presented following a neutral water stimulus. One-tailed Wilcoxon Signed Rank Tests generally showed a significant difference (p<0.05) in the amount of time males in all three groups spent investigating the third presentation of one odor and the first presentation of the next consecutive odor in all four tests. The exceptions to this rule were for males with partial AOB lesions: In these males there was only a non-significant trend for a difference in investigation times when ovariectomized female urine was presented after estrous female urine (Fig. 2B) and when male urine was presented after estrous female urine (Fig. 3B). One-tailed tests were applied because previous experiments [2,21] showed that male mice readily discriminate between the pairs of odors presented in the current experiment.

Male mice with either partial or complete lesions of the AOB often spent significantly less time than sham-operated control males investigating urinary volatiles from estrous females.

Thus overall between-groups comparisons using a Kruskal-Wallis One Way ANOVA on Ranks showed a significant effect of group on investigation of estrous female urinary volatiles in Fig. 2A (H=5.875, df=2, p=0.05), in Fig. 3A (H=6.193, df=2, p<0.05), and in Fig. 3B (H=5.882, df=2, p=0.05). One-tailed Mann–Whitney Rank Sum Test *post-hoc* comparisons of pairs of groups were subsequently made because we had a strong hypothesis based on previous studies [21,22] that males would be more interested in investigating estrous female urine than either ovariectomized female or intact male urine. These *post-hoc* comparisons showed that males with partial AOB lesions spent less time than sham-operated controls investigating

estrous female urinary odors in Fig. 2A (U=20, p<0.02), Fig. 3A (U = 20, p <0.02) and Fig. 3B (U = 22, p =0.03); similar differences between males with complete AOB lesions and sham-operated controls were seen in Fig. 3A (U=22, p=0.03) and Fig. 3B (U=21, p=0.02).

3.2. Mating behavior

Mating test results (Table 1) were analyzed with parametric one-way ANOVAs. There were no statistically significant differences in latency and number of anogenital investigations, mounts with and without intromission, mount rates, and ejaculations among the three groups. It is noteworthy that 100% of mice with complete AOB lesions displayed mounts with intromission and 80% of males in this group also showed ejaculation. This level of mating performance in males with complete AOB lesions, if anything, tended to exceed that of sham-operated controls.

4. Discussion

Male mice with either partial or complete AOB lesions resembled sham-operated control males in their ability to discriminate volatile urinary odors from ovariectomized vs. estrous females as well as urinary volatiles from male vs. estrous female mice. This result resembles that of a previous study [21] in which VNO removal failed to disrupt the ability of male mice to discriminate urinary volatiles from male vs. estrous female mice. Mice in which function of the MOE was disrupted were unable to locate or discriminate between different conspecific urinary odors in home-cage habituation/dishabituation tests [15,17]. Taken together, the results of these studies suggest that the ability of mice to use distal olfactory cues (urinary volatiles) to discriminate either sex or endocrine status depends on the detection and processing of these odors by the main as opposed to the accessory olfactory system. A similar conclusion can be drawn from the results of another prior study [28] in which bilateral AOB lesions failed to disrupt the ability of female opossums to discriminate between body odors of male and female conspecifics. To our knowledge, there are no previous studies assessing the effects of AOB lesions on olfactory sex discrimination in any other vertebrate species.

Male mice with either partial or complete AOB lesions spent significantly less time than sham-operated males investigating urinary volatiles from estrous females, with this effect being particularly notable when this stimulus was presented in sequence (either after or before) with male urine during home-cage habituation/dishabituation tests. The fact that AOB lesions selectively decreased the time spent investigating estrous female urinary volatiles, as opposed to volatiles from either ovariectomized females or gonadally intact males suggests that the deficit was ‘motivational’ as opposed to ‘sensory’. Thus, all 3 types of urinary volatiles were successfully detected by all 3 groups of males. However, only males with AOB lesions were less motivated to persist in their investigation of estrous urinary volatiles. It is interesting to note that this particular effect of AOB lesions differed from the effect of VNO removal in males [21], which had no effect on the time spent investigating urinary volatiles from males vs. estrous females. By contrast, VNO removal did reduce the time that males spent in direct nasal contact with urine spots from estrous females regardless of whether they were presented simultaneously [21] or sequentially [22] with urine spots from males. These latter results imply that male subjects’ motivation to remain in close proximity to non-volatile components of opposite-sex (female) urine depends on the processing of these odor cues by the accessory olfactory system following their detection by the VNO. The discrepant effects of VNO removal and AOB lesions on the motivation of male mice to remain close to the source of urinary volatiles from estrous females suggest that inputs from VNO to the AOB are not responsible for the contribution of the latter structure to attraction towards this odor stimulus.

A possible alternative source of odor inputs to the AOB is via the main olfactory projection to the amygdala. As explained in the Introduction, a recent study from our laboratory [17] found

that urinary volatiles from estrous female (but not male) mice stimulated Fos expression in the AOB mitral and granule cell layers of male subjects. This AOB Fos response to opposite-sex urinary volatiles was blocked in male subjects that previously received intranasal infusions of the toxin, zinc sulfate, raising the possibility that the MOE, and not the VNO, had detected the urinary odors responsible for AOB Fos activation in the mice in which this response occurred. Exposure to estrous female urinary volatiles provoked a significant activation of glomeruli in the olfactory bulb of male subjects, in addition to stimulating Fos in subdivisions of the medial amygdala as well as in the hypothalamus [17]. Several previous studies [24,26] have established that MOB mitral cells project to cortical subdivisions of the amygdala, in addition to cortical regions, and a recent study (N. Kang, J.A. Cherry, and M.J. Baum, unpublished data), using both anterograde and retrograde tracing methods, has established that mitral cells located in the ventral MOB of mice project directly to the medial amygdala. Previous research in mice [1] and hamsters [5] identified centrifugal input pathways to the AOB from the medial amygdala as well as the bed nucleus of the accessory olfactory tract. More research is needed to determine whether any of these centrifugal input pathways to the AOB are activated by urinary volatiles from opposite-sex conspecifics and whether these inputs motivate mice to seek out and maintain proximity to these reproductively significant volatile odor cues.

Whereas AOB lesions attenuated males' motivation to investigate urinary volatiles from estrous females when they were presented in isolation during habituation/dishabituation tests, they had no effect on the display of mating behavior by male mice when directly confronted by an estrous female just as AOB lesions failed to disrupt the display of sexual behavior in female rats [7]. Our present results are consistent with the previous finding [21] that VNO removal had no disruptive effect on mating performance in male mice, even when they lacked previous sexual experience. These outcomes contrast with the disruptive effect of VNO removal on mating behavior of sexually naïve male hamsters [19]. The absence of any effect of either VNO or AOB damage on mating in male mice also contrasts greatly with the profound disruption of this behavior reported in male mice (either sexually naïve or experienced) following disruption of main olfactory function by genetic mutations [16], or systemic [27] or intranasal [8] administration of toxic drugs that damage the MOE.

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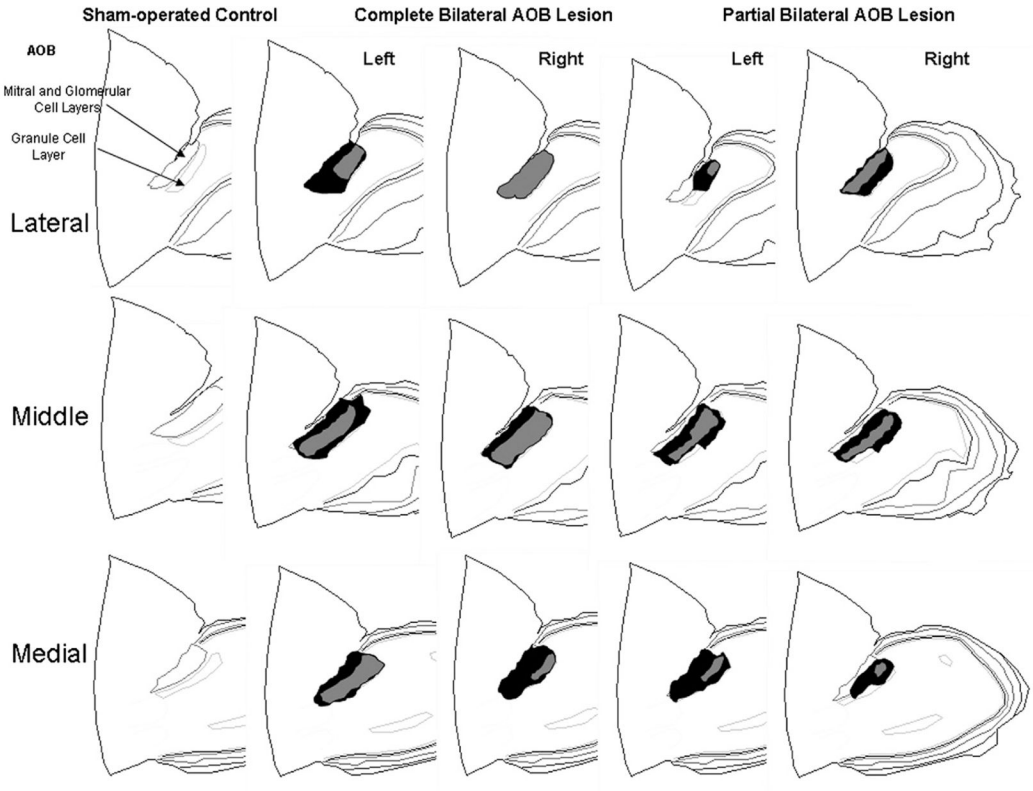

Fig. 1.

Schematic representations of complete (*n*=5) as well as partial (*n*=5) bilateral lesions of the accessory olfactory bulb (AOB) in male mice. Drawings of the glomerular, mitral and granule cell layers of the AOB from a sham-operated control male are shown in sagittal sections taken from the lateral, middle, and medial segments of the entire olfactory bulb. AOB lesion reconstructions are also shown from the left and right hemispheres. Gray shading represents the area of overlap of AOB lesions in all members of each group. Black shading represents the area of greatest extent of the AOB lesion in any member of each group.

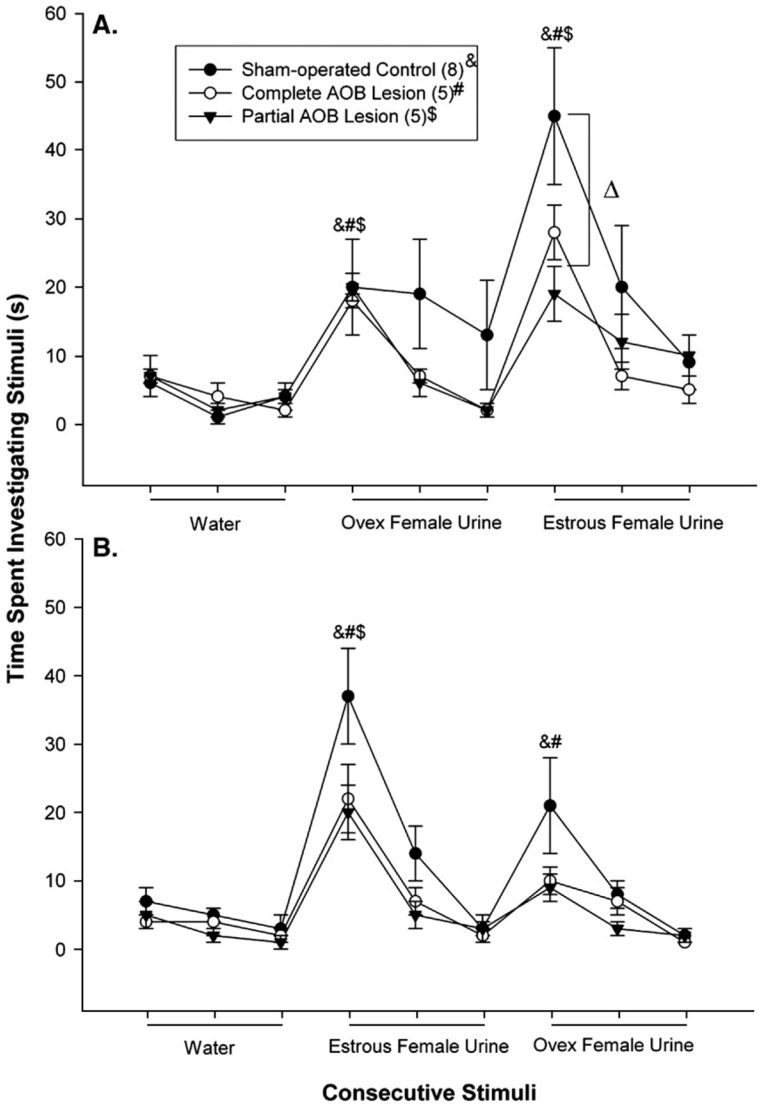

Fig. 2.

Effect of accessory olfactory bulb lesions on the time spent by male mice investigating urinary volatiles from ovariectomized female vs. estrous female mice in home-cage habituation/ dishabituation tests. The sequence of urinary odor stimuli was reversed in the two tests (Panels A and B) given on consecutive days. Wilcoxon Signed Rank Tests were used to make within groups comparisons of the time spent investigating the third presentation of one odor and the first presentation of the next odor: &, #, $ indicate a significant difference ($p<0.05$) in investigation times for males in the sham-operated, complete AOB lesion, and partial AOB lesion groups, respectively. Δ indicates a significant overall group difference in investigation times by a Kruskal Wallis One-Way ANOVA on ranks. Data are expressed as mean±SEM; group numbers are given in parenthesis.

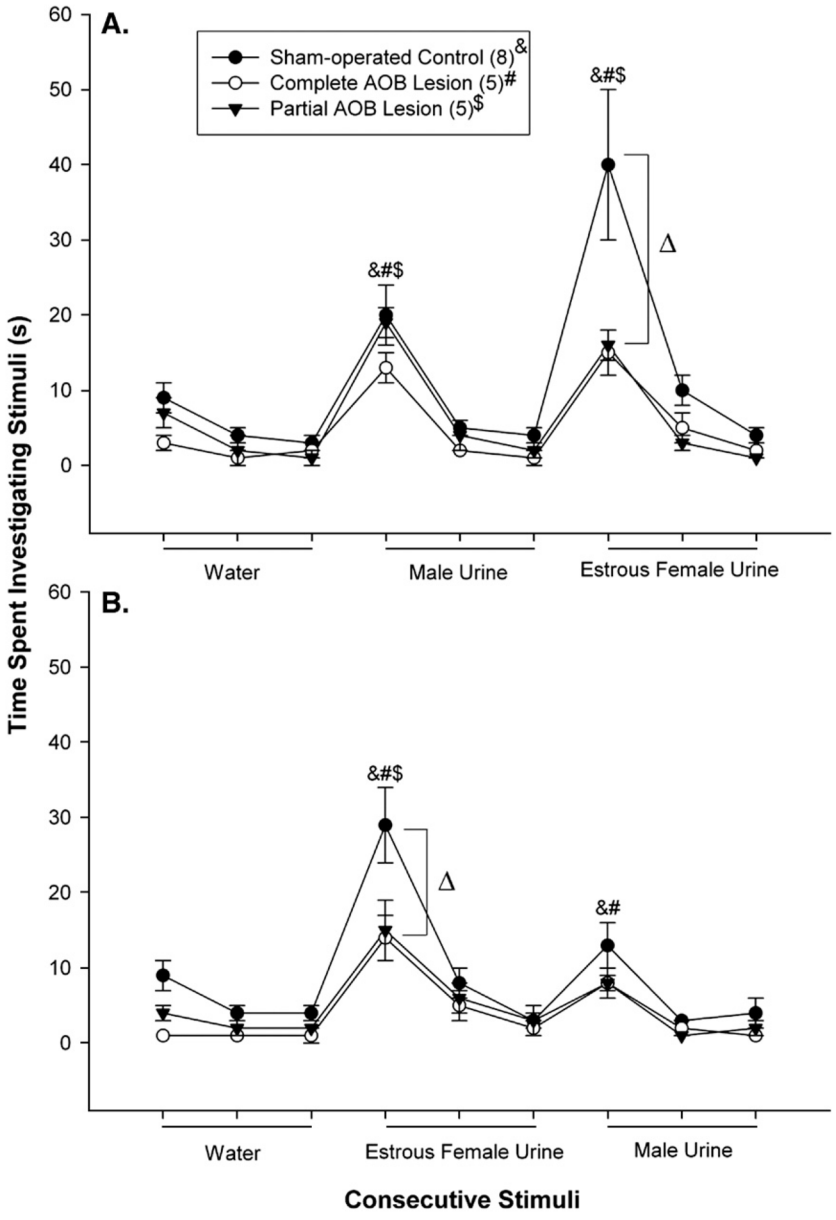

Fig. 3.

Effect of accessory olfactory bulb lesions on the time spent by male mice investigating urinary volatiles from gonadally intact male vs. estrous female mice in home-cage habituation/dishabituation tests. The sequence of urinary odor stimuli was reversed in the two tests (Panels A and B) given on consecutive days. Wilcoxon Signed Rank Tests were used to make within groups comparisons of the time spent investigating the third presentation of one odor and the first presentation of the next odor: &, #, $ indicate a significant difference ($p<0.05$) in investigation times for males in the sham-operated, complete AOB lesion, and partial AOB lesion groups, respectively. Δ indicates a significant overall group difference in investigation times by a Kruskal Wallis One-Way ANOVA on ranks. Data are expressed as mean±SEM; group numbers are given in parenthesis.

Table 1

Effect of bilateral accessory olfactory bulb (AOB) lesions on mating behavior in male mice

Behavior	Sham-operated Control (*n*=8):	Complete AOB Lesion (*n*=5):	Partial AOB Lesion (*n*=5):
Anogenital investigation:			
Percentage showing (%):	100	100	100
Latency (min):	1.4±0.3	1.5±0.2	1.3±0.2
Number per test:	7.0±1	7.0±1	6.0±1
Mounts (with and without intromission):			
Percentage showing (%):	100	100	100
Latency (min):	15.3±4.7	23.4±9.7	37.1±22.5
Number per test:	55.0±15	50.0±5	82.0±29
Mounts per Minute:	0.4±0.2	0.4±0.1	0.6±0.2
Mounts (with intromission):			
Percentage showing (%):	85.7	100	100
Latency (min):	12.9±3.0	95.8±37.6	40.0±21.9
Number per test:	31.0±11	29.0±6	45.0±12
Percentage of mounts leading to intromission:	53.3±10.4	60.0±12.7	59.8±11
Ejaculations:			
Percentage showing (%):	42.9	80	60
Latency (min):	177.6±39.6	117.0±29.1	122.1±47.8

The data are expressed as mean±SEM.