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Extracts from Salivary Glands Stimulate Aggression and Inositol- 1, 4, 5-Triphosphate (IP₃) Production in the Vomeronasal Organ of Mice

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Abstract

Mammals use chemical cues to coordinate social and reproductive behaviors. Chemical cues are detected by the VNO organ (VNO), which is a cartilage-encased elongated organ associated with the vomer bone in the rostral nasal cavity. The resident intruder paradigm was utilized to examine the ability of saliva and its feeder exocrine glands, the submaxillary, parotid, and sublingual glands to mediate aggression in mice. Saliva and extracts from submaxillary and parotid glands, but not extracts from sublingual glands of male CD-1 mice, induced a greater number of attacks and lower latencies to sniff and attack ($p < 0.05$) and significantly increased IP₃ production ($p < 0.05$) versus vehicle (PBS) in CD-1 male mice VNO. We further show that CD-1 male mouse saliva and submaxillary gland extract induced significantly more attacks and a lower latency to attack in lactating female CD-1 mice and produced significantly more inositol triphosphate (IP₃), indicative of phospholipase C_β signaling which mediates pheromonal activity, in CD-1 female VNO compared to PBS. Castrated CD-1 male mouse saliva, and exocrine gland extracts induced significantly less IP₃ production in male VNO and less aggression by CD-1 males and lactating females compared to responses to normal CD-1 male mouse saliva and gland extracts. Thus, chemical cues present in saliva, submaxillary and parotid glands of CD-1 male mice are capable of stimulating aggression in male and female congenic mice which are correlated with significant production of IP₃ in the VNO. Additionally, these stimulations of aggression and IP₃ production are shown to be androgen-dependent.

Keywords

IP₃; Exocrine glands; salivary glands; VNO; aggression

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1.0 Introduction

The survival of animal species is dependent upon the ability to identify members of an individual animal's own species and the ability to distinguish genders for procreation [1]. To mediate these tasks, many mammals and other animals exchange pheromones which are water-soluble volatile and non-volatile chemicals used to communicate intraspecies cues which induce various social and reproductive behaviors [2]. Pheromones are generally thought to elicit innate responses which occur in naïve animals without prior learning and are significantly characterized by their size and polarity, which determine their volatility in air and solubility in water, respectively. Attractant and alarm pheromones, which act at a distance, are typically small and volatile. Methylthiomethanethiol (MTMT), which is present in male mouse urine and attracts investigation by females, is a good example. By contrast, pheromones which convey information about specific individuals are likely to be relatively non-volatile proteins or peptides such as major histocompatibility complex (MHC) class I peptides which do not disperse and can be more reliably associated with the producer [3].

Two multigene families of G protein-linked receptors (V1R and V2R), distinctively expressed in apical and basal regions of the Vomeronasal organ (VNO), respectively, have been identified. The V1 receptors (V1Rs) are linked to $G_{\alpha_{i2}}$, have a relatively short NH_2 -terminal, and have greatest sequence diversity in their transmembrane domains. The V2Rs are linked to G_{α_o} and comprise a family of about 140 genes distinguished by their long extracellular NH_2 -terminal which is thought to bind ligands [4 and references therein]. While volatile pheromonal signals are thought to be mediated by V1Rs in apical VNO neurons, evidence suggests that V2Rs located in the basal VNO mediate non-volatile signaling and are thus responsible for behaviors attributed to non-volatiles such as pregnancy block (failure of implantation caused by a non-congenic male that occurs in recently impregnated females mated to congenic males; also called the "Bruce Effect") as mediated by MHC class I peptides [5].

Most of the studies based on identifying the ligands for pheromone receptors have focused on chemical signals such as urine, seminal fluid and vaginal secretions [6] which are initially produced in anogenital areas of the animal. Although pheromones initially produced by anogenital areas might be transferred to the head by grooming, observed differences in responses of some neurons to explorations of the facial area are significantly contributed to by semiochemicals from scent glands of the facial area, including exocrine skin glands and the salivary glands. In fact, investigations between test animals and either anesthetized or behaving stimulus animals typically involve the face and mouth areas more frequently and for longer periods than the anogenital area. Recordings suggest that semiochemicals from glands in the facial area are accessible to the VNO and can communicate gender and genetic identity with fidelity similar to that of anogenital sources of pheromones [7].

Saliva and salivary glands are thus thought to contain substances which are pheromonal in nature. Laukaitis et al., [8] observed the expression of many androgen binding proteins (Abps) in various glands in the head and neck including the lacrimal and salivary glands. Salivary Abp mediates mate preference [8] and saliva is involved in sexual communication [9] indicating the presence of pheromones in the glands involved in saliva production.

Currently, no data are available on the presence of volatile pheromones in saliva as has been shown for urine [10]. However, further evidence supporting the presence of non-volatile pheromones in saliva comes from findings of mouse saliva containing high concentrations of lipocalins, which are very similar to the major urinary proteins (MUPs) [10, 11]. Like urinary proteins, which are synthesized in the liver as well as in the submaxillary, lachrymal, sublingual, parotid, and mammary glands under the control of various developmental and hormonal stimuli [12], lipocalins are also testosterone-dependent chemosignals which signal the presence of a reproductively active male [13] and are strongly implicated in intraspecies chemical communication [14]. Just as the functions of MUPs are not limited to transport of hydrophobic molecules but also include mediation of aggressive behavior in male mice [15], puberty acceleration [16], and induction and synchronization of estrus cycles [17], it is similarly reasonable to propose that salivary proteins are also involved in pheromonal activity.

Gender or sex-related differences in tissue expression of genes is also indicative of potential pheromonal activity within a species. Many structural and functional differences that are sex-related have been identified in the submandibular, sublingual, and parotid glands in a variety of species [18, 19] in a tissue-specific manner [19, 20, 21, 22]. For example, the gene encoding the steroidogenic enzyme 17 β -HSD3 was up-regulated in the male submandibular gland, but was not differentially expressed in the sublingual or parotid glands; this enzyme affects the peripheral synthesis of all active estrogens and androgens [23]. The fact that so many sex-associated differences exist, and in a tissue-specific manner, may relate not only to structural and functional differences among the major salivary glands, but also to their responsiveness to sex steroid hormones and other sex-related factors [24] which are central to pheromonal activity.

There are examples of other exocrine glands being involved in pheromonal activity. Chemical cues produced in the preputial glands and secreted into the urine have been shown to modify aggressive interactions [25]. Furthermore, socially-dominant male mice were shown to have larger preputial glands than do subordinate males while preputial-glandectomized males failed to establish social dominance as readily as sham-operated males. Interesting, an adaptive, self-protective mechanism seems to have evolved in which recipients of robust preputial signals appear to delay their involvement in aggressive behavior, thus reducing the recipient's risk of physical injury resulting from fighting with a dominant male [26]. A more recent study conducted by our laboratory [6] confirmed the involvement in aggression of the preputial gland and demonstrated for the first time that the intraorbital lacrimal gland was also involved in male aggression in the mice [26]. Previous studies indicate that activation of phospholipase C β (PLC β) by receptor-ligand binding and subsequent G-protein signaling that leads to hydrolysis of phosphoinositol bis-phosphate (PIP₂) into inositol-(1, 4, 5)-triphosphate (IP₃) and diacylglycerol (DAG) is the operative signal transduction pathway which mediates pheromonal activity in the VNO [60]. Thus, this study also connected aggression to inositol-(1, 4, 5)-triphosphate (IP₃) production in male and female mouse VNO. Kimoto et al., [27] has also identified a male specific 7 kDa protein produced by the extraorbital lacrimal gland which induced electrical activity in female VNO neurons. This protein is encoded by a member of a family of at least 23 related

genes which are expressed by other glands in the head region, including the salivary and Harderian glands; behavioral effects of this family of proteins are currently unknown [27].

The studies discussed above support the idea that exocrine glands found in the facial region of the mouse are capable of stimulating the VNO. Hence, in this study we explored whether other exocrine glands located at the rostral region can stimulate production of IP₃ in the VNO and elicit behavioral responses. We hypothesized that salivary glands and saliva contain compounds which can stimulate the VNO and elicit behavioral responses. To test this hypothesis, we used salivary gland extracts and saliva to stimulate the VNO of male and female mice with a dummy mouse swabbed with the individual gland extract or with saliva to test for inter-male and maternal aggression using the resident-intruder paradigm.

2.0 Materials and Methods

2.1 Animals

CD-1 mice *Mus musculus* L. were originally obtained from Charles River Laboratories (Kingston, NY, USA) 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-inspected and approved facilities and cared for according to the NIH Guide for Care and Use of Laboratory Animals (1997). Animals were maintained in a 12:12 light: dark reverse light cycle, with food (Harlan TEKLAD Rodent diet) and tap water provided *ad libitum*. All mice were individually housed in Naglene cages 26 cm × 21 cm × 14 cm, at 25°C room temperature without filtration. All testing was conducted during the dark part of the light: dark cycle in recognition of the nocturnal behavior patterns of mice.

2.2 Membrane preparations

VNOs from male or female mice, were dissected from their crevices in the nasal cavity, removed from the cartilaginous capsule, and frozen on dry ice. The tissues were then minced with a razor blade, crushed with a Teflon pestle and subjected to sonication for 2–5 min in ice-cold PBS in a Bransonic bath sonicator. The resulting suspension was layered on a 45% (wt/wt) sucrose cushion and centrifuged at 4 °C for 30 min at 40,000 rpm in a Beckman SW55Ti rotor. The membrane fraction on top of the sucrose was collected and centrifuged as before for 15 min. to pellet the membranes. The membranes were resuspended in 100 µl of ice-cold PBS. Protein concentrations were determined according to the method of Lowry *et al.* (28), using bovine serum albumin (BSA) as standard.

2.3 Second messenger assays

For IP₃ assays, stimulants (CD-1 male urine, salivary gland extracts, or saliva) at 10% v/v in reaction buffer (25 mmol l⁻¹ Tris-acetate buffer pH 7.2, 5 mmol l⁻¹ magnesium acetate, 1 mmol l⁻¹ dithiothreitol (DTT), 0.5 mmol l⁻¹ adenosine triphosphate (ATP), 0.1 mmol l⁻¹ CaCl₂, 0.1 mg ml⁻¹ bovine serum albumin (BSA), 10 µmol l⁻¹ guanosine triphosphate (GTP)) were incubated and allowed to equilibrate to 37°C in a water bath. Reactions were started by adding a volume containing 20 µg VNO membrane protein to the reaction solution containing stimulants and reaction buffer and incubating the reaction for 1 min at 37°C in the water bath; total reaction volume was 120 µl. Reactions were terminated by the

addition of 1 mol l⁻¹ trichloroacetic acid (TCA). IP₃ concentration was measured with a kit from Perkin Elmer, Inc. (Boston, MA, USA) according to the manufacturer's instructions, based on displacement of [³H]IP₃ from a specific IP₃ binding protein. Differences between experimental and control animals were analyzed by analysis of variance (ANOVA). Statistical differences shown by ANOVA were further analyzed for differences between pairs using the Student t-test. Statistical significances were accepted as $p < 0.05$ (the probability of random occurrence of less than 5 % or $p < 0.05$).

2.4 Exocrine gland extracts preparation

The submaxillary, sublingual, and parotid glands were dissected from adult male mice. The salivary glands are located cranio-ventral to the throat region. The paired submaxillary salivary glands and sublingual salivary glands are removed from the ventral cervical region. The submaxillary glands are the largest of these tissues and lie caudal to the sublingual glands. The diffuse, pink parotid salivary gland is located ventral to the base of the ear. The main portion of the parotid gland, although not a compact structure was found laterally, and there was an extension in close association with the superficial surface of the other two glands. The three glands were separated easily along fascial planes. The individual glands from five adult male mice were homogenized with 500 µl of ice cold PBS (1 ml of ice cold PBS was needed to homogenize five submaxillary glands) using a Teflon pestle, centrifuged at 4 °C for 10 min at 8500×g in a Beckman Coulter Microfuge 22R rotor, aliquoted and stored at -80 °C till ready to be used. Saliva and urine were collected from adult males; urine was pooled, spun for 5 min at 5000×g and decanted to be used immediately while saliva was stored at -80 °C till ready to be used. Castrated male urine and glandular extracts were collected within one month post-castration; castration was performed by the animal supplier upon adult males which were received in our laboratory at 30 days old. Protean assays were performed on all extracts and on saliva.

2.5 Aggression Tests (Behavioral Test)

2.5.1 Inter-male Aggression Test—This test was based on a “resident-intruder” paradigm. Sexually naive 7 to 10-week-old males were used as “residents.” Resident males were individually housed for 1 week prior to the test session, with bedding unchanged to allow establishment of territory. A dummy mouse to which 20 µl of a glandular extract, saliva, urine, or PBS had been applied by pipette tip was then introduced as “intruders.” The “intruder” was placed into the resident's cage for a maximum of 5 minutes, and the session was video-taped. Recorded parameters include latency to sniff, latency to first attack, cumulative attack duration, and number of attacks (bites). The number of bites, cumulative attack time, and latency to first attack were measured as aggression parameters. An attack is a subset of aggressive behavior which contains contact between the resident and the intruder such as biting or wrestling. Aggression tests were conducted in an isolated, darkened room using a 40-W red bulb for visibility. To facilitate the delivery of only the fluid or gland's components, a dummy mouse was constructed using the toe portion of a white cotton sock, shaped in a characteristic manner resembling a mouse, and sewn closed. New, unused white socks were used as “dummy” mice. Using clean laboratory gloves to avoid odor and chemical contamination, packages of white socks used in these studies were opened and prepared (cut to size and stuffed with cotton) just prior to initiating behavior studies. Though

no control for background odors was assessed, use of socks from the similar, commercially sealed packages minimized the possibility of extra or dissimilar odorants or chemicals being present on different socks. “Dummy” mice were used within a short period of time (< 10 seconds) after application of body fluids or extracts; they were used once and discarded after use. Several sizes were made in order to be sized-matched with the resident mouse at the time of presentation. Extracts were applied in a rostral location and the dummy mouse placed in the opposing corner with rostral end towards the resident male.

The dummy mouse was chosen over castrate males for the following reasons: (1) although castrate males produce no androgen-dependent pheromones, other chemosensory cues are present such as the MHC class I peptides, extralacrimal secretory protein 1 (ESP 1), and possibly others as yet unidentified, (2) parotid glands and saliva have not been previously been shown to be androgen dependent, (3) the use of the dummy mouse allowed the application of chemical cues from one specific source without influence from other venues, and (4) the use of dummy mouse is not that different than using a cotton swab to introduce single odorants into the experiment for habituation-dishabituation studies (6). Experiments using castrated mice were performed as follow-up studies and to validate the previous experiments. Differences between experimental and control animals were analyzed by ANOVA. Differences between pairs of stimulants or animal groups were further analyzed with the Student t-test. Statistical differences were accepted as $p < 0.05$.

2.5.2 Maternal Aggression Test (Assay)—Female mice were paired with males for 10 days and then housed individually in polypropylene cages with a 12/12 light/dark schedule. The date of birth was considered postpartum day 0, and litters were culled to ten pups to decrease variability in maternal aggression [29]. On postpartum days 6, 7 and 8, female mice were tested for maternal aggression in a manner similar to males i.e., by the introduction of a dummy mouse to which 20 μ l of a glandular extract, saliva, urine, or PBS had been applied by pipette tip as “intruder males” for 10 min; since comparisons were to be made between male and lactating females, data sets were evaluated at 5 and 10 minutes of the 10 protocol and only 5 minute data is reported herein. Behavioral testing occurred between 9.00 A.M and 5.00 P.M. The pups were removed from the cage 3 min before the behavioral test. Removal of the pups from a dam just before an aggressive test does not diminish the expression of maternal aggression in mice [30]. Non-lactating, non-pregnant CD-1 female mice were also individually housed for a week without a change of bedding and tested using the “resident intruder” paradigm for aggression. Non-lactating female aggression served as a negative control for the maternal aggression study; ovariectomized (OVX) CD-1 female were similarly tested using male urine and saliva. For quantification of maternal aggression, latency to attack, number of attacks, and duration of attacks were examined. Differences between experimental and control animals were analyzed by ANOVA with statistical differences accepted as $p < 0.05$; groups of animals and stimulants that showed statistically significant differences by ANOVA were further analyzed for differences between individual groups or stimulants by the Student t-test.

3.0 Results

3.1 Submaxillary gland, parotid gland, and saliva stimulate the production of IP₃ in the male VNO organ

In order to assess the role of chemical cues that are potentially found in secretions that are found in saliva and produced by the salivary glands, we incubated normal male VNO membrane preparations with extracts from submaxillary gland, parotid gland, sublingual gland or saliva or urine of normal and castrated male mice. A significant increase in the production of IP₃ was obtained with urine, saliva, and extracts from submaxillary, and parotid of normal male mice ($p < 0.05$, Figure 1). IP₃ production by sublingual gland extract of normal male mice or extracts from submaxillary gland, parotid gland, sublingual gland, or saliva or urine of castrated male mice was significantly less than that produced by normal males ($p < 0.05$) and showed no significant difference compared to PBS (Figure 1). In these experiments, male urine was used as a positive control and PBS as a negative control. These results show that there are compounds being produced in the submaxillary gland, parotid gland, and saliva of normal male mice which are capable of inducing significant IP₃ elevation indicative of signal transduction and action potential propagation in the CD-1 male VNO; the production of these compounds is apparently androgen-dependent.

3.2 Saliva and submaxillary gland of male stimulate the production of IP₃ in the CD-1 female VNO organ

In order to further assess the role of these chemical cues which stimulate the VNO and are produced by the salivary glands, we incubated non-pregnant, non-lactating CD-1 female VNO membrane preparations with saliva or extracts from submaxillary gland, parotid gland, or sublingual gland of normal CD-1 male mice. A significant increase in the production of IP₃ ($p < 0.05$) was obtained with extracts from saliva and submaxillary gland extract of male mice. Parotid or sublingual gland extracts of normal male mice did not produce significant amounts of IP₃ (Figure 1). In these experiments, CD-1 male urine was used as a positive control and PBS as a negative control. These results show that there are compounds being produced in the submaxillary gland and secreted into saliva of male mice which are capable of stimulating the female VNO.

3.3 Submaxillary gland, parotid gland, and saliva elicit aggression in male mice

To determine whether saliva and gland extracts from the submaxillary, parotid, or sublingual elicit male-male aggression, a dummy mouse swabbed with saliva or exocrine gland extract was introduced as an intruder into a resident male cage. We found that when the resident male was exposed to a dummy mouse swabbed with a normal CD-1 male urine, saliva, submaxillary or parotid gland extracts, the latency to sniff (Figure 2) and latency to attack (Figure 3) decreased significantly ($p < 0.05$), while the number of attacks (Figure 4) and duration of attacks (Figure 5) significantly increased. Exposure to urine, saliva, extracts from submaxillary gland, parotid gland, or sublingual gland of castrated CD-1 male mice and sublingual gland extract of normal CD-1 male mice had no effect relative to PBS on the latency to attacks, number of attacks, and duration of attacks (gradient-filled bars, Figures 3, 4, and 5).

3.4 Saliva and submaxillary gland of male mice elicit maternal aggression in female mice

To determine whether CD-1 male saliva and gland extracts from the submaxillary, parotid, and sublingual glands elicit maternal aggression in lactating CD-1 female mice, a dummy mouse swabbed with PBS, urine, saliva or exocrine gland extract was introduced as an intruder into a resident lactating CD-1 female cage from which the pups had been recently removed (see Materials and Methods). We found that when the resident female was exposed to a dummy mouse swabbed with male urine, saliva, or submaxillary gland extract, the latency to attacks (Figure 3) decreased significantly, while the number of attacks (Figure 4) and duration of attacks (Figure 5) significantly increased ($p < 0.05$ for all). Exposure to extracts from parotid gland and sublingual gland of CD-1 male mice had no effect on the latency to attack, number of attacks, and duration of attacks relative to PBS. When the resident lactating female was exposed to a dummy intruder swabbed with urine, saliva, or submaxillary gland extract of castrated CD-1 male mice, none of the stimulants induced significant changes in any indexes of aggressive activity relative to PBS (Figures 2–5). These results indicate that, just as in the case of CD-1 male aggression, aggression in lactating females mediated by normal CD-1 male saliva and salivary gland extract is androgen-dependent since exposure to castrated mouse saliva or submaxillary extract does not incur significant activity.

Comparisons of male and lactating female data indicate that levels of aggressivity are generally similar between males and lactating females but have differences in the responses to urine versus saliva and its glandular extracts. Latency to attack, the number of attacks, and the duration of attacks were not significantly different between the groups when the resident mouse was stimulated by normal male urine. However, saliva and glandular extracts of parotid, or sublingual gland elicited a significant increase in the number and duration of attacks in lactating female CD-1 mice relative to normal CD-1 males ($p < 0.05$ for all, Figures 2–5); numbers and duration of attacks during stimulation with male submaxillary gland extract were increased for lactating female versus male mice but the differences were not quite statistically significant ($p \approx 0.05$ for both). Thus, there appears to be a gender related effect of saliva and salivary glandular extracts on aggressive behavior in CD-1 mice; a similar effect is not observed with CD-1 male urine.

3.5 Saliva and salivary gland extracts do not elicit aggression in Non-lactating and OVX CD-1 Female Mice

In order to investigate the role of lactation in female aggression, a dummy mouse swabbed with CD-1 male urine, saliva, or extracts from the submaxillary, parotid, or sublingual gland were introduced as an intruder into a resident cage containing a normal CD-1 female mouse that was not pregnant (non-lactating) and parameters of aggressive behavior were observed for 5 minutes; similarly, urine or saliva was introduced into a resident cage containing an OVX CD-1 female mouse for behavior observation. Interestingly, only saliva elicited attacks in non-lactating females. However, the number of saliva mediated-attacks was not significantly greater than those induced by PBS in non-lactating females ($p > 0.11$, Figure 7). By contrast, lactating females significantly increased the number of attacks evoked by saliva relative to PBS at 5 minutes ($p < 0.01$, Figure 4). Urine tended to increase the number of attacks ($p = 0.07$, Figure 7) while both urine and saliva tended to increase the duration of

attacks ($p \approx 0.06$ for both, Figure 8) in OVX CD-1 female mice relative to PBS. Latency to attack was not different from vehicle (PBS) for either urine or saliva in OVX mice (Figure 6). Vaginal smears to determine estrus cycle phase were performed on non-lactating females immediately prior to behavior testing. Leukocyte numbers were counted and estrus phase were determined to correlate potential cycle-dependent aggression responses. There was no correlation found between estrus phase and aggressivity in non-lactating female mice (since there is no significant aggression observed in these mice) which indicates that there was no cycle-dependent effect on aggression in non-lactating female mice (data not shown).

Comparison of non-lactating female and normal male mice responses to male saliva (shown above as the only stimulant that induced any aggression, although insignificant, in females) demonstrates that females have significantly elevated latency to attacks ($p < 0.01$, Figures 3 and 6), fewer number of attacks ($p < 0.001$, Figures 4 and 7) and diminished duration of attacks ($p < 0.03$, 5 and 8) when compared to normal males. As earlier results indicated, normal male CD-1 mice also showed significant aggressive responses to male urine and submaxillary and parotid gland extracts whereas non-lactating females showed virtually no aggressive response to any of these substances. Thus, non-lactating female mice are phenotypically non-aggressive whereas normal males display an aggressive profile in response to urine and salivary components under the circumstances of the resident intruder paradigm. Maternity and childbirth drastically changes the behavior of the normally docile female to more closely resemble that of the normally aggressive male.

3.6 Overview of Stimulant Profiles for Normal Male and Lactating Female Mice

In general, an obvious pattern is observed when analyzing stimulant profiles, which include latencies to sniff and attack, the numbers of attacks, and durations of sniffs and attacks in male and female mice. First of all, there exists an inverse relationship between latencies to sniff and attack and the number and duration of attacks that are observed in both normal male and lactating female mice: shorter latencies correlate with increased aggression (numbers and duration of attacks). Secondly, latencies to sniff are invariably shorter than latencies to attack consistent with the two step model of pheromonal activity which basically states that olfactory cues (i.e., volatile chemosignals) necessarily precede pheromonal exposure and subsequent behavioral activity. Thirdly, shorter latencies to sniff coupled with increased duration of sniffs (duration of sniff data was compiled but not included) correlates with relatively fewer aggressive events. Thus, normal male mice generally displayed similar latencies to sniff to those of lactating females (except, notably, for a longer latency for saliva) but displayed numerically longer durations of sniffs for all stimulants (data not shown). This correlated with greater numbers and duration of attacks for lactating female versus normal male mice for normal male saliva, and extracts of normal male submaxillary, parotid, and sublingual gland. Thus, profile analysis gives an empirical perspective for the differential effect of saliva and salivary glands on female versus male aggression as discussed above. These gender-related effects of saliva and its glandular contributors do not appear to be due to hormonal influences mediated by lactation in females because the differences are not global in nature as urine-mediated responses are not different between genders. Possible explanations are (1) that the same component or components, i.e., pheromones, of saliva have differential effects for male and female, (2) salivary aggression

is induced by different salivary pheromones in male versus female mice, or (3) a combination of these possibilities. A prime candidate for the gender-related differences may be the androgen binding proteins which are not found in urine.

4.0 Discussion

Until recently, little has been done to identify the roles of exocrine glands as a source of the chemosignals responsible for behavioral responses in mice. The findings described in this study indicate that at least some of the chemosignals responsible for aggression are produced by the salivary glands and saliva of the male mouse. Our results demonstrate that incubation of male VNO membranes with extracts from the submaxillary gland, parotid gland, or saliva stimulates significant IP_3 production in the male VNO (Figure 1) and that this stimulation is androgen-dependent since glands from castrated male mice produced significantly less IP_3 and incurred significantly less aggressive behavior when compared to normal male mice. We also demonstrated that incubation of female VNO membranes with male extracts from the submaxillary gland or saliva stimulates IP_3 production in the female VNO (Figure 7). We further observed that while parotid gland extract is able to stimulate the production of IP_3 in the male VNO, there is no significant production of IP_3 in the female VNO by male parotid gland extract. This demonstrates that the VNO organ responds to parotid stimuli in a sex dependent manner, which may be an important cue in coordinating social and reproductive behaviors. This study represents the first report of aggressive behavior in rodents which is mediated by saliva and the submaxillary and parotid salivary glands.

There are several studies that have shown sexual dimorphism in the vertebrate VNO organ in terms of size of the VNO or even number of neurons in the VNO since there is evidence that more neurons are present in the VNO of males than female rats [34]. Thompson et al. [35] showed that presentation of 2-heptanone or 2, 5-dimethylpyrazine, which are mice urine derivatives, was able to stimulate the production of IP_3 in the female VNO but not in the male VNO. Zufall et al. [36] found that female mouse VNO neurons' are extremely sensitive to 2-heptanone and 2, 5-dimethylpyrazine, as measured by an electrovomeranosogram (EVG), and highly selective as individual neurons appear to recognize only one or very few pheromones [36]. Holy et al. [37] using a multielectrode array to record from sheets of VNO neuroepithelium found that there are neurons in male and female VNOs which respond uniquely to pheromones of one sex. There is further evidence that neurons within defined subdivisions of the VNO respond differentially to pheromones and moreover the responses differ in the VNO of females versus male VNOs [38]. This is an interesting observation which matches behavioral data showing that the parotid gland has specific pheromonal effects which are observed in males but not in females. Our results suggest that males may respond to specific chemicals within the parotid gland. This is unlike other systems such as in insects where the same chemical compound solicits different pheromonal effects depending on the sex of the recipient.

Previous studies on the salivary glands have associated the whole salivary gland to estrus stimulation [9], saliva with mate selection [10] and submaxillary gland with mate selection [39] and male/male aggression [40]. Sublingual and parotid glands have not been correlated to any specific behaviors. Yamashita et al. [40] reported that goldthioglucose (GTG)-

induced obese mice exhibited lower inter-male aggression against an unfamiliar mouse, and had smaller submaxillary and preputial glands than lean controls. These results suggest that the absence of typical intraspecific social behavior in male GTG-obese mice was associated with the total or partial failure to release a factor or factors from the submaxillary glands. Androgen binding proteins which are secreted by the mouse submaxillary gland and found in saliva, were shown to be involved in some behavioral responses such as mate selection [8, 39] and shown not to be secreted in urine [41]. Kimoto et al., [27] found that extract from submaxillary gland induced robust expression of c-fos in female VSNs. These results in total suggest that non-volatile male specific cues are secreted from the submaxillary gland and ultimately into saliva. Thus, although saliva and salivary glands have been implicated in several pheromone-associated behaviors such as estrus stimulation and mate selection, our study provides the first evidence of direct involvement of saliva and salivary glands in aggression in mice.

Aggressive behavior results from a complex interplay between innate hormonal regulators and environmental cues. In males, aggressive behaviors require androgens during a critical period of development, presumably to establish the requisite neural circuitry [42, 43]. Circulating androgens are also required during adulthood to facilitate the aggressive response [43]. In adult male red deer, for example, aggression is cyclical during the year and the aggressive cycle is causally related to changing androgen levels [44]. Male mice show high levels of aggression toward intruders throughout the year. In contrast to males, female mice attack behaviors are largely restricted to periods of nursing, again revealing a hormonal dependence of aggressivity [45]. Because this temporal expression of aggression, termed maternal aggression, is highly conserved among mammals, it has been hypothesized that this behavior increases the likelihood of survival of the offspring [46]. Female mice can also show a type of territorial aggression toward other females, but this aggression is much less fierce than that shown toward males during maternal aggression and is thought to be neurally similar to male aggression [47].

Leypold et al. [48] stated that aggression in both sexes requires a functional VNO. Transient receptor potential channel 2 (Trp2, a non-selective, gated plasma membrane calcium channel found almost exclusively in the VNO of rodents including mice) mutant mice do not exhibit characteristic aggressive responses to an intruder male and this docile behavior is not altered by fighting or mating experience. The simplest interpretation of this is that males express a pheromone recognized by the VNO which elicits attack behavior in other mice. This behavioral response is innate but is regulated internally by hormonal status and externally by pheromones in the environment. Superimposed on innate endocrine regulators are environmental cues which assure that aggressive behaviors are elicited only in appropriate situations. Maternal aggression is decreased in mice missing either neuronal nitric oxide synthase (Nos1) [49], a subset of pheromone receptors [50], or the trp2 ion channel which transduces pheromonal inputs [48]. Conversely, maternal aggression is significantly increased in mice missing estrogen receptor β [51]. Low levels of a serotonin agonist can eliminate female-female aggression and male aggression but have no effect on maternal aggression [47]. Steroid hormones, such as estradiol and progesterone, released during pregnancy enable female rats to express maternal behaviors, including aggression [52, 53, 54].

Our behavioral studies show that submaxillary gland extracts, parotid gland extracts, or saliva can stimulate aggressive behaviors in male mice and this stimulation is androgen dependent (Figure 2–6), while only saliva and submaxillary gland extracts are able to stimulate aggressive behaviors in female mice (Figure 7 and 8). We found that when the resident males were exposed to a dummy mouse swabbed with submaxillary gland extracts, parotid gland extracts, or saliva from a normal male, the latency to sniff (Figure 2) and latency to attacks (Figure 4) decreased significantly ($p < 0.05$), while the number of attacks (Figure 3) and duration of attacks (Figure 5) significantly increased. On the other hand, latency to sniff, latency to attacks, number of attacks, and duration of attacks shown by a normal male towards a dummy mouse swabbed with submaxillary gland extracts, parotid gland extracts, or saliva from a normal versus castrated male were significantly different (Figure 6). We also found that when the resident lactating females were exposed to a dummy mouse swabbed with submaxillary gland extracts or saliva from a normal male, the latency to attacks (Figure 7) decreased significantly ($p < 0.05$), while the number of attacks (Figure 8) and duration of attacks (Figure 9) significantly increased. Differences observed in behavioral responses of males and females to a specific pheromone may be due to differences in central processing or to sex differences in steroid-sensitive centrifugal inputs mediating the VNO response to pheromones [38]. A pheromonal effect has to be conducted through the glomeruli of the auxiliary olfactory bulb to the amygdala and hypothalamus and an appropriate response initiated. Perhaps, it is this sexual dimorphism which allows differences in the behavior responses to a particular pheromone. Experiments using immediate-early gene (IEG) protein expression as markers of neuronal activity have shown that chemical signals in soiled bedding stimulate a sexually dimorphic pattern of c-fos protein immunoreactivity in central sites along the VNO projection pathway [38, 55, 56, 57].

These studies suggest that chemosignals found in submaxillary gland extracts, parotid gland extracts, or saliva are an additional source of mice olfactory signals which are capable of stimulating spontaneous aggressive behaviors in mice. This stimulation is hormonal dependent and may help to explain why direct contact between mice evokes a more pronounced effect.

In conclusion, the prediction derived from our hypothesis that salivary glands and saliva contain compounds which can stimulate the VNO and elicit behavioral responses was supported. The specific behavior attributes of various volatile compounds, especially those found in urine, have been documented much more so than are behavioral activities mediated by non-volatile pheromones. Indeed, to date only two non-volatile pheromones, ESP1 [27, 58] and MHC class I peptides [59] have been identified with the latter being the only one shown to mediate a specific behavior activity (pregnancy block – “Bruce Effect”). It is reasonable to suggest that non-volatiles constitute a significant portion of the pheromonally active components found in saliva or extracts from salivary glands. Identification of these proteins would add greatly to our knowledge and understanding of pheromonal activity mediated by the VNO organ. Furthermore, our results suggest that male saliva may exert sexually-dimorphic effects upon female mice since the constituents of one of the salivary glands, the parotid, stimulates isolated VNO preparations and affects aggression in a sex-related manner. Therefore, further work is required to identify the chemosignals which are found in submaxillary gland, parotid gland, and saliva which stimulate aggression and which

may mediate other social intra-species interactions between mice or other mammalian species.

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Figure 1.

Salivary fluids and urine from CD-1 normal male mice stimulate the production of IP₃ in CD-1 male and female vomeronasal organs (VNOs). Reactions were performed without stimulus (phosphate buffered saline, PBS), or in the presence of 10% v/v of male urine, saliva, submaxillary gland (SMG) extract, parotid gland (PG) extract, or sublingual gland (SLG) extract. **NorM-Norm** represents normal CD-1 male mice VNO exposed to normal CD-1 male mice fluids. **NorM-Cas** represents normal CD-1 male mice VNO exposed to castrated CD-1 male mice fluids. **NonLac-Norm** represents non-lactating female CD-1 mice VNO exposed to normal CD-1 male fluids. * indicates that significant differences exist between an individual group and basal (PBS) responses such that $p < 0.05$. ! indicates that significant differences exist between a normal male group response to individual normal versus castrated CD-1 male fluids such that $p < 0.05$. The data points and error bars represent the averages of at least nine independent experiments.

Latency to Sniff

Figure 2.

CD-1 male and lactating female mice are differentially and highly responsive to male urine, saliva, and male salivary glandular extracts. Resident normal male mice or resident lactating female mice were exposed to a dummy mouse swabbed with PBS, male urine, saliva, SMG, PG, or SLG extracts for five (5) minutes. **Lac-Norm** represents lactating CD-1 female mice exposed to normal CD-1 male fluids. **Lac-Cas** represents lactating CD-1 female mice exposed to castrated CD-1 male fluids. **NorM-Norm** and **NorM-Cas** and acronyms for male fluids are the same as defined in Figure 1. Designations of statistically significant differences are the same as in Figure 1. The data points and error bars represent the averages of five or more independent experiments.

Figure 3.

Normal CD-1 male and lactating female mice rapidly responded to male urine, saliva, or glandular extracts from male salivary glands. Figure legend labels and acronyms for male fluids are the same as defined in previous figures. Designations of statistically significant differences are the same as in Figure 1. The data points and error bars represent the averages of five or more independent experiments.

Figure 4.

Both CD-1 male and lactating female mice showed increased, but differential aggressive behavior when exposed for five (5) minutes to dummy mouse swabbed with male urine, saliva or extracts from male salivary glands. Figure legend labels and acronyms for male fluids are the same as defined in previous figures. Designations of statistically significant differences are the same as in Figure 1. The data points and error bars represent the averages of five or more independent experiments.

Figure 5.

Normal CD-1 male and lactating female mice demonstrated longer durations of attacks, relative to vehicle control (PBS), with dummy mice swabbed with male urine, saliva, or glandular extracts including male SMG (males and lactating females) or PG (male only). Figure legend labels and acronyms for male fluids are the same as defined in previous figures. Designations of statistically significant differences are the same as in Figure 1. The data points and error bars represent the averages of five or more independent experiments.

Figure 6.

CD-1 non-lactating and ovariectomized (OVX) female mice demonstrated significantly longer latencies to attack compared to lactating female mice in response to normal CD-1 male fluids. Resident female mice were exposed to a dummy mouse swabbed with PBS, urine, saliva, or with extracts from SMG, PG, or SLG for five (5) minutes; OVX mice were exposed only to urine or saliva but not to salivary gland extracts. * indicates that significant differences exist, with $p < 0.05$, between lactating or OVX versus non-lactating female mice responses to the indicated male fluid. ! indicates that significant differences exist, with $p < 0.05$, between OVX versus lactating female mice responses to normal male saliva or urine, respectively. Although lactating female responses to castrated male fluids are included in Figures 6–8, statistical differences are not indicated since they have already been shown in earlier figures. **NonLac-Norm** represents non-lactating female mice exposed to normal CD-1 male fluids. **OVX-Norm** represents ovariectomized CD-1 female mice exposed to normal CD-1 male fluids. Other figure legend labels and the acronyms for male fluids are the same as defined in previous figures. The data points and error bars represent the averages of five (5) or more independent experiments.

Figure 7.

CD-1 non-lactating and OVX female mice demonstrated significantly fewer numbers of attacks compared to lactating female mice in response to normal CD-1 male fluids. Resident female mice were exposed to a dummy mouse swabbed with PBS, urine, saliva, or extracts from SMG, PG or SLG glands for five (5) minutes; OVX mice were exposed only to urine and saliva but not to salivary gland extracts. Figure legend labels and the acronyms for male fluids are the same as defined in previous figures; designations of statistically significant differences are the same as in Figure 6. The data points and error bars represent the averages of five (5) or more independent experiments.

Figure 8.

CD-1 non-lactating and ovariectomized female mice demonstrated significantly shorter duration of attacks compared to lactating female mice in response to a five (5) minute exposure to a dummy mouse swabbed with normal CD-1 male fluids. Figure legend labels and the acronyms for male fluids are the same as defined in previous figures; designations of statistically significant differences are the same as in Figure 6. The data points and error bars represent the averages of five (5) or more independent experiments.