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Effects of androgens on behavioral and vomeronasal responses to chemosensory cues in male terrestrial salamanders (*Plethodon shermani*)

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Abstract

Chemosensory stimuli and sex steroid hormones are both required for the full expression of social behaviors in many species. The terrestrial salamander, *Plethodon shermani*, is an emerging nonmammalian system for investigating the nature and evolution of pheromonal communication, yet little is known regarding the role of sex steroid hormones. We hypothesized that increased circulating androgen levels in male *P. shermani* enhance chemoreception through morphological, behavioral, and physiological mechanisms. Experimental elevation of plasma androgens increased development of cirri, morphological structures thought to enhance the transfer of chemosensory cues from the substrate to the vomeronasal organ (VNO). Elevated plasma androgens also increased expression of a chemo-investigatory behavior (nose tapping) and increased preference for some female-derived chemosensory cues. Male-produced courtship pheromones activated a large number of cells in the VNO as measured by the method of agmatine uptake. However, androgen levels did not affect the total number of vomeronasal cells activated by male-produced courtship pheromones. Future studies will determine whether androgens potentially modulate responsiveness of the VNO to female-derived (as opposed to male-derived) chemosensory cues.

Keywords: Pheromone; Androgen; Testosterone; Amphibian; Salamander; Olfaction; Vomeronasal; Odorant; Chemosensory; Steroid

Introduction

Chemosensory stimuli are important to the full expression of reproductive and social behaviors in many vertebrate species (Johnston, 1983; Sachs and Meisel, 1988; Vandenbergh, 1988; Wingfield et al., 1994; Wood and Newman, 1995). Nonvolatile chemosensory stimuli are typically detected by receptors expressed by sensory neurons of the vomeronasal organ (VNO) (Halpern and Martinez-Marcos, 2003). Sensory neurons of the VNO project axons to the accessory olfactory bulb. In turn, neurons of the accessory olfactory bulb project to areas of the brain involved in reproductive behaviors and physiology, such as the medial amygdala, bed nucleus of the stria terminalis, the preoptic area, and the ventromedial hypothalamus (Scalia and Winans, 1975; Schmidt and Roth, 1990). Sex steroid hormone receptors are found in these brain areas, and the integration of hormonal and chemosensory information in the brain is required for the expression of male mating behavior (Wood, 1998; Wood and Newman, 1995).

Much information concerning chemosensory signals is available for mammals. We focus here, however, on a more basal tetrapod group: terrestrial salamanders. The nature of chemosensory signals involved in reproduction has been examined in this group, in particular for Shermani's salamander, *Plethodon shermani*, and related *Plethodon* species. These animals are nocturnal and rely on chemical communication to mediate social interactions. Information about species, sex, and individuality are conveyed via chemosensory cues (Dawley, 1984; Gillette et al., 2000; Jaeger and Gergits, 1979; Mathis, 1990; Palmer, 2004). In particular, a combination of studies at multiple levels indicates that *P. shermani* is an excellent model for understanding the nature and evolution of vertebrate pheromonal communication (Feldhoff et al., 1999; Houck and

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Arnold, 2003; Palmer et al., 2005; Rollmann et al., 1999; Watts et al., 2004; Wirsig-Wiechmann et al., 2002). In particular, a blend of proteinaceous pheromones is produced by an exocrine gland (mental gland) on a male's chin and is applied to the nose of a female during courtship. These courtship pheromones activate VNO cells as indexed by agmatine labeling and increase female receptivity. Several pheromones in the blend have been biochemically characterized and genetic analyses reveal that the pheromones have experienced rapid, selection-driven evolution.

Despite the wealth of information about chemosensory communication in *P. shermani* and related *Plethodon*, almost nothing is known about the role of sex steroid hormones in chemical communication. We hypothesized that androgens enhance the ability of males to respond to conspecific chemosensory cues. Our hypothesis was based on the observation that androgens – both testosterone and dihydrotestosterone (DHT) – are highest during the breeding season when males are searching for female mating partners and interacting with rival males (Woodley, 1994). We predicted that elevation of androgen levels in adult males would (1) increase the size of cirri (protuberances that surround each nasolabial groove and extend below the upper lip), which transport nonvolatile chemosensory cues from the substrate into the lumen of the VNO (Dawley and Bass, 1989); (2) increase "nose tapping", a chemo-investigatory behavior that brings the cirri in contact with the substrate so that cues from the substrate are drawn into the lumen of the VNO; (3) increase preferences for conspecific chemosensory cues over neutral cues; and (4) increase the number of VNO cells that physiologically respond to chemosensory cues.

Methods

We used chemosensory stimuli derived from the whole body of conspecific stimulus animals to measure behavioral responses to chemosensory cues. In contrast, we used extract from male mental glands (a source of pheromones used in courtship) to measure VNO responsiveness to chemosensory cues. Ideally, we would have used the same types of chemosensory stimuli for testing both behavioral and VNO responsiveness. However, for tests of VNO responsiveness, we decided to use male mental gland extract because, at the time, it was the only chemosensory stimulus demonstrated to activate the VNO (Wirsig-Wiechmann et al., 2002). Also, male mental gland extract was chemically characterized and could be prepared with a known concentration and purity (Feldhoff et al., 1999; Rollmann et al., 1999). This extract was available in amounts sufficient for VNO tests, but not in the quantity needed for chemosensory stimuli in behavioral tests. Thus, for behavioral tests, we used chemosensory stimuli derived from whole-body rinses of stimulus animals because large amounts could be prepared easily.

Animals

All methods were approved by Duquesne University's Institutional Animal Care and Use Committee. Animals were collected with the appropriate permits from North Carolina Department of Wildlife. Test subjects were collected from a single location (Wayah Bald, Macon County, NC, 83° 39′ 30″ N longitude; 35° 19′ 49″ W latitude) in August 2003 and August 2004. Shortly after capture, animals were anesthetized and mental glands were surgically removed (see below; procedure approved by OSU ACUP to LDH) to ensure that an animal's own mental gland secretions did not contribute to responses to chemosensory stimuli. In the laboratory, subjects were individually housed at 16°C on a 14L:10D photoperiod in 16×16×5 cm plastic boxes lined with moist paper towels and fed wax worm larvae. Average body length (snout-vent length) did not differ between animals in the 2 treatment groups.

Implants and surgery

Implants were made from Dow Corning Silastic Laboratory Grade Tubing (1.02 mm ID, 2.16 mm OD). Implants were 13 mm long, of which 10 mm were packed with crystalline testosterone propionate (TP) (Sigma #T1875). Ends were sealed with Sylgard 184 silicon elastomer. Control (BLANK) implants were similar, but contained no TP.

Animals were randomly assigned to treatment groups although we distributed animals caught in different years equally between the two treatment groups. Before surgery, animals were anesthetized in 0.5% MS222. The implant was inserted into the body cavity via a single 3-mm long incision in the abdominal wall and the incision was closed with a suture. A surgical adhesive and topical antibiotic were applied to the incision. Surgeries were performed in May 2005 and animals were allowed 3 weeks to recover before further testing.

Cirrus size

Six weeks after surgeries, we assessed cirrus size by taking digital images of the right-lateral view of each animal's head at 3× magnification. The length of the cirrus from the ventral margin of the external naris to the ventral tip of the cirrus along the nasolabial groove was measured with Image-Pro Plus imaging software. In addition, images were sorted into 4 groups based on overall size of the cirrus: not visible, small, medium, or large. The investigator was blind to treatment group when assessing cirrus size.

Behavioral tests

General

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

Chemosensory stimuli used in behavioral tests

For behavioral tests, we used (1) whole-body rinses and (2) substrates that were scent-marked by females in reproductive condition because previous studies showed that these sources of chemosensory cues elicited behavioral responses in *P. shermani* and *P. cinereus* (a congeneric of *P. shermani*) (Palmer, 2004; Sullivan et al., 2003).

We tested whole-body rinses derived from nonreproductive males ($n=5$), reproductive females ($n=5$), and nonreproductive females ($n=5$). To obtain body rinses, individual animals were placed in 50 ml of ddH$_2$O in glass containers for 48 h at 16°C. Body rinses from the same category (e.g., males) were pooled and diluted with ddH$_2$O to a volume of 520 ml. Body rinses were frozen at −20°C in 4 aliquots until use in behavioral tests. Control stimuli were prepared in an identical manner as for body rinses except that an animal was not placed in the ddH$_2$O. Aliquots were coded so the investigator was blind to the nature of the different chemosensory stimuli. Body rinses were used in behavioral tests within 7 days of collection. Animals used to obtain body rinses were not used as test subjects.

Substrates scent-marked by reproductive females were prepared by placing moistened paper towels on the bottom of females' home boxes for 48 h. The scent-marked substrates were then immediately used in behavioral tests. Control substrates were prepared by lining clean home boxes (without a female) with moistened paper towels for 48 h.

Response to chemosensory stimuli: nose tapping and locomotor activity

In plethodontid salamanders, nonvolatile substances gain access to the VNO via two nasolabial grooves running from the upper lip to the external nares (Dawley and Bass, 1989). Each nasolabial groove is contained within a cirrus, a fleshy protuberance that is thought to increase transport of nonvolatile substances via the nasolabial groove. Animals behaviorally control access of nonvolatile substances to the VNO by tapping their noses (and thereby the nasolabial cirri and grooves) to the substrate. The behavior is termed "nose tapping" and is an unambiguous and easily scored behavior. We measured nose tapping and as well