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The effects of sex on chemosensory communication in a terrestrial salamander (*Plethodon shermani*)

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ABSTRACT

Although much evidence reveals sexually dimorphic processing of chemosensory cues by the brain, potential sex differences at more peripheral levels of chemoreception are understudied. In plethodontid salamanders, the volume of the vomeronasal organ (VNO) is almost twice as large in males as compared to females, both in absolute and relative size. To determine whether the structural sexual dimorphism in VNO volume is associated with sex differences in other peripheral aspects of chemosensation, we measured sex differences in chemo-investigation and in responsiveness of the VNO to chemosensory cues. Males and females differed in traits influencing stimulus access to VNO chemosensory neurons. Males chemo-investigated ("nose tapped") neutral substrates and substrates moistened with female body rinses more than did females. Compared to females, males had larger narial structures (cirri) associated with the transfer of substrate-borne chemical cues to the lumen of the VNO. These sex differences in chemo-investigation and narial morphology likely represent important mechanisms for regulating sex differences in chemical communication. In contrast, males and females did not differ in responsiveness of VNO chemosensory neurons to male mental gland extract or female skin secretions. This important result indicates that although males have a substantially larger VNO compared to females, the male VNO was not more responsive to every chemosensory cue that is detected by the VNO. Future studies will determine whether the male VNO is specialized to detect a subset of chemosensory cues, such as female body rinses or female scent marks.

Introduction

Males and females often differ in their behavioral responsiveness to chemosensory cues emitted by conspecifics. For example, frontalin, a pheromone secreted by mature male elephants, elicits different behavioral responses in other elephants depending on gender and reproductive state (Rasmussen and Greenwood, 2003). Sodefrin, a pheromone released by breeding male Japanese newts, attracts sexually active females but not males or nonreproductive females (Kikuyama and Toyoda, 1999; Toyoda et al., 1995). Sex differences in responsiveness to conspecific chemosensory cues are typically thought to be due to the actions of sex steroid hormones early in development or in adulthood (Bakker, 2003; Xiao et al., 2004). Sex differences in responsiveness to chemosensory cues could be the result of sex differences in the processing of chemosensory cues by the brain, and/or sex differences in detection of chemosensory cues by the olfactory sensory organs.

Although sex differences in processing of chemosensory cues by the brain have been well studied (Bakker, 2003; Baum and Everitt, 1992; Kelliher et al., 1998; Wersinger and Baum, 1997), less is understood about potential sex differences in the chemosensory organs, such as the main olfactory epithelium or the vomeronasal organ (VNO). Both the main olfactory epithelium and the VNO detect chemosensory cues used in signaling among members of the same species, although the VNO has been studied the most in this context (Restrepo et al., 2004; Spehr et al., 2006). VNO ablations often produce different behavioral outcomes in males versus females (Kimchi et al., 2007) but it is unclear whether these behavioral sex differences are due to sex differences in filtering of sensory information by the VNO or due to differential responsiveness of brain circuits to the sensory information transmitted by the VNO. Kimchi et al. (2007) argued that the VNO modulates chemosensory information in a sexually dimorphic manner. Indeed, there is evidence in mice (Halem et al., 2001; Halem et al., 1999), axolotl salamanders (Park et al., 2004), and snakes (Huang et al., 2006) that the VNO is differentially responsive to chemosensory cues depending on sex.

Here, we examine sex differences in VNO activation by chemosensory cues as well as behavioral and morphological traits associated with VNO function in a plethodontid salamander, *Plethodon shermani*. Terrestrial salamanders in the family Plethodontidae are nocturnal, voiceless, and rely on chemical communication to mediate social interactions. Information about species, sex, and individuality are

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conveyed via chemosensory cues (Dawley, 1984; Gillette et al., 2000; Jaeger and Gergits, 1979; Mathis, 1990; Palmer, 2004). The nature of chemosensory signals involved in reproduction has been examined in this group, in particular for Sherman's salamander, *P. shermani*, and related plethodontid species. Courtship pheromones from the mental gland in males have been particularly well studied. Secretions from the mental gland are applied by males to the nares of reproductive females during courtship (Houck, 1998). Mental gland secretions have been biochemically and molecularly characterized and also have been shown to influence female receptivity (Feldhoff et al., 1999; Palmer et al., 2007a, 2005, 2007b; Rollmann et al., 1999; Watts et al., 2004).

In *P. shermani*, and a related species, *Plethodon cinereus*, the volume of the VNO epithelium (but not main olfactory epithelium) is almost twice as large in males as compared to females, even though males are slightly smaller in body size (Dawley, 1992; Dawley and Crowder, 1995; Woodley, 2007). There is no sex difference in cell density, suggesting that males have more VNO cells overall (Dawley, 1992; Dawley and Crowder, 1995; Woodley, 2007). We reasoned that the larger VNO reflects increased responsiveness of the VNO in males compared to females. The male VNO could be more responsive to all chemosensory cues detected by the VNO, or could be more responsive to a subset of chemosensory cues detected by the VNO. To test these hypotheses, we measured responsiveness of vomeronasal sensory neurons to chemosensory cues in males versus females.

Furthermore, in many species, animals express specific chemo-investigative behaviors that facilitate entry of chemosensory cues into the lumen of the nasal cavities housing the chemosensory neurons (Dawley and Bass, 1989; Halpern and Martinez-Marcos, 2003). We hypothesized that the larger VNO in male *P. shermani* reflects increased sampling of the chemosensory environment and we predicted that males would express more chemo-investigative behavior (nose tapping) than females and possess larger cirri, narial structures that facilitate uptake of nonvolatile chemical cues into the lumen of the VNO (Dawley and Bass, 1988; Dawley and Bass, 1989).

Methods

Animals

All methods were approved by Duquesne University's Institutional Animal Care and Use Committee. Animals were collected from Macon County, NC (83° 30′ 30″ N longitude; 35° 10′ 49″ W latitude) in August 2005 under appropriate permits from the North Carolina Department of Wildlife. All males used in this experiment had their mental glands surgically removed upon collection in North Carolina (see below; procedure approved by Oregon State University ACUP to L.D. Houck). Animals were housed for two months at Oregon State University, during which time they participated in behavior experiments that involved mating (data not shown). Animals arrived at Duquesne University in October 2005 and were housed individually at 16 °C on a 14L:10D photoperiod in 16×16×5 cm plastic boxes lined with moist brown paper towels. Animals were fed wax worms. In this species, adult females breed every other year such that during the breeding season, both reproductive and nonreproductive adult females are present. These studies compared males to both reproductive and nonreproductive females. Female reproductive condition was determined by looking through the ventral abdominal body wall for the appearance of yolky ova (reproductive female: REP), or absence of yolky ova (nonreproductive female: NONREP). Sex and reproductive state were confirmed later by dissection. Experiments examining chemo-investigative behavior, behavioral discrimination between different substrates, and vomeronasal function were begun in early November and finished by mid-December 2005. Sample sizes were 24 males, 24 REP females, and 24 NONREP females. In this species, males are slightly smaller in average body length than females. The body lengths (snout-vent lengths) were: REP females: 59.3±0.7 mm; NONREP female: 57.8±0.6 mm; males: 56.3±0.7 mm.

Chemosensory cues

We examined behavioral and VNO responses to several chemosensory cues. Ideally, we would have used the same types of chemosensory stimuli for testing both behavioral and VNO responsiveness, but this was not always possible. For tests of VNO responsiveness, we used an extract from male mental glands and female skin secretions. For tests of behavioral responses, which require large volumes of chemosensory cues, we used female skin secretions and body rinses from males and females. Below we describe each chemosensory cue in more detail.

Mental gland extract was used to test VNO responsiveness because it has a clear behavioral effect, is chemically characterized (Feldhoff et al., 1999; Rollmann et al., 1999), can be prepared with a known concentration and purity, and was demonstrated previously to activate sensory neurons of the VNO (Schubert et al., 2006; Wirsig-Wiechmann et al., 2002). Mental gland extract was available in amounts sufficient for VNO tests, but not for behavioral tests, which required large volumes of chemosensory stimuli. To obtain male mental gland extract, mental glands were surgically excised from anesthetized male salamanders and extracted in 0.8 mM acetylcholine chloride as described previously (Wirsig-Wiechmann et al., 2002). Acetylcholine chloride was removed by ultra-filtration with a 3KDa cutoff to ensure that levels of acetylcholine chloride were below the level of physiological responsiveness. Extracts were pooled, standardized to concentrations of 2.0 μg/μl in 0.5X phosphate buffered saline (PBS), and frozen at −20 °C until use. The diluent, 0.5× PBS, was designed to mimic natural osmotic concentrations of typical bodily secretions. A concentration of 2.0 μg/μl was used because it had increased behavioral receptivity and activated vomeronasal cells in *P. shermani* in previous studies (Rollmann et al., 1999; Schubert et al., 2006; Wirsig-Wiechmann et al., 2002).

Female skin secretions are released from dorsal tail granular glands when animals are handled (Largen and Woodley, submitted) and may serve to deter predators (Brodie and Howard, 1973; Brodie et al., 1979; Evans and Brodie, 1994; Hensel and Brodie, 1976). Skin secretions also have pheromonal properties, as indicated by behavioral responses by conspecifics and activation of the VNO after exposure to female skin secretions (Woodley, unpublished data). Skin secretions were collected from reproductive females by placing a female in 100 ml of ddH_2O in a glass container and gently pressing the animal's tail with blunt-nosed forceps. This procedure produced copious amounts of secretions. For the study of VNO responsiveness, an aliquot from one individual female was removed and stored at −20 °C until use. For behavioral studies, skin secretions from 8 reproductive females were pooled and then stored at −20 °C in aliquots until use in testing. Protein concentration of the skin secretions was 0.5 μg/μl, as determined by a protein assay. All aliquots were coded so that the investigator was blind to the chemosensory stimulus during behavioral tests. Skin secretions were used within 7 days of collection and animals used to provide stimuli were not used as test subjects.

Whole-body rinses from males and reproductive females were used to test behavioral responsiveness to chemosensory cues because large amounts could be prepared easily and behavioral responses to these rinses had been shown previously (Schubert et al., 2006). Whole-body rinses were collected from reproductive females ($n=10$) and males ($n=10$). Reproductive condition of each female used as a stimulus animal was determined by examining the ventral abdominal body wall for presence of yolky ova, and was confirmed later by dissection. Body rinses were collected by placing a single animal in 75 ml of ddH_2O in glass containers at 16 °C on a 14 L: 10D photoperiod for 48 h. In this volume, animals were not submerged. Body rinses were pooled for each category of animal (e.g., reproductive females or males). Control stimuli were prepared similarly except that animals were not placed in the water. Rinses were frozen at −20 °C in aliquots (coded so that the investigator would be blind to the chemosensory cue) until use in behavioral testing. Rinses were used within 2 weeks of preparation. The protein concentrations (as determined by a BCA assay) of reproductive female whole-body rinse and male whole-body rinse were 0.01 μg/μl and 0.03 μg/μl, respectively.

Behavioral tests

Scan sampling methods (Martin and Bateson, 1993) were used to quantify behavioral responses to chemosensory stimuli. All behavioral tests were conducted in the evening during the dark period of the photoperiod when animals normally are most active. Tests were conducted at 25 °C under dim incandescent light. A single investigator performed all the testing and was blind to the experimental treatments of the subjects.

Chemosensory investigation

In *P. shermani*, non-volatile chemosensory cues are sampled via nose tapping, a behavior whereby an animal physically touches the distal tip of the upper lip to a substrate. Chemosensory cues are transported from the substrate into the lumen of the VNO via the nasolabial groove, which runs from the distal tip of the upper lip into the nasal cavity (Dawley and Bass, 1988). Nose tapping is an easily scored and unambiguous behavior that provides an assay by which to evaluate levels of chemosensory investigation by the animal. Nose tapping is typically expressed as animals move about; therefore, we also measured locomotory activity.

To measure nose tapping and locomotory activity, each individual was placed in a 23×23×2 cm plastic test chamber with the bottom lined by brown paper towel moistened with 10 ml of a chemosensory stimulus, and scan sampling was used to observe behavioral responses. Each animal was scanned for approximately 2 s every 60 s for a period of 60 min. During each scan, the occurrence of nose tapping (1=nose tapping observed; 0=no nose tapping observed) was recorded, as well as the general location of the animal in the chamber, i.e., in which quadrant the animal's head was positioned. Upon completion of observations, each animal was given two scores: 1) total number of nose taps observed (maximum=60) and 2) general locomotor activity, as measured by the number of times the animal moved from one quadrant (noted in the previous scan) to a different quadrant (maximum=60).

Over the period of one week, each animal was tested once on each of the 4 prepared chemosensory stimuli. Each animal was tested with a single stimulus on a given night, with one night of rest between trials. The order of stimulus presentation was counterbalanced across animals such that each of the 4 different chemosensory stimuli was presented to 25% of the animals each night.