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Firing Properties of Accessory Olfactory Bulb Mitral/Tufted Cells in Response to Urine Delivered to the Vomeronasal Organ of Gray Short-Tailed Opossums

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Abstract

In comparison with many mammals, there is limited knowledge of the role of pheromones in conspecific communication in the gray short-tailed opossum. Here we report that mitral/tufted (M/T) cells of the accessory olfactory bulb (AOB) of male opossums responded to female urine but not to male urine with two distinct patterns: excitation followed by inhibition or inhibition. Either pattern could be mimicked by application of guanosine 5′-*O*-3-thiotriphosphate and blocked by guanosine 5′-*O*-2-thiodiphosphate, indicating that the response of neurons in this pathway is through a G-protein–coupled receptor mechanism. In addition, the inhibitor of phospholipase C (PLC), U73122, significantly blocked urine-induced responses. Male and female urine were ineffective as stimuli for M/T cells in the AOB of female opossums. These results indicate that urine of diestrous females contains a pheromone that directly stimulates vomeronasal neurons through activation of PLC by G-protein–coupled receptor mechanisms and that the response to urine is sexually dimorphic.

Key words: accessory olfactory bulb, opossum, urine, vomeronasal organ

Introduction

Pheromonal information, which elicits innate reproductive and social behaviors within a species, is detected by the sensory neurons in the olfactory and vomeronasal (VN) epithelia (for review, see Halpern and Martinez-Marcos 2003). In the VN system, that information is transmitted to the accessory olfactory bulb (AOB) (Halpern 1987; Kaba and Nakanishi 1995). For several species, pheromones have been shown to be present in urine (Krieger et al. 1999; Leinders-Zufall et al. 2000; Hurst et al. 2001). In vitro, dilute urine can activate VN sensory neurons (Inamura et al. 1999; Holy et al. 2000). In a previous study, urinary pheromones delivered to the female pig vomeronasal organ (VNO) stimulated VN receptor neurons via a receptor-mediated, G-protein–dependent increase in inositol 1,4,5-trisphosphate (IP_3) levels (Wekesa and Anholt 1997). G-proteins are specialized for a signal transduction function, namely, activation of phospholipase C (PLC) (Taylor et al. 1991) and have been implicated in VN signal transduction in a number of species (see Halpern and Martinez-Marcos 2003).

Recent behavioral evidence indicates that female gray short-tailed opossums do not respond to male opossum urine, whereas male opossums vigorously investigate urine of diestrous females (Zuri et al. 2003, 2005). However, response properties of neurons in the gray short-tailed opossums' accessory olfactory system to urine and their molecular mechanisms are currently unknown. Here, using extracellular single-unit recordings of mitral/tufted (M/T) cells of the AOB of male and female opossums, we directly measured responses of M/T cells to urine delivered to the VNO.

Materials and methods

Preparation

Gray short-tailed opossums (200–500 g, obtained from the Southwest Foundation for Biological Research, San Antonio, TX, or raised from birth in the animal care facility at the Downstate Medical Center) were housed individually in standard plastic cages with wood shavings and provided with a cylindrical plastic box for nesting. Dry fox food (Milk Specialties Co., New Holstein, WI) and water were given ad libitum. Fruits were added to the opossum diet twice a week. Lights were maintained on a reversed 12:12 h light:dark cycle. Because female opossums are induced ovulators, females

were placed in cages at least 3 m away from the nearest male to prevent them from entering estrus. We therefore assume that the urine used as stimuli in these experiments was from diestrous females. Animals were anesthetized with urethane (1.5 g/kg, intraperitoneally) and placed in a stereotaxic apparatus. Animals were freely breathing. For urine and chemical perfusion of the VNO, under a dissecting M900 microsurgery microscope (Seiler Instruments Model MC-M900, St Louis MO), the dorsal aspect of the bone above the VNO, VN vein, and associated nonsensory epithelium were removed using a dental drill and fine forceps. To record from M/T cells of the AOB, the bone and dura overlying the AOB and lateral olfactory tract (LOT) were removed.

Stimulation and recording

Single-unit activity of M/T cells was recorded extracellularly with glass microelectrodes filled with 3 M sodium chloride (5–12 MΩ). Under a dissecting microsurgery microscope (see above), a recording electrode was inserted at an angle of 50° subtended to the coronal plane passing vertically through the ear bars so as to be oriented in a direction perpendicular to the laminae of the AOB (Figure 1).

More apical than basal VNO neurons are activated by pheromones (Campenhausen and Mori 2000). Neurons in the apical zone of the VNO project their axons to the anterior AOB (Jia and Halpern 1996). In these experiments, single-unit recordings were made from the anterior AOB. For the recordings, the coordinates of the anterior part the AOB were lateral 500–1200 μm from the midline and 1–300 μm rostral to the anterior edge of the sulcal vein that runs over the AOB and from 200–800 μm below the surface depending on the location of the recording. To ascertain that the recording microelectrode was in the mitral cell layer of the AOB, we monitored the configuration of LOT-evoked responses. Stimulation of the LOT, through which axons of the AOB output neurons travel, results in a sharp spike following a negative field potential in the mitral cell layer (Rall and Shepherd 1968; Murakami et al. 2005). We assumed that when such a profile was obtained, we were recording from a M/T cell (Figure 1).

Recordings were made using an electrometer amplifier (Intra 767, World Precision Instruments, Sarasota, FL). The signal unit was band-pass filtered at 300 Hz–5 kHz (MDA-2, BAK Electronics, Germantown, MD) and passed through a window discriminator (DIS-1, BAK Electronics). Data were acquired with Digidata 1200A and recorded on a PC computer with pClamp software 8 (Axon Instruments, Inc., Molecular Devices Corporation, Sunnyvale, CA).

During the experiments, the VNO was perfused constantly with Ringer's solution at a rate of 1.5 ml/min using a peristaltic pump (Peristaltic P-3, Pharmacia Fine Chemicals, Sweden) attached to capillary tubing with a 200-μm tip diameter placed at the anterior end of the VNO. Another tube was placed at the posterior end of the VNO to provide

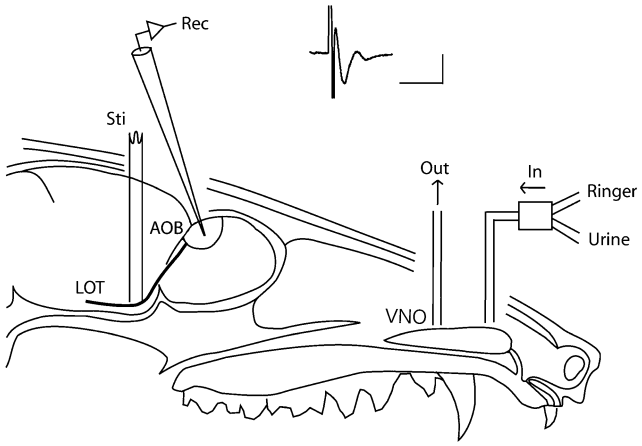

Figure 1 Schematic representation of the experimental setup. Rec, recording electrode; Sti, stimulating electrode. Top trace is an example of a field potential in the AOB resulting from antidromic stimulation of the LOT. Calibration: 5 ms, 0.2 V.

negative pressure, that is, suction (Figure 1). Urine was diluted 10-fold with Ringer's solution to provide the stimulating solution. Infusion of Ringer's solution was stopped during urine and drug infusions into the VNO. Dilute urine and drugs were infused for 2 s (50 μl) using a homemade injection system.

Urine was collected from female and male gray short-tailed opossums by placing each donor opossum in a restriction plastic bag and pressing its abdomen to get a few drops of urine. Urine samples of 2 ml were centrifuged for 5 min at 3000 rpm to discard occasional cell debris and then dialyzed overnight in a cellulose tubular membrane (pore range 6000–8000 da; Membrane Filtration Products, Inc., Seguin, TX) against 1 l of Ringer's solution at 4 °C overnight. Urine dilutions of 10-fold were prepared daily in Ringer's solution, which served as the control vehicle in all conditions.

All compounds and chemicals were obtained from Sigma-Aldrich (St Louis, MO), except for U73122, which was purchased from BioMol, Plymouth Meeting, PA.

Data analysis

The activity of the single units 10 s before stimulus delivery (control period) and 10 s after stimulus perfusion were analyzed. The firing rates were determined using programs in the DigiData 1200A using pClamp software 8. Single units were analyzed using analysis of variance followed by Scheffé *F*-test for comparison with controls, using the statistical package Statview (SAS Institute, Cary, NC).

Results

Eleven male and five female adult gray short-tailed opossums were used for single-unit recording. Typically, firing frequency of M/T cells in anesthetized opossum was 1–4 Hz. Sixteen of 23 M/T cells of male opossums responded to female urine but not to male urine. Two distinct firing patterns

were observed. Seven of 23 male M/T cells responded to female urine with increased firing frequency followed by a period of inhibition (Figure 2A). Nine M/T cells decreased the rate of action potential firing in response to dilute female urine (Figure 2B). In the remaining cells, the frequency of firing was unchanged by urine stimulation.

For cells displaying an increase in firing frequency, the increase lasted for 9.3 ± 1.3 s followed by an inhibitory period that persisted for 19.0 ± 3.8 s. The average latency of response to urine stimulation of the VNO was 1.3 ± 0.2 s. To summarize the multiunit response recorded from individual cells that showed an increased firing rate following stimulation with female urine, we calculated the average change in rate between two time intervals (the 10 s before and the 10 s after stimulation). The mean firing rate increased significantly compared with control (Ringer's solution) stimulation ($P < 0.005$) (Figure 2C). For neurons exhibiting a decrease in firing after urine stimulation, inhibition began 1.9 ± 0.3 s after stimulation and action potential firing was almost completely abolished for 16.5 ± 1.7 s (Figure 2B). The mean firing decrease was significant compared with control (Ringer's solution) stimulation ($P < 0.005$) (Figure 2D).

To study the transduction pathways activated by urine, we delivered G-protein guanosine 5′-*O*-3-thiotriphosphate (GTPγS) and the inhibitor guanosine 5′-*O*-2-thiodiphosphate (GDP-β-S) to the VNO. Urine-induced responses were mimicked by 100 μM GTPγS and blocked by 100 μM GDP-β-S (Figure 3). GTPγS of 100 μM significantly ($P < 0.005$)

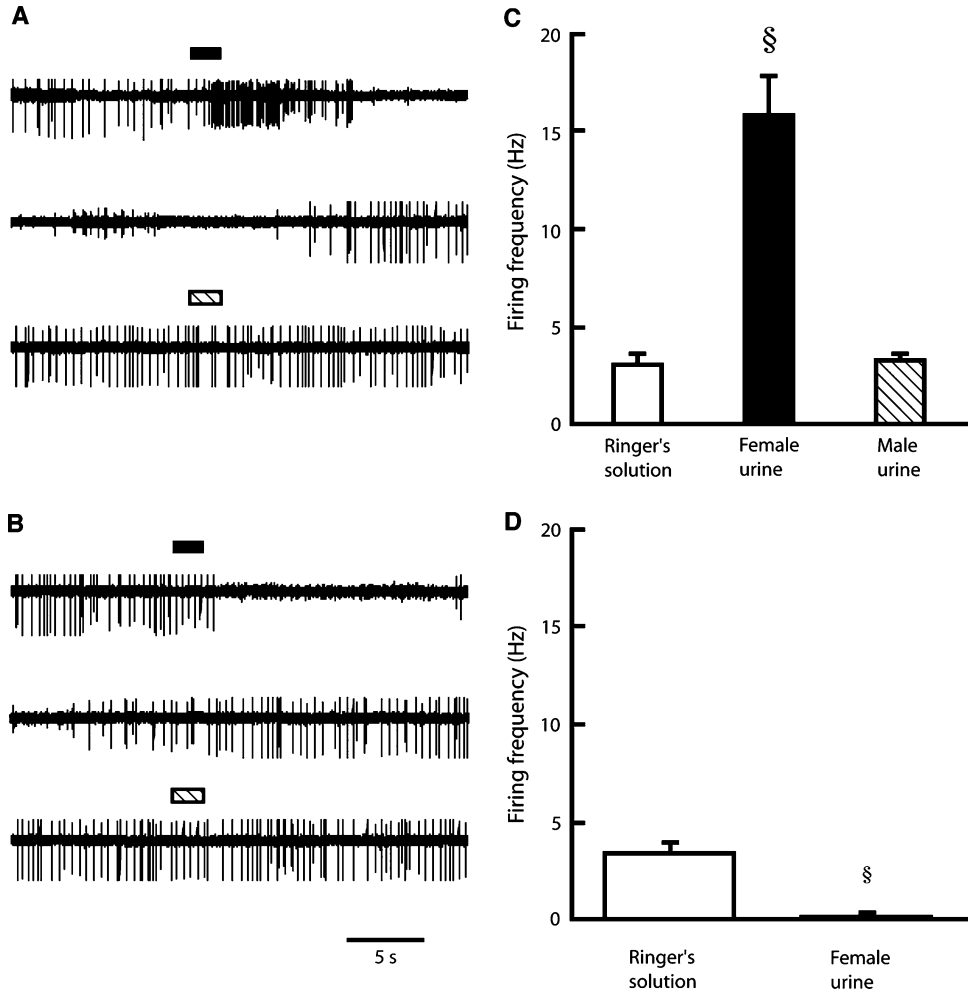

Figure 2 Female urine produces two types of responses in M/T cells of male opossums: excitation followed by inhibition (**A**, top trace) or inhibition (**B**, top trace). **(A)** and **(B)** Bath application of 10-fold dilution of female urine (black bars) or male urine (hatched bars) for 2 s. In cell (**A**), control (Ringer's solution) firing rate was 1–3 Hz; after female urine (50 μl) application firing rate was 9–18 Hz. In cell (**B**), control firing rate was 2–4 Hz; after female urine firing rate was 0–0.5 Hz. Male urine (50 μl) produced no change in firing rate. **(C)** Comparison of effect of 10-fold dilution of female and male urine on firing frequency of M/T cells of male opossums. For seven neurons that increased their firing rate, the average frequency of firing was increased significantly after the application of female urine (control, 3.1 ± 0.5 Hz; after female urine, 15.8 ± 1.9 Hz, $n = 7$), §$P < 0.005$ but not after application of male urine (3.3 ± 0.4 Hz, $n = 7$). **(D)** For nine neurons from male opossums that decreased their firing rate, the average frequency of firing was significantly decreased after the application of 10-fold dilution of female urine (control, 3.4 ± 0.6 Hz; after female urine, 0.2 ± 0.1 Hz, §$P < 0.005$, $n = 9$).

increased rate of action potential firing compared with control (Ringer's solution). We also observed that urine-induced responses were blocked with a highly selective, cell-permeable inhibitor of PLC (U73122, 50 μM) (Figure 3). Delivery of female urine with 100 μM GDP-β-S or U73122 (Figure 3) resulted in significantly ($P < 0.01$) reduced responses compared with female urine alone. Based on these findings, we suggest that male VNO neurons probably respond to stimuli in female urine via activation of PLC by G-proteins, resulting in either increases or decreases in M/T cell action potentials.

Under the same conditions as described above, we recorded action potential firing from six M/T cells from five female opossums. We found neither an increase nor a decrease in firing frequency of M/T cells to male or female urine, although all six cells responded to GTPγS (Figure 4). The GTPγS-induced excitation of female M/T cells was significantly different from control (Ringer's solution) ($P < 0.005$). No M/T cells responded to stimulation with Ringer's solution.

Discussion

The VNO plays a key role in detecting pheromones that regulate sexual behavior (for reviews, see Halpern 1987; Halpern and Martinez-Marcos 2003). By recording the activity of M/T cells of the AOB in anesthetized opossums during urine delivery to the VNO, we found that the male opossum VNO is stimulated by female opossum, but not male opossum, urine and transmits that excitation to M/T cells in the AOB. Unlike the male opossum VNO, the female opossum VNO does not

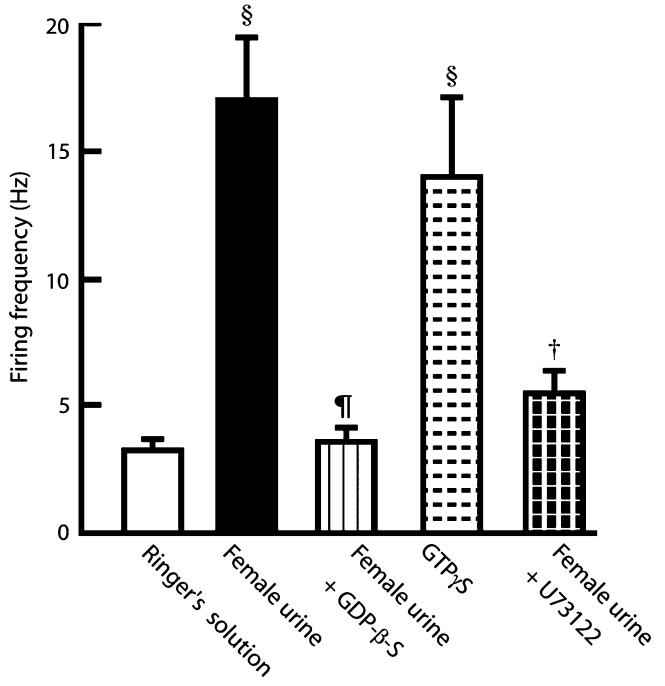

Figure 3 The role of GDP-β-S and U73122 on the excitatory effects of 10-fold dilution of female urine on the firing rate of five neurons in the male opossum (control, 3.2 ± 0.4 Hz; after urine, 17.0 ± 2.4 Hz, §$P < 0.005$ between Ringer solution and urine group; after urine in the presence of GDP-β-S and U73122, 3.6 ± 0.6 Hz and 5.3 ± 0.8 Hz, respectively, $n = 5$, $P < 0.01$ urine versus and ¶GDP-β-S and †U73122 group). The effect of female urine was mimicked by GTPγS (after GTPγS, 14.0 ± 3.0 Hz, $n = 5$, §$P < 0.005$ between Ringers' solution and GTPγS).

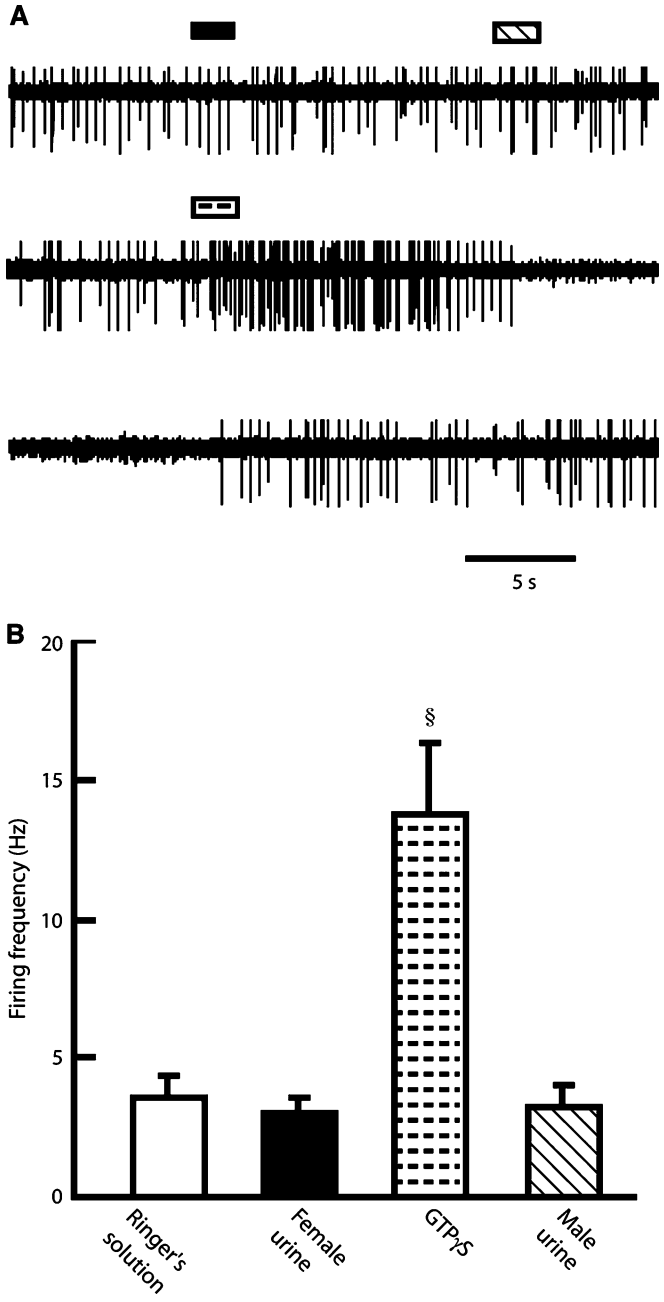

Figure 4 **(A)** Unit responses of an M/T cell from a female opossum to delivery of female urine (black rectangle), male urine (hatched rectangle), or GTPγS (dashed rectangle). Note that this cell responded to GTPγS with an initial increase in firing rate followed by an inhibitory interval. **(B)** No M/T cell recorded from a female opossum responded to either male or female urine. Increased responses were observed to delivery of GTPγS, from 3.7 ± 0.8 Hz to 13.7 Hz ± 2.5 Hz, §$P < 0.005$ between Ringer's solution and GTPγS group ($n = 6$).

appear to respond to either male or female urine. These electrophysiological results support behavioral observations that male opossums respond with vigorous investigation to the urine of diestrous females but that female opossums do not respond to male urine (Zuri et al. 2003, 2005).

Application of female urine to the VNO led to changes in M/T cell action potential firing with increases followed by decreases or decreased responses. Our results are similar to the results by Luo et al. (2003) in their recording of excitatory and inhibitory responses from the AOB of behaving mice during natural investigative behaviors. Inhibition was also frequently observed immediately following strong excitatory responses. Although it is uncertain whether urine can directly produce inhibitory responses, the results of increases followed by decreases are consistent with the hypothesis that when dendrites of M/T cells are activated by VN nerve inputs, they depolarize adjacent granule cell dendrites by releasing the neurotransmitter glutamate at excitatory synapses. Depolarized dendrites of granule cells in turn release the inhibitory neurotransmitter γ-aminobutyric acid (GABA) at inhibitory synapses and hyperpolarize the M/T cell dendrites (Brennan 2001). Hence we cannot exclude the possibility that we recorded some M/T cells that did not receive direct input from VNO receptor cells activated by urine and only received inhibition from activated granule cells that induced the inhibitory responses. Araneda and Firestein (2006) reported that noradrenaline decreases the frequency of firing of M/T cells in cell-attached patches and that dopamine controls the GABAergic inhibitory processing of odor signals (Brünig et al. 1999). It is possible that urine-induced inhibitory responses of M/T cells are mediated by centrifugal inputs such as noradrenaline and dopamine.

In the VNO, G-protein–coupled receptors, located on the microvillar surface of receptor cells (Wekesa and Anholt 1997), participate in axonal targeting, sensory function, and possibly cell survival (Mombaerts et al. 1996; Zhao et al. 1998). Previous studies demonstrated that the PLC system is a critical pathway for VNO neuron detection of pheromones (Holy et al. 2000). In the present study, we looked at the effects of G-protein and PLC inhibitors in the signal transduction pathway. Our single-unit recordings showed that urine stimulation was mimicked by GTPγS and blocked by GDP-β-S. Furthermore, use of a membrane-permeable inhibitor of PLC, U73122, significantly blocked the urine-induced responses. Our results are consistent with findings that VNO responses to components of urine are blocked by pharmacological inhibitors of PLC in mice (Inamura et al. 1997; Holy et al. 2000). It is possible that opossum urinary pheromonal transduction may act in a manner similar to that in mice. Many VNOs utilize PLC signaling (Runnenburger et al. 2002) to cleave phosphatidyl-inositol *bis*-phosphate into diacylglycerol (DAG) and IP_3 second messengers (Berridge and Irvine 1984). Therefore, it is possible that pheromonal signal transduction in the opossum VNO is via a G-protein–coupled mechanism that leads to stimulation of PLC, elevation of IP_3 and/or DAG, activation of TRP2 channels, and membrane depolarization.

The present results of an absence of urine-induced responses in female opossums are unlike those reported for activation of VN sensory neurons in female rats or female mice (Inamura et al. 1997; Holy et al. 2000). In 1997, using female golden hamsters, Petrulis and Johnston (1997) found that female golden hamsters did not investigate male urine more than clean vials. In addition, Zuri et al. (2003) reported that female gray short-tailed opossums responded to odor of male suprasternal gland but not conspecific urine. The present results support the idea that there may be differences in conspecific communication between the male and female opossums. Urinary pheromones do not appear to be potent chemosignals for female gray short-tailed opossum.

This study of *in vivo* single-unit recording has made two important observations: 1) male opossum VNO neurons detecting female urinary pheromones transmit this information to M/T cells in the AOB, and this response is sexually dimorphic and 2) urine-induced responses are mediated through stimulation of PLC by a G-protein–coupled receptor mechanism.

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