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Removal of the vomeronasal organ impairs predator odor detection in female golden hamsters

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Abstract

The main and accessory olfactory systems of mammals are involved in the detection of predator odor; however, the relative importance assigned to each varies between studies. We examined the behavioral responses of vomeronasal organ removed (VNOx) and sham operated (VNOi) female golden hamsters (*Mesocricetus auratus*) to urine odor from a predator, the ferret (*Mustela putorius furo*). We quantified the duration and frequency of the defensive behaviors vigilant rearing and concealment, and the neutral behaviors grooming and investigation, in female golden hamsters in a visible burrow system. We found that VNOx females exposed to predator urine odor spent significantly less time in concealment and more time rearing than VNOi females. Further experiments in which females were exposed to formaldehyde odor showed that their behavioral responses to ferret odor differed from those to formaldehyde odor. This shows that the female hamsters' behavioral responses are not adverse reactions to noxious odors, but typical reactions to predators. Golden hamsters have been laboratory-reared for over 80 years, but defensive behavior in females appears to be innate and can be impaired by surgical removal of the vomeronasal organ. Therefore, the accessory olfactory system, via the vomeronasal organ, plays a role in the detection of predator odor by golden hamsters, even though it has not been found to function in conspecific odor discrimination.

Keywords

Accessory olfactory system; chemosensory cues; defensive behavior; odor discrimination; predator odor detection; vomernasal organ

Introduction

The accessory olfactory system (AOS), which comprises the vomeronasal organ (VNO), accessory olfactory bulb (AOB) and vomeronasal-recipient amyg-

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daloid structures (Halpern & Martínez-Marcos, 2003), is essential for detecting pheromones and mediating social behavior in mammals (Meredith, 1998; Pankevich et al., 2006; Jakupovic et al., 2008; Martel & Baum, 2009). Recent research using functional magnetic resonance imaging shows that the accessory olfactory system also responds to volatile compounds (Xu et al., 2005), and an increasing body of literature suggests that the main olfactory and vomeronasal systems combine to detect pheromonal compounds in mice (Sam et al., 2001; Luo et al., 2003; Lin et al., 2005; Xu et al., 2005; Spehr et al., 2006). The VNO is also thought to detect relatively non-volatile molecules including pheromones. Several researchers have demonstrated that large molecules are taken up by the VNO and not by the main olfactory mucosa (Ladewig & Hart, 1980; Wysocki et al., 1980). Moreover, the AOB of rats (*Rattus norvegicus*; the prey) can be activated by cat (*Felis catus*; the predator) odor derived from fur and skin (McGregor et al., 2004; Staples et al., 2008a, b). Similar studies using fox (*Vulpes vulpes*) urine, however, have shown that predator odor activates the main olfactory bulb (MOB) rather than the AOB (Funk & Amir, 2000; Day et al., 2004; Fendt et al., 2005).

Sexual dimorphism exists in the structure of the AOS (Guillamón & Segovia, 1997; Halpern & Martínez-Marcos, 2003). The structural volume, neuroepithelium volume and the number of olfactory receptors (bipolar neurons) are greater in male rats than in females (Segovia & Guillamón, 1993). These morphological differences in the AOS of males and females indicate that the function of AOS may differ between the sexes. Surgical removal of VNO has been shown to impair male hamsters' individual discrimination and preferences for odors derived from flank glands, vaginal secretions and urine of females, but may not affect those two behaviors in female hamsters (Petrulis et al., 1999; Johnston & Peng, 2000). A similar result is found in mice, yet VNO is required for the expression of lordosis behavior (Keller et al., 2006). A recent study showed that chemosensory cues in the urine from both conspecifics and allospecifics (including predators) can be sensorily encoded by VNO in vivo by mice (Ben-Shaul et al., 2010). However, many researchers have used only males to investigate the function of AOS in detecting predator odor (Staples et al., 2008a, b; Papes et al., 2010; Liu et al., 2014). Therefore, it is not clear how the sexual dimorphism in the VNO's function affects the ability of females to detect predator odor.

Variation in the conclusions of different researchers concerning the role of the accessory olfactory system may also derive from their use of odor derived from different parts of a predator and from their use of different artificial odors. Different sources of odor represent different levels of risk to prey and elicit different defensive strategies. Odors from predator fur and skin collected from a collar or cloth are perceived as more dangerous than odors from gland secretions, urine and feces (Apfelbach et al., 2005). Previous studies into the role of the accessory olfactory system in mediating predator odor have favored fur and skin odor over urine odor, but the low volatility of fur and skin odor complicates experimental design. For example, rats need to be within five centimeters of a cat collar before they increase

the time they spend hiding (Dielenberg et al., 1999). For prey animals to be in such close proximity to a predator odor before assessing and responding to the perceived danger is unrealistic and unrepresentative of natural conditions. In contrast to odor from fur and skin, odor from the urine of predators is commonly encountered by prey in the wild. The effect of predator urine as a repellent for prey has been demonstrated both in the field and in the laboratory (Epple et al., 1993; Bramley & Waas, 2001). For these reasons, and because this odor represents a more natural context, experiments using predator urine odor should provide more consistent results and allow better resolution of the role of the accessory olfactory system in the detection of predator odors.

Ferrets (*Mustela putorius furo*) are domesticated polecats; wild polecats are common predators of rodents (Rusiniak et al., 1976; Apfelbach, 1978; Anisman et al., 1997; Masini et al., 2006). Ferret urine has been shown to disturb the estrous cycle in Campbell's hamster (*Phodopus campbelli*: Apfelbach et al., 2001) and elicit a predator-threat effect in mice (*Mus musculus*: Roberts et al., 2001; Zhang et al., 2007). Gland secretions from ferrets have also been shown to induce a threat response in golden hamsters (*Mesocricetus auratus*: McPhee et al., 2010). Golden hamsters are well-studied laboratory animals which were first housed in laboratories over 80 years ago. How golden hamsters respond to ferret predator urine odor remains unclear, and even less is known about whether the VNO is involved in detecting predator urine odor in this species.

To answer these questions, we designed experiments to test whether surgical removal of VNO impairs the behavioral responses of female golden hamsters to ferret urine odor. We used a visible burrow system to observe the behavioral responses of VNO-removed golden hamsters to ferret urine odor, and compared their behavior to that of control animals. We hypothesized that the VNO is necessary for female golden hamsters to detect predator urine odor, in this case derived from a ferret, and that animals without a VNO would respond differently to odor stimuli than intact animals. Our findings will contribute to the broader literature on olfaction in small mammals, while also revealing the role of the accessory olfactory system in odor detection by this model species.

Material and methods

Animals and VNO surgery

Female golden hamsters at nine weeks of age were acquired from Weitong-Lihua Experimental Animal Company (Beijing, China) and raised in our laboratory. We chose to perform these experiments on females because we are aware that other research groups use only males (Masini et al., 2010; Papes et al., 2010; Liu et al., 2014). Hamsters were housed individually in plastic cages (32 × 20 × 16 cm) and maintained under a reversed 14:10 light:dark cycle (lights on at 19:00 h). Food and water were available *ad libitum*. One month was allowed for animals to habituate to their environment, after which 40 adult females at 12 weeks of age were ran-

domly allocated to either the VNO removal group ($N = 16$) or the sham group (control group, $N = 24$). Eight hamsters from the sham group were also used in the formaldehyde exposure experiment.

Hamsters in the VNO removal group underwent bilateral surgery to remove the VNO (VNOx). The control group underwent the same surgery without removal of the VNO (VNOi). Surgical protocols are described elsewhere (Johnston & Peng, 2000; Keller et al., 2006; Liu et al., 2010, 2014), but in brief, animals were first anaesthetized by an intraperitoneal injection of 30% urethane (30 g/100 ml per hamster), then placed on their back in a head-holder at an angle of 60 degrees. The lower jaw was then opened and the VNO removed by making a midline incision in the soft palette extending rostrally from behind the first palatal ridge to the incisors. The vomer bone, which is attached to the VNO, was exposed by blunt dissection. Tweezers were used to cut the caudal and rostral ends of the bone. To remove the VNO, we carefully cauterized all tissues seen within the area behind the incisors. The surgical procedure for VNOi animals included the midline incision but the vomer bone was not cut. The incision was closed with absorbable sutures. The operation took between 30 and 40 min to complete after a series of practice on twelve hamsters before the real surgeries and tests. The surgical techniques were performed under sterile conditions and animals were allowed to recover for one week before the experiments. Animals in the VNOx group were overanesthetized with sodium pentobarbital (1 mg/10 g body weight) immediately after the tests for further examining the VNO was removed successfully under anatomic microscope. Both the brain tissues and the noses of the VNOx animals were also collected and post-fixed separately, and the disconnection of the VNOx group was further confirmed under microscope. Our results of microscope examination showed that all individuals' VNOs in the VNOx group were completely removed without any harm to the main olfactory epithelium.

Animal ethics

All subjects were healthy and displayed normal estrous cycles before surgery and experimentation. The use, management and welfare of these animals met the Chinese Animal Care Regulations for Captive and Laboratory Animals as outlined in the 1988 National Regulation on Laboratory Animal Research issued by the Ministry of Science and Technology. Our protocols were approved by the Animal Research Ethics Board of Beijing Normal University.

Predator scent collection

Ferret urine used as predator odor was collected from four males housed at the Beijing Zoo in a cage ($120 \times 70 \times 75$ cm) under natural photoperiod. Ferrets at the zoo are fed beef and sometimes fruit and vegetables. We collected urine from a male housed individually by placing a Petri dish (12 cm diameter) containing four-layer gauze in the corner of the cage where the ferret urinated. This animal was later

removed from the zoo, so we collected urine from a further three males housed together. This urine was mixed to form one sample. Once we observed urination, the dish was immediately removed from the cage. Samples were transported to our laboratory in an ice-cooler; this took 15 min. Gauze samples were stored together at −20°C and cut into 4 cm^2 square pieces prior to use. Each gauze piece was placed in a 2 ml centrifuge tube sealed by parafilm. Gauze squares of the same size (4 cm^2) in 1 ml distilled water were used as the control.

Odor exposure apparatus

A visible burrow system has been used to investigate the responses of rats to cat odor (Dielenberg et al., 1999). Our apparatus consisted of an open rectangular arena (35 × 65 × 60 cm) with a small enclosed chamber (35 × 15 × 60 cm). The burrow entrance was a semicircular hole with a diameter of 5 cm; natural burrow entrances in golden hamsters are 4-5 cm in diameter (Gattermann et al., 2001). We modified the visible burrow system by adding a platform for odor presentation. This platform comprised a ceramic cylinder (5 × 2.5 × 3 cm) fixed to the bottom of the wall opposite the chamber entrance, in a location similar to that of ferret urination in the wild and in captivity. The platform had a hole designed to hold a 2 ml centrifuge tube facing the burrow entrance. Gauze containing ferret urine (or control gauze with distilled water) was presented in the centrifuge tube. Our design ensured that subjects were exposed to the stimulus from a standardized distance, and were unable to bite the tube.

Exposure procedure

All experiments were performed during the dark cycle from 09:00-12:00 h. Each odor exposure experiment ran for six days and consisted of a five-day adaptation phase and a one-day stimulation test. During the adaptation phase, a piece of clean gauze (4 cm^2) with distilled water was placed in the platform, and each subject was permitted to move freely in the visible burrow system for 30 min per day to minimize behavioral changes caused by the novelty of the apparatus. Then, in the stimulation test, hamsters from the VNOi and VNOx groups were randomly selected for testing individually with a gauze by adding either ferret urine or distilled water. Our four experimental groups VNOi-water, VNOi-urine, VNOx-water and VNOx-urine each contained eight hamsters.

The apparatus was carefully cleaned with 50% ethanol following each trial. A 25 W red light suspended 1 m above the apparatus provided light. Two video recorders (Field Television System, Fuhrman Diversified Inc., Seabrook, USA) installed in the two chambers were used to record behavioral responses. Four behaviors were recorded during the 30 min odor stimulation test: concealment (hamster conceals itself in the enclosed chamber); vigilant rearing (hamster stands on its rear legs with forelimbs retracted for at least 3 s); investigation (hamster sniffs the environment); and grooming (hamster licks paws or scratches fur with paws).

Concealment and vigilant rearing are typical defensive behaviors directed towards predators in a visible burrow system (Blanchard & Blanchard, 1989). Investigation and grooming are considered non-defensive behaviors. Behaviors were coded using Observer 3.0 (Noldus Information Technology, Wageningen, The Netherlands) by an experimenter who was blind to the experimental conditions.

To exclude the possibility that ferret urine induced a response in hamsters simply because of its noxious properties, we compared the behavior of hamsters exposed to ferret urine ($N = 8$) with that of hamsters exposed to 4% formaldehyde-saline solution ($N = 8$). Formaldehyde has a putrid fish-like smell and has an adverse effect on many mammals (Gieroba et al., 1994). Eight VNOi hamsters not used in the first experiment were used for the formaldehyde exposure experiment. The experimental protocols were similar to those of the first experiment. The stimulus used was 1 ml 4% formaldehyde-distilled water solution on 4 cm^2 gauze.

Statistical analyses

We quantified the duration and number of bouts of the four measured behaviors, pooled across animals within each experimental group. We tested for normality by using the Kolmogorov-Smirnov test (all variables had a normal distribution, $P > 0.05$). A two-way ANOVA was used for each behavioral variable: the factors were stimulus (urine odor or distilled water) and treatment (VNOx or VNOi). Independent t tests were used to test for significant differences between groups exposed to ferret urine or formaldehyde. Alpha was set at 0.05. We used SPSS v15.0 (SPSS, Inc., Chicago, USA) for all analyses.

Results

We found a significant effect of VNO removal (VNOx vs. VNOi) on the time hamsters spent in concealment in the visible burrow system ($F_{2,29} = 8.74$, $P = 0.006$; fig. 1), and within the VNOi group, control animals (exposed to water) spent less time in concealment behavior than those exposed to ferret urine ($F_{2,29} = 7.17$, $P = 0.012$). An interaction between treatment and odor was also found ($F_{2,29} = 15.44$, $P = 0.001$). VNO removal also affected the time spent engaged in vigilant rearing ($F_{2,29} = 5.79$, $P = 0.023$). VNOx hamsters exposed to ferret urine spent significantly less time in vigilant rearing than VNOi hamsters also exposed to predator urine ($F_{2,29} = 5.62$, $P = 0.025$). An interaction between treatment and odor was found ($F_{2,29} = 5.79$, $P = 0.023$). VNO removal had no effect on grooming or investigation behavior (all $P > 0.05$).

Independent t tests showed that the hamsters were in fact reacting to the urine as an indicator of the presence of predators, and not to its noxiousness (fig. 2). Golden hamsters exposed to predator odor spent more time concealed ($t_8 = 5.915$, $P = 0.0009$, $N = 8$), increased the duration of their vigilant rearing ($t_8 = 2.444$, $P = 0.042$, $N = 8$), displayed more bouts of vigilant rearing ($t_8 = 2.229$, $P = 0.043$, $N = 8$) and grooming ($t_8 = 2.158$, $P = 0.049$, $N = 8$), and displayed fewer

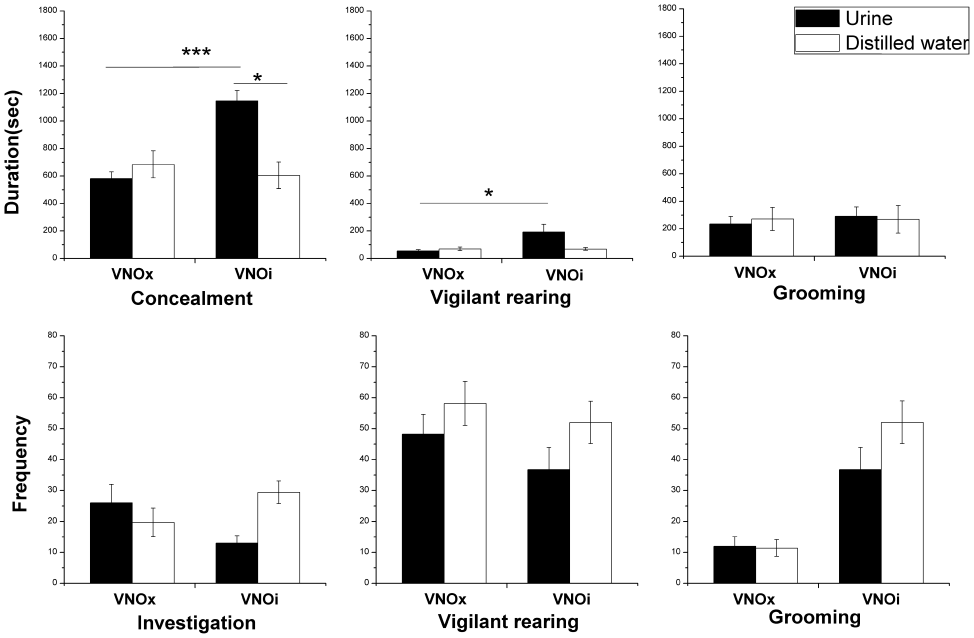

Figure 1. Duration (seconds) and frequency (number of events) of grooming, vigilant rearing and investigation/concealment in VNOx and VNOi female golden hamsters ($N = 8$ for each group) exposed to ferret urine (solid) and distilled water (blank). All values are means ± SE, and the asterisks indicate the significance level (*, $P < 0.05$; ***, $P < 0.001$). Abbreviations; VNOx, surgically removed VNO group; VNOi, VNO sham operated group.

bouts of investigating behavior ($t_8 = -2.396$, $P = 0.031$, $N = 8$) than hamsters exposed to the formaldehyde solution.

Discussion

It appears that the VNO is necessary for the detection of predator odor in female golden hamsters. Concealment and vigilant rearing are common behaviors in rodents faced with an increased risk of predation, and are often observed in frightening or stressful situations (Blanchard & Blanchard, 1969; McGregor et al., 2002). Hamsters exposed to predator odor avoided the stimulus by remaining concealed in the burrow, and displayed increased risk-assessment behavior (rearing), but only if their VNO was intact. Our observations are consistent with those of other researchers who have utilized a visible burrow system (Blanchard & Blanchard, 1989; Dielenberg et al., 1999; McGregor et al., 2002). The decrease in concealment and vigilant rearing in VNOx hamsters clearly shows the importance of the VNO in detecting allospecific odor, in this case the odor of ferret urine.

Exposure of female hamsters to low-concentration formaldehyde revealed that ferret urine is not simply an aversive odor but one which is associated with predators. Although exposure to noxious odors can cause avoidance behavior in animals,

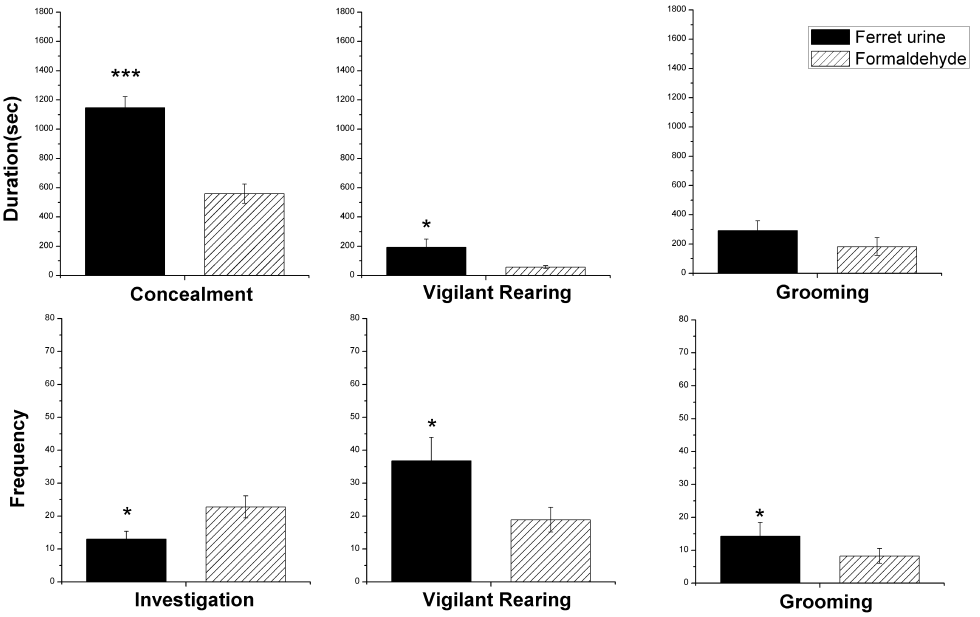

Figure 2. Duration (seconds) and frequency (number of events) of grooming, rearing and investigation in female golden hamsters exposed to formaldehyde (twill) and ferret urine (solid) ($N = 8$ for each group). All values are means ± SE, and the asterisks indicate the significance level (*, $P < 0.05$; ***, $P < 0.001$).

this response can be distinguished from avoidance of a predator (Wallace & Rosen, 2000). Rats exposed to cat odor retreated to their nest boxes, reduced their locomotor activity, and displayed head-out behavior; rats exposed to 4% formaldehyde responded differently (McGregor et al., 2002). In our golden hamsters, 4% formaldehyde induced a reduction in grooming behavior, but only ferret urine elicited specific defensive behaviors. This suggests the presence of a qualitative component of ferret urine that codes for predator-related information. Possible volatile compounds include quinoline, 2,5-dimethylpyrazine, and 4-hepatanone. These compounds are present in male ferret urine and, in combination, are as effective as raw male ferret urine in reducing investigation time in mice (Zhang et al., 2007).

We did not observe a change in grooming and investigation behavior in female golden hamsters, nor did we detect freezing behavior of any kind following exposure to predator urine, though these responses have been found in studies of rats (Blanchard & Blanchard, 1989; Dielenberg et al., 2001; McGregor et al., 2002). Experiments in which live predators or fur and skin odors are used to elicit intense fear stimulation in prey, because of the perceived immediate risk. It is possible that, when exposed to these odors, prey are unable to do anything but cease all movement, because they experience high muscle tension and parasympathetic activation (Nijsen et al., 1998). In contrast, urine or glandular odors may indicate that the predator is present but not necessarily at close range (Blanchard et al., 2003), and

may therefore elicit a less intense reaction in prey than predator fur and skin odor (Dielenberg et al., 2001; McGregor et al., 2002; Masini et al., 2006).

Our results may be considered inconsistent with previous research suggesting that the VNO is not necessary for the discrimination of individual odors by female golden hamsters (Petrulis et al., 1999; Johnston & Peng, 2000). However, firstly, the paradigms we used differed from those used by the other researchers. A habituation-dishabituation paradigm with two stimuli tests was used by Johnston & Peng (2000) and Petrulis et al. (1999); in contrast, we exposed the female hamsters directly to one stimulus in each trial. It is a challenging task for the animals to discriminate different chemosensory cues by memory (R. E. Johnston, personal communication). Secondly, discrimination between and preferences for conspecifics' odors may not be achieved by the same process as detecting and discriminating the odors of potential predators. Thirdly, Johnston & Peng (2000) and Petrulis et al. (1999) showed that the VNO is not essential for the discrimination of conspecific odor or for preferences for male over female odor, but did not rule out the possibility that it is essential for the discrimination of allospecific odor.

VNO removal significantly impaired the behavioral responses of female golden hamsters to predator urine odor. The VNO is the first station of the AOS, in which the AOB receives projections from the VNO and then projects to other brain regions. Removing the VNO impaired signal transformation within the AOS circuit; results suggest that the VNO is necessary in the detection of olfactory information contained in predator urine. Golden hamsters innately recognize ferrets as predators, even after 80 years of inhabiting predator-free laboratories. Golden hamsters also avoid the anal gland secretions of ferrets (McPhee et al., 2010) and wild Siberian weasels (*Mustela sibirica*: Zhang et al., 2003). Studies of the chemical composition of weasel anal gland secretions show that sulfur compounds present in the genus *Mustela* elicit innate avoidance behavior in prey (Mappes et al., 1998; Zhang et al., 2003). Quinoline, a major volatile compound in ferret urine, is an effective repellent of mice, ants, spiders, cockroaches and frogs (Eisner et al., 1997; Zhang et al., 2007). It is likely that quinoline and other constituents of urine are common to various predator species and are responsible for eliciting innate reactions in prey species.

A limitation of our experimental design was that we did not control the concentration of ferret urine used for odor exposure. The concentration might affect subjects' behavioral responses. However, the zoo animals which provided our urine samples had a regular daily diet and management regime, which may have helped to keep their urine concentration stable. In addition, any effects of variations in urine concentration may have been reduced by the small volume of the burrow system we used.

In summary, although the VNO is not involved in the discrimination of conspecific odors (Petrulis et al., 1999; Johnston & Peng, 2000), the accessory olfactory system, via the vomeronasal organ, may play a role in the detection of predator odor in female golden hamsters.

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