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Removal of the Vomeronasal Organ Blocks the Stress-Induced Hyperthermia Response to Alarm Pheromone in Male Rats

Yasushi Kiyokawa[1,2], Takefumi Kikusui[2], Yukari Takeuchi[2] and Yuji Mori[2]

[1]Research Fellow of the Japan Society for the Promotion of Science, 1-8 Chiyoda-ku, Tokyo 102-8472, Japan and [2]Laboratory of Veterinary Ethology, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

Correspondence to be sent to: Yasushi Kiyokawa, Laboratory of Veterinary Ethology, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan. e-mail: kiyokawa-ns@umin.ac.jp

Abstract

Previously, we reported that male Wistar rats release alarm pheromone from their perianal region, which aggravates stress-induced hyperthermia (SIH) in pheromone-recipient rats. The subsequent discovery that this pheromone could be trapped in water enabled us to expose recipients to the pheromone in their home cages. Despite its apparent influence on autonomic and behavioral functions, we still had no clear evidence as to whether the alarm pheromone was perceived by the main olfactory system (MOS) or by the vomeronasal system. In this study, we investigated this question by exposing 3 types of recipients to alarm pheromone in their home cages: intact males (Intact), vomeronasal organ–excised males (VNX), and sham-operated males (Sham). The Intact and Sham recipients showed aggravated SIH in response to alarm pheromone, whereas the VNX recipients did not. In addition, the results of the habituation/dishabituation test and soybean agglutinin binding to the accessory olfactory bulb verified the complete ablation of the vomeronasal organ (VNO) with a functional MOS in the pheromone recipients. These results strongly suggest that male rats perceive alarm pheromone with the VNO.

Key words: alarm pheromone, anxiety, stress-induced hyperthermia, VNX, vomeronasal organ

Introduction

Pheromones were first proposed by Karlson and Luscher (1959) as ''substances which are secreted to the outside by an individual and received by a second individual of the same species, in which they release a specific reaction, for example, a definite behaviour or a developmental process.'' The vomeronasal organ (VNO), which is the peripheral organ of the vomeronasal system, is a bilateral, tubular structure within a hard bony and cartilaginous capsule that lies at the base of the nasal septum (Wysocki 1979). In rodents, the VNO perceives several pheromones. For example, a pheromone released from male mice accelerated puberty in female mice (Vandenbergh 1969), and this response was blocked by the removal of the VNO from the subject mice (Lomas and Keverne 1982). A male pheromone can block pregnancy (Bruce 1960), and the vomeronasal system was clearly shown to be involved in this response (Kaba et al. 1989). However, accumulating findings indicate that not all pheromones are perceived by the vomeronasal system and that the main olfactory system (MOS) can also perceive several pheromones (Schaal et al. 2003; Lin da et al. 2005).

Previously, we reported that stressed male Wistar rats released alarm pheromone, which caused increased body temperature (stress-induced hyperthermia, SIH) in recipient rats (Kikusui et al. 2001). Then, we discovered that this pheromone was released from the perianal region of the donor rat in a testosterone-independent manner (Kiyokawa et al. 2004a, 2004b). Subsequently, we reported that alarm pheromone was water soluble, as water droplets collected from the ceiling of a box in which alarm pheromone was released reproduced all the responses seen in recipients directly exposed to the pheromone (Kiyokawa et al. 2005a). The studies conducted so far using this pheromone solution have suggested that the primary effect of alarm pheromone is to increase the anxiety and that the various responses evoked by the alarm pheromone, such as aggravated SIH in the home cage (Kiyokawa et al. 2005a), increased defensive and risk assessment behaviors in a modified open-field test (Kiyokawa et al. 2006), and enhanced acoustic startle response (unpublished data), can be ascribed to elevated level of anxiety in recipient animals.

Despite its apparent influence on autonomic and behavioral functions, we still had no clear evidence as to whether alarm pheromone was mediated by the MOS or by the vomeronasal system. Although we had reported in one study (Kiyokawa et al. 2005a) that alarm pheromone increased Fos expression in the mitral/tufted cell layer of the accessory olfactory bulb (AOB), this increment was not consistently observed in another study (Kiyokawa et al. 2005b). In addition, the finding that alarm pheromone simultaneously aggravated SIH and increased Fos expression in the AOB of the recipients does not imply causality. Therefore, a more direct study was needed to reveal the role of the vomeronasal system in alarm pheromone perception.

To investigate this question, we prepared 3 types of pheromone-recipient rats: intact males (Intact), surgically vomeronasal organ–excised males (VNX), and sham-operated males (Sham). The autonomic and behavioral responses to alarm pheromone presented in their home cages allowed us to evaluate their abilities to perceive alarm pheromone. In addition, we prepared a second cohort of animals to verify that our surgical procedure completely eliminated the vomeronasal system while preserving a functional MOS.

Materials and methods

Recipient animals

Experimentally naive male Wistar rats were purchased from Clea Japan (Tokyo, Japan). The rats were housed 3–4 animals per cage at a constant temperature (24 ± 1 °C) and humidity (45 ± 5%). Food and water were available ad libitum, and the animals were kept under a 12-h light/12-h dark cycle (lights on at 0800) throughout the experiment. This experiment was approved by the Animal Care and Use Committee of the Faculty of Agriculture, University of Tokyo. Some of the recipients underwent the VNX or sham surgery (Wysocki CJ and Wysocki LM 1995) 17–24 days before pheromone presentation. For surgery, the animal was placed in a head-holder under anesthesia with sodium pentobarbital (50 mg/kg, i.p. [intraperitoneally], Nembutal; Abbott Laboratories, North Chicago, IL) and its mouth was opened. After making a midline incision in the palate and retracting the tissue in order to access the VNO, the wound was closed for the sham surgery. For the VNX surgery, the rostral end of the VNO was exposed by drilling, and the caudal end of the vomer bone was cut. Then, the VNO was removed bilaterally using forceps. Bleeding was controlled using cotton swabs, and then the wound was closed. Postoperatively, the animals were housed individually in standard polycarbonate rat cages (28 × 44 × 18 cm) in a colony room.

All the recipient rats had a telemetry transmitter (TA11CTA-F40; Data Sciences International, St Paul, MN) implanted i.p. under anesthesia with ether 10–11 days before pheromone presentation. Postoperatively, the rats were housed individually in standard polycarbonate rat cages placed on an antenna board (RPC-1; Data Sciences International) in a soundproof chamber (36 × 54 × 35 cm; Muromachi Kikai, Tokyo, Japan) located in a room maintained at a constant temperature (22 ± 1 °C) under a 12-h light/12-h dark cycle (lights on at 0800). All the recipient rats were handled for 5 min/day, beginning 6–7 days before pheromone presentation.

We prepared a second cohort of animals (Intact: $n = 9$; VNX: $n = 9$; and Sham: $n = 9$) to assess whether our VNX surgery completely incapacitated the vomeronasal system while preserving a functional MOS. These rats underwent exactly the same procedures as the first cohort, except no telemetry transmitters were implanted.

Pheromone preparation

We prepared pheromone samples using an established method (Kiyokawa et al. 2005a, 2006). Briefly, we prepared adult male Wistar rats as pheromone donors and sprayed purified water (5 ml) on the ceiling of an acrylic box (20 × 20 × 10 cm). A donor rat was anesthetized (50 mg/kg, i.p., Nembutal; Abbott Laboratories), and intradermal needles (27 G) for electrical stimulation were placed in the neck or perianal region. The rat was placed in the box and given 3 electrical stimuli (10 V for 1 s) at 1-min intervals, to either the neck or perianal region. The electrical stimulation of the perianal region induced the release of alarm pheromone; stimulation of the neck region was conducted to provide a similar number of olfactory stimuli that affected neither SIH nor behavioral responses (Kiyokawa et al. 2004a, 2005a). After being stimulated in this manner, the donor was removed, and the water droplets on the ceiling were collected using forceps and 2 sheets of filter paper (5 × 5 cm). Water droplets collected from a box in which no animal had been placed were used as a vehicle control. The pheromone donors were used 2–3 times, with at least 2 weeks between uses. The pheromone box was washed in hot water with a cleanser and wiped with a paper towel before each use.

Pheromone presentation

Pheromone samples were presented to 10-week-old recipients using an established method (Kiyokawa et al. 2005a). Immediately after preparation, 1 of the 3 types of pheromone samples was brought into the room in which the recipients were kept postoperatively. The recipients with a stable baseline, that is, a body temperature below 37.5 °C, were used for the experiment. The wire mesh ceiling of the recipient's home cage was replaced with a perforated acrylic board, and sheets of filter paper were placed on both walls at the same time. Then, the home cage was placed on an antenna board in a soundproof chamber and left there for 30 min. The recipients were randomly assigned to one of the following 9 groups according to VNO status and pheromone exposure: Intact Alarm pheromone ($n = 9$), Neck odor ($n = 9$), Control ($n = 9$); VNX Alarm pheromone ($n = 9$), Neck odor ($n = 10$),

Control (n = 10); and Sham Alarm pheromone (n = 10), Neck odor (n = 10), Control (n = 10). Then, the behavior of the recipient was video recorded (DCR-TRV18; Sony, Tokyo, Japan) through a window in the chamber wall. Body temperature was transmitted via the antenna board placed under the home cage, and the values obtained were recorded by a data acquisition system (Dataquest A.R.T. Silver 3.10; Data Sciences International). After pheromone presentation, the lack of the base of the nasal septum, including the VNO, in all the VNX recipients was visually confirmed. All the pheromone presentation trials were conducted between 0900 and 1800.

Habituation/dishabituation test

The second cohort of animals underwent a habituation/dishabituation test 17 days after VNX or sham surgery, which was equivalent to the shortest recovery period allowed for the pheromone recipients. This test assessed the ability of an animal to discriminate 2 olfactory stimuli using the tendency of laboratory animals to show interest in or be attracted to a novel stimulus (Johnston et al. 1993). As an odor becomes more familiar to laboratory animals, the time spent investigating the odor stimulus will decrease over successive presentations. The subsequent presentation of a different stimulus will result in a longer investigation time if the new stimulus can be discriminated from the first. The increased investigation time indicates the ability of the subject to discriminate the 2 odor stimuli. Urine collected from 2 mouse strains was used as the odor stimuli. The urine was collected from approximately 15-week-old gonadally intact male ICR mice and approximately 10-week-old castrated male Balb/c mice (castrated at 3 weeks old) by holding the animals over an acrylic plate and gently pressing the abdominal area. The collected urine was aliquoted and frozen at −20 °C until use.

Subjects were placed in an acrylic test box (27.5 × 20 × 27 cm) in the colony room. After a 90-s acclimation period, the odor stimulus was presented to the subjects by hanging a cotton swab soaked with 50 µl of the odor stimulus from the ceiling. The subject received three consecutive 1-min presentations of purified water, followed by three 1-min presentations of the first urine sample, and finally three 1-min presentations of the second urine sample, at 30-s intervals. The order of the 2 urine presentations was counterbalanced, and the behavior of the subject was video recorded (DCR-DVD403; Sony) for later analyses.

Histology

To verify the complete removal of the VNO, we observed the glomerular layer of the AOB, which is known to disappear following a successful lesion (Wysocki CJ and Wysocki LM 1995). After the habituation/dishabituation test 17 days after the surgery, 2–4 animals (rats) were kept per cage for 14 days because a previous study (Matsuoka et al. 2002), as well as our preliminary observations, showed that at least 30 days were required for the disappearance of the AOB glomerular layer after the peripheral lesion to the vomeronasal system in rats. Each subject was deeply anesthetized with sodium pentobarbital (Nembutal; Abbott Laboratories) and perfused intracardially with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The brain was removed, immersed in the same fixative overnight, and then placed in 30% sucrose/phosphate buffer for cryoprotection. The olfactory bulb was cut into 30-µm-thick sagittal sections, and every section containing the AOB from randomly chosen side, whereas every fifth section from the other side of the olfactory bulb, was mounted on glass slides. After being washed in phosphate-buffered saline (PBS) for 10 min, the sections were incubated with soybean agglutinin (SBA)–fluorescein isothiocyanate conjugate (20 µg/ml; Vector Laboratories, Burlingame, CA) in PBS for 20 min, and rinsed with PBS for 10 min. Sections were then coverslipped with Fluoro-Guard Antifade Reagent (Bio-Rad Laboratories, Hercules, CA) and observed under the fluorescence microscope (BX51; Olympus, Tokyo, Japan) equipped with a digital camera (DP30BW; Olympus).

Data analysis and statistical procedures

The data were analyzed using StatView J 5.0 software (SAS Institute, Cary, NC; no longer available).

A researcher who was blind to the experimental conditions analyzed the behavior of the recipient using Microsoft Excel–based Visual Basic software for recording each parameter. The number of steps taken with the hind paws (walking) and the durations of digging (digging the bedding with the forelimbs or nose), grooming (face washing, oral grooming, and scratch grooming), rearing (rising to its hind paws), sniffing (regular movement of vibrissae), freezing (immobile posture, with cessation of skeletal and vibrissae movements except during respiration), resting (small movement of the vibrissae, closed eyes, and relaxed posture of the body), and contact (direct contact with the filter paper) were recorded during the 30-min presentation period. For the precise definitions of these behaviors, see our previous studies (Kikusui et al. 2001; Kiyokawa et al. 2004c, 2005a). All behavioral data were analyzed statistically using 2-way multivariate analysis of variance (Hotelling's trace) followed by the Tukey–Kramer post hoc test.

Body temperature was recorded continuously, and the values were stored as the average obtained for a 5-s period during each minute. The individual baseline values were defined as the average body temperature recorded in the home cage during the 5-min period just before the measurement. The changes from baseline were analyzed using repeated 3-way analysis of variance followed by the Tukey–Kramer post hoc test.

The duration of the investigation time for each odor stimulus was recorded in the habituation/dishabituation test

using the same software mentioned above. The investigation time was defined as the time the rat spent sniffing toward the stimulus with its nose within 1 cm of the stimulus. The investigation times between the third water presentation and the initial presentation of the first urine, and between the third presentation of the first urine and the first presentation of the second urine were analyzed using Wilcoxon's signed-ranks test.

Results

All the recipients showed behavioral responses to environmental manipulations, such as moving their home cage and replacing the ceiling, and the presence of the pheromone did not influence recipient's behavior ($F(16,138) = 1.37$, $P = 0.166$). Although the VNO status significantly affected the behavior ($F(16,138) = 2.76$, $P < 0.01$), the interaction between these 2 factors was not significant ($F(32,274) = 0.979$, $P = 0.504$). The post hoc test revealed no significant differences, with one exception; the VNX Alarm pheromone group showed reduced grooming behavior compared with the Intact Control group (Table 1).

Although the baseline body temperature values were virtually identical among the groups (data not shown), the magnitude of SIH was significantly influenced by the presence of the pheromone ($F(2,77) = 12.5$, $P < 0.01$), the VNO status ($F(2,77) = 5.85$, $P < 0.01$), and time ($F(35,2695) = 160$, $P < 0.01$). The interactions between all pairs of factors were significant in all analyses (presence of pheromone × VNO status: $F(4,77) = 3.46$, $P < 0.05$; presence of pheromone × time: $F(70,2695) = 4.96$, $P < 0.01$; and VNO status × time: $F(70,2695) = 3.81$, $P < 0.01$), and the interaction among all 3 factors was also significant ($F(140,2695) = 2.90$, $P < 0.01$). The post hoc test revealed that neither VNX nor sham surgery affected SIH in the control groups. Alarm pheromone significantly aggravated SIH compared with the control group when the VNO was intact ($P < 0.05$; Figure 1, top). The VNX surgery caused recipients to loose the ability to perceive alarm pheromone (Alarm pheromone vs. Control: $P > 0.05$; Figure 1, middle), whereas the sham surgery did not (Alarm pheromone vs. Control: $P < 0.05$; Figure 1, bottom). In contrast to alarm pheromone, neck odor did not affect SIH regardless of the recipient's VNO status.

In the habituation/dishabituation test, the investigation time significantly increased after changing from the third water stimulus to the initial presentation of the first urine (Intact: $P < 0.05$; VNX: $P < 0.05$; and Sham: $P < 0.05$) and from the third presentation of the first urine to the first presentation of the second urine (Intact: $P < 0.05$; VNX: $P < 0.05$; and Sham: $P < 0.05$) regardless of the VNO status (Figure 2).

The AOB of the second cohort of animals was processed using SBA, which specifically binds to the vomeronasal axons and the glomeruli of the AOB (Key and Giorgi 1986). All the animals in the Intact group had complete glomeruli in the AOB (Figure 3, top), whereas these structures were absent in all the VNX animals (Figure 3, middle). Sham surgery did not affect these structures in any animals (Figure 3, bottom).

Discussion

In this study, we first corroborated the previous finding (Kiyokawa et al. 2005a) that alarm pheromone presentation in a recipient's home cage evoked an autonomic stress response (as shown by aggravated SIH) with no effects on the behavioral response in intact male recipients. VNX surgery completely blocked the effects of the pheromone on the autonomic response in recipients, whereas sham surgery had no effect. In contrast to the autonomic response, neither VNX nor sham surgery affected the behavioral response in recipients. These results suggest that the VNO plays a crucial role in alarm pheromone perception in male rats.

We prepared a second cohort of animals to verify the effectiveness of our VNX surgery because histological verification is more definitive than visual inspection of the nasal cavity (Wysocki CJ and Wysocki LM 1995) and because

Table 1 Behavioral responses of recipient rats

Recipient	Sample	Grooming	Digging	Rearing	Walking	Sniffing	Freezing	Resting	Contact
Intact	Control (9)	616 ± 25	53.2 ± 21.2	369 ± 27	198 ± 11	508 ± 36	0.07 ± 0.07	246 ± 69	143 ± 33
	Neck (9)	574 ± 37	106 ± 23	316 ± 34	200 ± 14	479 ± 46	0.09 ± 0.09	218 ± 79	180 ± 35
	Alarm pheromone (9)	492 ± 42	174 ± 36	368 ± 26	243 ± 20	502 ± 37	2.48 ± 1.69	188 ± 67	152 ± 22
VNX	Control (9)	521 ± 43	159 ± 65	330 ± 27	224 ± 16	507 ± 37	1.36 ± 1.22	184 ± 58	181 ± 36
	Neck (10)	504 ± 44	147 ± 30	300 ± 34	187 ± 12	463 ± 36	0.94 ± 0.49	262 ± 85	165 ± 32
	Alarm pheromone (10)	381 ± 49	171 ± 53	308 ± 23	185 ± 13	438 ± 31	2.00 ± 1.03	361 ± 83	155 ± 40
Sham	Control (10)	545 ± 55	44.6 ± 11.6	358 ± 25	212 ± 15	521 ± 26	0.11 ± 0.11	186 ± 72	180 ± 31
	Neck (10)	528 ± 38	97.9 ± 22.9	375 ± 34	231 ± 13	494 ± 38	0 ± 0	166 ± 62	163 ± 34
	Alarm pheromone (10)	495 ± 30	120 ± 24	427 ± 33	234 ± 14	586 ± 41	0 ± 0	89.6 ± 45.1	212 ± 24

Data are expressed as means ± SEMs. The number of subjects is given in parentheses.

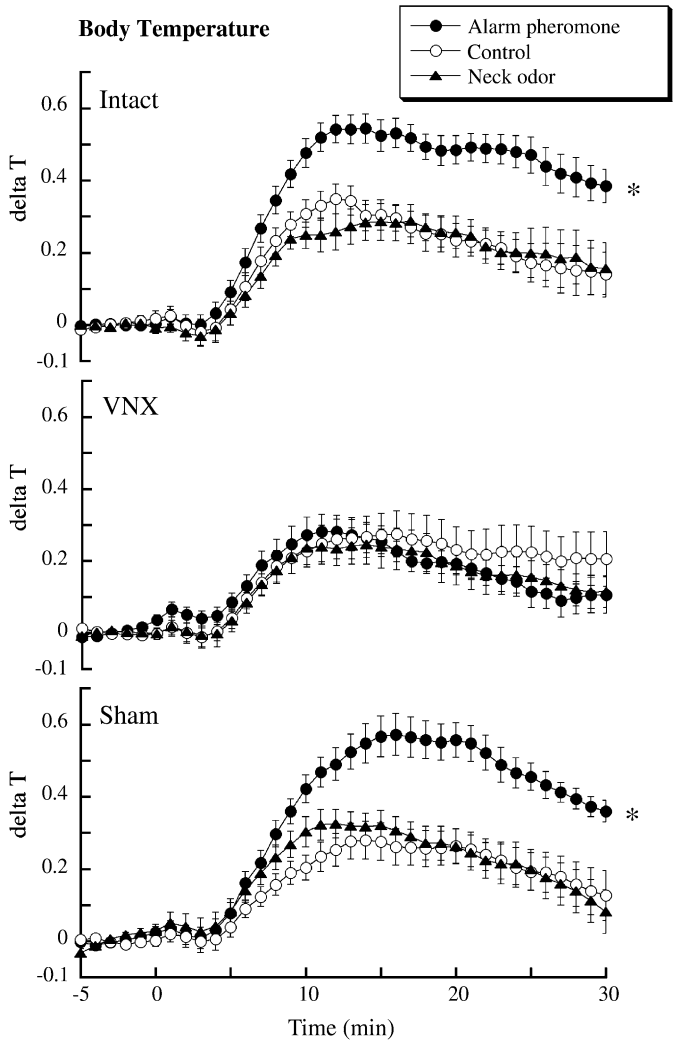

Figure 1 Time-dependent changes in the body temperature of Intact(top), VNX (middle), and Sham (bottom) recipient rats that were exposed to water collected from a box in which either alarm pheromone (Alarm pheromone: Intact, $n = 9$; VNX, $n = 9$; and Sham, $n = 9$) or neck odor (Neck odor: Intact, $n = 9$; VNX, $n = 10$; and Sham, $n = 10$) had been released from an anesthetized donor rat. The water collected from a box in which no animal had been placed was used as the control stimulus (Control: Intact, $n = 9$; VNX, $n = 10$; and Sham, $n = 10$). *$P < 0.05$ compared with the control group in the same recipient group with repeated 3-way analysis of variance followed by the Tukey–Kramer test (mean ± SEM).

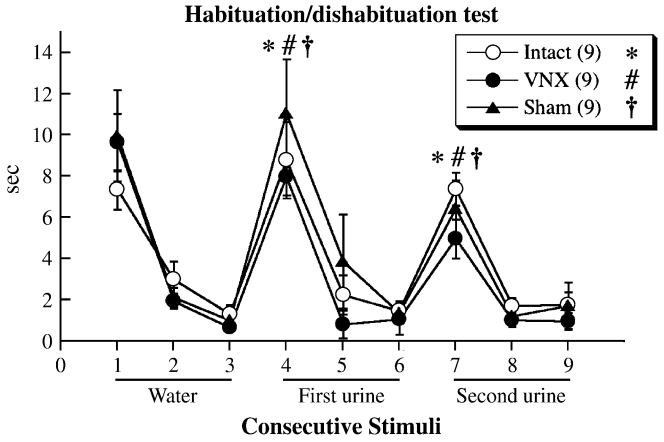

Figure 2 The investigation time for urine from 2 types of mice (presented after 3 consecutive presentations of purified water) by Intact, VNX, and Sham subjects. The number of subjects is given in parentheses. *, #, †: $P < 0.05$; Wilcoxon's signed-ranks test comparisons with the third presentation of the previous stimulus for Intact, VNX, and Sham animals, respectively (mean ± SEM).

more time is required for the glomeruli of the AOB to disappear in rats after the surgery (Matsuoka et al. 2002). The results with the second cohort indicate that the disappearance of the autonomic response to the pheromone in the VNX recipient was attributable to the lack of a VNO rather than the destruction of both olfactory systems by the VNX surgery. According to the habituation/dishabituation test, VNX surgery did not affect the ability to detect or discriminate urine odors, suggesting that VNX recipients retained a functional MOS. In addition, the histological study using SBA showed that the VNO had been completely removed during VNX surgery. Therefore, the VNX recipients, which completely lacked the VNO, were considered to have a functional MOS when they were exposed to the pheromone. Taken together, the present results strongly suggest that alarm pheromone is perceived by the VNO in male rats.

The present study has provided evidence that the alarm pheromone is unique in being perceived by the VNO due to its volatile nature. The unidentified ligand molecule of the alarm pheromone may be a small volatile substance as alarm pheromone activity was trapped in water droplets sprayed on the ceiling after the pheromone was released into the ambient air. It is widely accepted that nonvolatile molecules are transferred to the VNO when an animal's nares directly contact a stimulus (Wysocki et al. 1980); however, the role of the VNO in perceiving volatile pheromones has not been well studied, despite the proposals of several volatile molecules as pheromone candidates.

A volatile pheromone released in the urine of group-housed female mice was shown to delay the timing of puberty (Drickamer 1974, 1982, 1986; Drickamer and Assmann 1981). A volatile molecule, 2,5-dimethylpyrazine (DMP), was identified in the urine of females only when they were group housed, and puberty was delayed when females were exposed to DMP dissolved in urine from adrenoectomized females (Novotny et al. 1986). The molecule itself delayed puberty only when applied at unnaturally high concentrations (Jemiolo and Novotny 1993, 1994). However, the role of the VNO in perceiving the puberty-delaying pheromone is still unclear because it was shown that the removal of the VNO itself, rather than the blocking of pheromone effects, resulted in delayed puberty (Lomas and Keverne 1982). Group-housed female mice also released an estrus-suppressing pheromone (Van Der Lee and Boot 1955; Champlin 1971), whose effect was mediated by the vomeronasal system (Reynolds and Keverne 1979). Although DMP has been

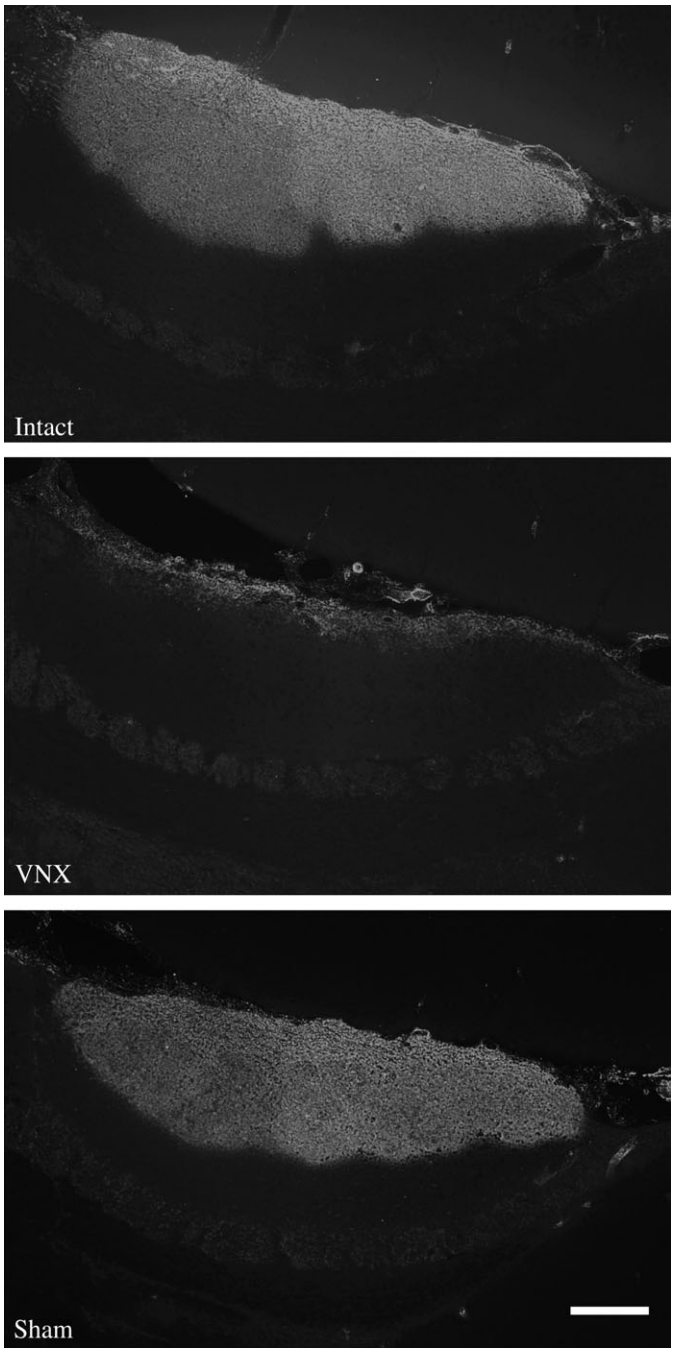

Figure 3 Photomicrographs of SBA binding in the AOB of Intact, VNX, and Sham rats. The rostral portion is shown on the left. The horizontal bar indicates 200 μm.

also proposed as a candidate molecule for this estrus-suppressing pheromone (Ma et al. 1998), it is unknown whether a single pheromone mediates the 2 phenomena or if the estrus-suppressing pheromone is volatile.

Although male volatile pheromones have been shown to induce estrus in female mice (Whitten 1956; Whitten et al. 1968) and 2 mixtures of male-derived volatile molecules, that of 3,4-dehydro-exo-brevicomin and 2-sec-butyl-4,5-dihydrothiazole (Jemiolo et al. 1986) and that of α- and β-farnesenes (Ma et al. 1999), were reported to induce estrus in female mice, the role of the vomeronasal system in this phenomenon remains ambiguous. In addition to these 2 mixtures (Novotny, Ma, et al. 1999), other male-derived volatile molecules such as 6-hydroxy-6-methyl-3-heptanone (Novotny, Jemiolo, et al. 1999), isobutylamine (Nishimura et al. 1989), isoamylamine (Nishimura et al. 1989), and the mixture of 2-heptanone, 2-hexanone, and 4-heptanone (only at unnaturally high concentrations) (Jemiolo et al. 1989) were reported to accelerate puberty in mice. The puberty-accelerating pheromone was shown to be released in the urine of male mice (Vandenbergh 1969; Colby and Vandenberg 1974), and its effect was mediated by the vomeronasal system in females (Kaneko et al. 1980; Lomas and Keverne 1982). Nevertheless, the pheromone released by male mice is considered nonvolatile because the acceleration of puberty occurred only when females directly contacted the male urine (Drickamer and Assmann 1981). In contrast, female-derived puberty-accelerating pheromone released into the urine of pregnant and lactating females as well as estrus females were identified as volatile (Drickamer and Hoover 1979; Drickamer 1982, 1986). The involvement of the VNO in perceiving these volatile pheromones is again unclear because the neural mechanisms of female pheromone perception are largely unknown, and among the male-derived molecules mentioned above, only 3,4-dehydro-exo-brevicomin has been identified in female urine at low concentrations (Schwende et al. 1984; Andreolini et al. 1987).

It was shown recently that the VNO responded to several volatile molecules in vitro (Leinders-Zufall et al. 2000). However, even if volatile molecules produce neuronal activities in the vomeronasal epithelium and if the vomeronasal receptor for a volatile molecule has been identified (Boschat et al. 2002), it does not necessarily follow that pheromone effects are mediated exclusively by the vomeronasal system because volatile molecules are simultaneously perceived by the MOS (Xu et al. 2005). Therefore, the alarm pheromone appears to be the first volatile pheromone shown to be both perceived and transmitted by the VNO. The identification of the alarm pheromone molecule should shed light on the mechanism of volatile pheromone perception by the VNO.

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