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Chemosensory cues from the lacrimal and preputial glands stimulate production of IP_3 in the vomeronasal organ and aggression in male mice

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Abstract

The social and reproductive behaviors of most mammals are modulated by chemosensory cues. The perception of some of these cues is mediated by the vomeronasal organ, which is a cartilage-encased elongated organ associated with the vomer bone in the rostral nasal cavity. Several studies have shown that chemosensory cues are present in urine, seminal fluid or vaginal secretions but only a few studies have focused on exocrine glands as a source of chemosensory cues. Here we show that chemosensory cues present in two exocrine glands, i.e., the preputial gland located at the caudal region and the lacrimal gland located at the rostral region, are capable of stimulating aggression in male mice. We further show that these extracts can stimulate the production of inositol-(1,4,5)-trisphosphate in the vomeronasal organ.
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1. Introduction

Most mammals use chemical signals (pheromones) to coordinate reproductive and social behaviors. These chemical signals can be classified into two categories: those with short-term effects on the behavior of the recipient (signaling pheromones), and those with long-term effects on the physiology of the recipient (priming pheromones) [1]. For example, signaling pheromones in urine or glandular secretions play a role in the initiation of copulatory behavior and aggression [2], whereas priming pheromones are responsible for puberty acceleration [3–7], and reproductive activation [8,9]. Although the perception of signaling pheromones may be mediated by the main olfactory system, the physiological effects of most priming pheromones are initiated in the vomeronasal organ (VNO). Recent evidence has shown that the detection of pheromones can rely on both the main olfactory system and the vomeronasal system [10,11]. The VNOs are paired, cartilage-encased elongated organs associated with the vomer bone in the rostral nasal cavity. The VNO contains a lumen which communicates via a duct with the oral or nasal cavity [12,13]. Therefore chemical stimuli in urine and glandular secretions of conspecifics can act upon the dendritic microvilli of bipolar chemosensory neurons in the VNO.

Molecular evidence has led to the isolation of two independent families of vomeronasal receptor genes (VR), known as V1Rs [14], and V2Rs [15–17] that encode putative pheromone receptors. V1Rs and V2Rs are expressed by distinct subpopulations of VNO neurons. These populations are non-overlapping and individual VNO neurons express only one receptor gene [14–17]. Neurons lining the apical layer of the VNO neuroepithelium express V1Rs [14] whereas neurons in the basal layer express V2Rs [15–17]. The neurons expressing V1Rs also express the alpha subunit of $G\alpha_{i2}$ and project to the anterior region of the accessory olfactory bulb (AOB), whereas the neurons expressing V2Rs also express the alpha subunit of $G\alpha_O$ and project to the posterior regions of the AOB [18–20]. The expression of two types of pheromone receptors supports the idea that they might be involved in different types of chemosensory information.

Most of the studies based on identifying the ligands for pheromone receptors has focused on chemicals present in urine, seminal fluid and vaginal secretions. However recent

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studies by Luo et al. [21] found that in their recording sessions, the first interactions between animals involved investigating the facial and mouth areas. They found that test animals investigated the face and head of stimulus animals more frequently and for longer time periods than the anogenital areas. These rostral areas also evoked the most robust responses by neurons in the AOB of behaving mice [21]. From these experiments they suggested that chemicals from glands found in the facial area such as the Harderian glands and the submaxillary salivary glands are capable of stimulating the VNO. A more recent study by Kimoto et al. [22] demonstrated that a peptide from the lacrimal gland of a male mouse was able to stimulate V2R-expressing vomeronasal sensory neurons in the female mouse. These studies suggest that exocrine compounds found in the facial region of the mouse are capable of stimulating the VNO. We therefore decided to explore whether other exocrine glands located at the caudal or rostral region can stimulate the VNO via the production of IP_3. Furthermore, we wanted to determine the behavioral role elicited by these exocrine compounds.

2. Methods and materials

2.1. Animals

CD-1 mice were originally obtained from Charles River Laboratories (Kingston, NY) and maintained in a breeding colony in the Department of Biological Sciences at Alabama State University. Animals were housed in Institutional Animal Care and Use Committee (IACUC) inspected and approved facilities and cared for according to the NIH Guide for Care and Use of Laboratory Animals. Mice were kept in Nalgene cages 26 cm × 21 cm × 14 cm, at 25 °C room temperature and a 12:12 h light/dark cycle. Food and water were provided *ad libitum*.

2.2. Membrane preparation

Male mice VNOs were dissected from their crevices in the nasal cavity, removed from the cartilaginous capsule and frozen on dry ice. The tissues were minced with a razor blade, crushed with a Teflon pestle and subjected to sonication for 2–5 min in ice-cold phosphate-buffered saline (PBS) in a Bransonic bath sonicator. The resulting suspension was layered on a 45% (w/w) sucrose solution and centrifuged at 4 °C for 30 min at 40,000 rpm in a Beckman SW55Ti rotor. The membrane fraction was collected and centrifuged as before for 15 min to pelletize the membranes. The membranes were resuspended in 100 μl of ice-cold PBS. Protein concentration was then determined according to the method of Lowry et al. [23]. The procedure used for the preparation of microvillar membranes is modeled after well-established methods for harvesting olfactory cilia from olfactory neuroepithelium [24,25]. The sonication of membranes results not only in the detachment of vomeronasal microvilli but also in the detachment of microvilli from sustentacular cells and plasma membrane fragments from other components of the neuroepithelium. Electron microscopic examination of these preparations revealed vesicles, axonemal structures devoid of a plasma membrane, and axonemal structures associated with membrane fragments [24,25]. The membrane preparation we refer to as "microvillar membranes" is, therefore, likely to contain contaminants derived from other components of the VNO, including microvillar membranes from supporting cells, and it is difficult to estimate the purity of the preparation precisely. However, our preparation appears to be sufficiently enriched in chemosensory membranes for the purpose of our studies [26–29].

2.3. Exocrine glandular extract preparation

The preputial gland, lacrimal gland, and the Harderian gland were dissected from adult male CD-1 mice. Preputial glands are located in front of the genitals and are thought to produce pheromones. The lacrimal glands are paired exocrine glands that sit alongside the eyeball within the orbit, nestled in the lacrimal fossa of the frontal bone and secrete lacrimal fluid. The Harderian glands are located near the eyes and produce a reddish-brown discharge when the animals are stressed. The glands from five adult male mice were dissected free and placed in ice-cold PBS. They were homogenized, centrifuged and aliquoted for storage. For our studies we used 20 μl which is estimated to be equal to one gland as was done in previous studies by Ma et al. [30]. Male urine was collected from adult males, pooled, spun for 5 min at 5000×*g* and decanted on the day of use.

2.4. Second messenger assay

For IP_3 assays, reactions were incubated for 1 min at 37 °C in 25 mM Tris–acetate buffer pH 7.2, 5 mM Mg–acetate, 1 mM DTT, 0.5 mM ATP, 0.1 mM $CaCl_2$, 0.1 mg/ml bovine serum albumin, 10 μM GTP, and 20 μg VNO membrane protein. 20 μl of stimulant, which is equivalent to 10% of the reaction volume, was used. Reactions were terminated by the addition of 1 M trichloroacetic acid. IP_3 was measured with a kit from Perkin Elmer, Inc. (Boston, MA) according to the manufacturer's instructions and is based on displacement of [^{3}H] IP_3 from a specific IP_3 binding protein. Differences between experimental and control animals were analyzed by analysis of variance (ANOVA).

2.5. Behavioral studies

In order to evaluate if the exocrine extracts induced aggression in males, we monitored the ability of males to initiate aggressive displays in response to pheromones using the resident–intruder paradigm. No conditioning or training period is required in this assay as it relies on innate and stereotyped behaviors initiated by chemosensory cues. After an isolated resident male mouse has established the cage as its territory, a dummy mouse to which 20 μl of a glandular extract or PBS had been applied was placed in the cage. The latency to sniff, duration of sniffs, and the number of attacks (bites) were recorded during a 5-min test period. Behavioral tests were conducted in an isolated, darkened room using a 40-

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