

# Female Snake Sex Pheromone Induces Membrane Responses in Vomeronasal Sensory Neurons of Male Snakes

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## Abstract

The vomeronasal organ (VNO) is important for activating accessory olfactory pathways that are involved in sexually dimorphic mating behavior. The VNO of male garter snakes is critically important for detection of, and response to, female sex pheromones. In the present study, under voltage-clamp conditions, male snake VNO neurons were stimulated with female sexual attractiveness pheromone. Thirty-nine of 139 neurons exhibited inward current responses (reversal potential:  $-10.6 \pm 2.8$  mV). The amplitude of the inward current was dose dependent, and the relationship could be fitted by the Hill equation. Under current-clamp conditions, application of pheromone produced membrane depolarizing responses and increases in firing frequency. These results suggest that the female pheromone directly affects male snake VNO neurons and results in opening of ion channels, thereby converting the pheromone signal to an electrical signal. The response to female pheromone is sexually dimorphic, that is, the pheromone does not evoke responses in VNO neurons of female snakes. An associated finding of the present study is that the female sex pheromone, which is insoluble in aqueous solutions, became soluble in the presence of Harderian gland homogenate.

**Key words:** patch clamp, pheromone, snake, VNO

## Introduction

The vomeronasal organ (VNO) of vertebrates is a chemoreceptive organ that has been implicated in the detection of, and response to, sex pheromones (Halpern, 1987; Wysocki and Meredith, 1987; Meredith, 1998; Keverne, 1999; Johnston, 2000; McClintock, 2002; Halpern and Martinez-Marcos, 2003). One of the few vertebrate pheromones that has been isolated, purified, and characterized is the sex pheromone of the red-sided garter snake (*Thamnophis sirtalis parietalis*) (Mason *et al.*, 1989, 1990). This pheromone, a mixture of 13 long-chain ( $C_{29}$ – $C_{37}$ ) saturated and monounsaturated methyl ketones, is expressed on the dorsal surface of adult female snakes during the mating season. When males encounter a female expressing the pheromone, they exhibit stereotyped courtship behaviors including chin rubbing, rapid tongue-flicks, and caudocephalic body undulations (Noble, 1937; F.M. Blanchard and F.C. Blanchard, 1941; Aleksuik and Gregory, 1974; Crews, 1976; Kubie *et al.*, 1978; Mason *et al.*, 1990). Male garter snakes deprived of a functional vomeronasal (VN) system are unable to detect or respond appropriately to this pheromone (Noble, 1937; Kubie

*et al.*, 1978; Halpern and Kubie, 1983). Although behavioral studies have established the critical involvement of the VN system in detection of this pheromone, the transduction mechanism by which the pheromone activates snake sensory neurons has yet to be elucidated. The present study was designed to examine the effects of the purified female snake sex pheromone on the membrane potential and firing properties of VNO sensory neurons. We used VNO neurons from male and female red-sided garter snakes, testing them under whole-cell voltage- and current-clamp protocols to identify neural responses to the purified pheromone.

Prior to conducting the electrophysiological study, we tested the effect of the female sex pheromone on the generation of  $IP_3$  in male garter snake VN sensory epithelium homogenates.  $IP_3$  is a known second messenger in the snake VN signal transduction pathway for prey chemicals (Luo *et al.*, 1994). The female sex pheromone is insoluble in aqueous solutions. Since solubility in the aqueous medium filling the VNO is required for odorant access to the VN sensory epithelium and the major source of fluid in the VNO is

derived from the Harderian gland (Rehorek, 1997; Rehorek *et al.*, 2000), the pheromone was solubilized in Harderian gland homogenate. Therefore, this paper describes the effects of female snake sex pheromone, solubilized in Harderian gland homogenate, on the membrane potential and firing properties of VN sensory neurons of male garter snakes.

## Methods

### Animals

Thirty-six male and four female red-sided garter snakes (*T. sirtalis parietalis*) were obtained from Manitoba, Canada, by R.T. Mason during the early portion of the mating season (early May) and transported to Brooklyn, NY, for the electrophysiological experiment. An additional 10 male snakes from the same source were used in a preliminary study to determine whether the pheromone would have the effect of increasing the production of IP<sub>3</sub>. The animals were maintained in a cool environment to prolong their sexually active period. Prior to electrophysiological experimentation, all males were tested for courtship behavior with females to ensure that they were still in mating condition. If males were not sexually active, they were placed in a cool refrigerator 4°C overnight to return them to mating condition.

### Preparation of garter snake VN sensory epithelial homogenate

A total 10 male animals (20 VN organs) were used for preparing VN homogenate. For each reaction, the amount of homogenate used was based on protein concentration only. Experimental animals received an overdose of Brevital sodium (0.01 g/g body weight). The VN organs were isolated immediately after the snakes were killed. The sensory epithelium of each organ was carefully dissected on ice. The pooled sensory epithelia were suspended in 200 ml of binding buffer (20 mM Tris/HCl, pH 8.0, 1 mM ethyleneglycol-bis(aminoethylether)-tetraacetic acid (EGTA), 1 mM phenylmethylsulphonylfluoride, 0.5 mg/ml antipain, 1 mg/ml leupeptin, 1 mg/ml aprotinin, 0.6 mg/ml chymostatin, and 0.6 mg/ml pepstatin) and homogenized with a 5-ml glass Dounce homogenizer. The homogenized material was centrifuged at 4°C for 5 min at 500 × *g* to remove the debris. The supernatant is referred to as “VN sensory epithelial homogenate.”

### Estimation of IP<sub>3</sub>

VN sensory epithelial homogenate (12 mg of protein) was incubated with snake pheromone (5 ml) in Harderian homogenate (124 mg protein) in a reaction solution (50 ml) of 25 mM Tris acetate, pH 7.5, 5 mM MgAc<sub>2</sub>, 0.5 mM adenosine triphosphate (ATP), 1 mM dithiothreitol, 0.01 mM guanosine triphosphate (GTP), 0.1 mM CaCl<sub>2</sub>, and 0.1 mg/ml bovine serum albumin. Harderian homogenate alone served as the control. The incubation was carried out for 2 min at 37°C. The reaction was stopped by adding 10 ml

of cold 10% HClO<sub>4</sub>, the reaction vials were placed on ice for 30 min, and then centrifuged at 20,000 rpm for 5 min at 4°C. The supernatants were transferred into new vials. To each vial, 16 ml of 1.5 M KOH in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer was added and placed on ice for 30 min and centrifuged at 20,000 rpm for 5 min at 4°C. The IP<sub>3</sub> content in the samples was estimated according to the manufacturer's instruction (IP<sub>3</sub> [<sup>3</sup>H] Biotrak Assay System; Amersham Biosciences, Piscataway, NJ). Three separate experiments were performed, each in duplicate.

### Protein determination

The concentration of protein was estimated using the Micro BCA protein assay reagent (Pierce, Rockford, IL).

### Preparation of female pheromone

Adult, sexually attractive female red-sided garter snakes of varying size (snout-vent length range: 50.0–78.0 cm; *N* = 36) were collected immediately upon emergence from communal den sites near Inwood, Manitoba, Canada from 5 May to 12 May 2005. The animals were killed with an overdose of Brevital sodium. Each snake was placed dorsal side down in a 500-ml glass beaker, covered with 25–50 ml of 100% hexane (C<sub>6</sub>H<sub>12</sub>) for 12 h. After removal of the animals, the excess solvent was removed under reduced pressure by rotoevaporation at 35°C. The resulting residues were resuspended in fresh hexane (1–2 ml) and sealed in 9-ml glass vials with polyethylene-lined caps for storage.

To isolate the methyl ketones composing the sexual attractiveness pheromone, we fractionated the skin lipid extracts using column chromatography (Mason *et al.*, 1989, 1990). Briefly, we loaded the skin lipid extracts onto glass columns (350-mm long × 22-mm internal diameter) packed with alumina (Activity grade III) and eluted the columns with hexane and ethyl ether (C<sub>4</sub>H<sub>10</sub>O) solutions of increasing polarity. For each sample, the fraction containing the appropriate methyl ketones (fraction 5; 98% hexane:2% ethyl ether) was collected and the excess solvent removed by rotoevaporation (35°C). The resulting methyl ketone residues were weighed on a digital scale (Mettler AT400) and resuspended in 1 ml fresh hexane. Each female yielded an average of 1.4 ± 1.1 mg of methyl ketones which is approximately 6.1 ± 3.4% of the total skin lipids collected from the female snakes. This pooled solution was subsequently used in the experiments as the stock pheromone solution.

### Preparation of Harderian gland homogenate

Harderian glands were isolated after the snakes were killed with an overdose of Brevital sodium and homogenized in binding buffer with a 5-ml Dounce glass homogenizer. The homogenized material was centrifuged at 500 × *g* for 5 min at 4°C. The supernatant is referred to as “Harderian gland homogenate.”

#### *Preparation of pheromone–Harderian gland homogenate mixture*

Pheromone, collected from female garter snake skin lipids, was dissolved in hexane. Pheromone of 2.5  $\mu$ l was mixed with 6  $\mu$ l of 5% NP-40 and 10  $\mu$ l Harderian homogenate (protein concentration = 33  $\mu$ g/ $\mu$ l), vortexed, and exposed briefly to helium gas stream before use to evaporate the hexane.

#### *Preparation of pheromone–Harderian gland homogenate control*

The control solution consisted of 100 ml Harderian gland homogenate, 6  $\mu$ l NP-40, and 10  $\mu$ l hexane. This mixture was vortexed and exposed to helium gas stream briefly before adding required aliquot to the reaction vial.

#### *Preparation of earthworm shock secretion*

Electric shock–induced earthworm secretion (ESS) was prepared as described elsewhere (Jiang *et al.*, 1990). Briefly, earthworms were rinsed in distilled H<sub>2</sub>O to remove adherent soil, dried, placed in an electric stimulator, and shocked with an electric current from a 9-V battery (applied as 20  $\times$  6 s bursts with 30 s intershock intervals). In response to this shock regime, earthworms secreted a mucus-like fluid that drained into a collection beaker. This secretion, known to contain chemoattractants for garter snakes (Halpern *et al.*, 1987; Jiang *et al.*, 1990), was diluted with Ringer's solution and used as a positive control stimulus in the experiments on female snakes described herein.

#### *Slice preparation*

Slices of VNO were prepared from garter snakes as described previously (Taniguchi *et al.*, 2000). Briefly, the animals were immobilized by cooling on ice for 30–40 min. The VN epithelium was dissected from decapitated snakes and rapidly moved into 0–4°C Ringer's solution containing (in mM): 119 NaCl, 4.1 KCl, 2.5 CaCl<sub>2</sub>, 1.5 MgCl<sub>2</sub>, 15 glucose, 5 Na-pyruvate, 10 HEPES–NaOH, pH 7.4. The VN epithelium was fixed to a carrot block with glue and cut into 150- to 200- $\mu$ m thick slices with a vibrating slicer (Vibratome 3000, Technical Products International Inc., St Louis, MO) in 0–4°C Ringer's solution. Cut slices were kept in 4°C Ringer's solution until use. The preparations were viewed with an up-right microscope (Optiphot UD-2, Nikon, Tokyo, Japan) using a 40 $\times$  water immersion lens (Carl Zeiss, Jena, Germany). The slice was placed in a glass-bottomed chamber and fixed in place with a grid of parallel nylon threads supported by a U-shaped silver wire weight. During the experiment, the slice in the recording chamber was perfused constantly with Ringer's solution at a rate of 1–1.5 ml/min using a peristaltic pump (Peristaltic P-3, Pharmacia Fine Chemicals, Sweden).

#### *Whole-cell recording and analysis*

Conventional whole-cell patch-clamp recordings were made on bipolar neurons using Axopatch 200B (Axon Instru-

ments, Foster City, CA). Data were acquired through a DigiData 1322A interface onto a personal computer using pClamp software 9.2 (Axon Instruments). The signal was low-pass filtered at 2 kHz and sampled at 5 kHz. Patch pipettes were pulled from borosilicate glass (1B150F-4; World Precision Instruments, Sarasota, FL) with a P-97 horizontal puller (Sutter Instrument, Novato, CA) and fire polished using a microforge (Narishige MF83, Tokyo, Japan). The electrodes had tip resistances ranging from 4 to 6 M $\Omega$  when filled with internal solution containing (in mM): 132.1 KCl, 0.1 CaCl<sub>2</sub>, 3.6 MgCl<sub>2</sub>, 1 EGTA, 10 HEPES–KOH, 2.5 Na<sub>2</sub>-ATP, pH 7.6. The series resistance was monitored every 20 s with hyperpolarizing voltage steps from a holding potential of –70 mV. Recordings with series resistance in the range of 12–20 M $\Omega$  were included in the analysis. All recordings were performed at room temperature (22–27°C). Off-line analysis was performed using Clampfit 9.2 (Axon Instruments). We estimated the magnitude of inward currents from just before the response to the peak. Liquid junction potentials were measured with a microelectrode containing 3 M KCl (Neher, 1992). All data in this report have been corrected for junction potentials. All values are reported as mean  $\pm$  SE. Statistical comparisons were determined using Student's *t*-test. Curve fitting was performed using Igor Pro 4.08J (WaveMetrics, Inc., Portland, OR).

#### *Chemostimulation*

A four-barrel quartz micromanifold (ALA Scientific Instruments, Westbury, NY) was used to apply substances at 4–5  $\mu$ l/s using a homemade injection system. The tip (200- $\mu$ m diameter) of the manifold was placed  $\sim$ 2 mm from the epithelium microvilli. Using sky blue dye, we determined that fluid from the micropipette tip reached the epithelial surface in less than 1 s. Infusion of 20–40  $\mu$ l was completed in 4–8 s.

## **Results**

As expected, due to the high molecular weight and the aliphatic chain length (C<sub>29</sub>–C<sub>37</sub>), we found that the nonpolar female sex pheromone was not soluble in aqueous solvent, even with small amounts (less than 0.3%) of detergents added, for example, NP40, Tween-20, and Tween-80. We tried to partition the nonpolar pheromone into more polar organic solvents by using a mixture of pheromone (in hexane) and ethanol, acetone, or dimethylsulfoxide, but none of these mixtures were successful in dissolving the pheromone in aqueous reaction buffer. The amount of detergent required to solubilize the pheromone would have been deleterious to the integrity of the VN sensory epithelium and would have rendered the tissue unusable. As indicated above, since Harderian gland secretions fill the VNO, and it is generally understood that secretions in the oral cavity coat the snake's tongue, it was reasonable to suppose that Harderian gland secretions might normally act to facilitate transfer of female sex pheromone to VNO receptor cells under normal

conditions. We found that Harderian gland homogenate added to female pheromone with a small amount of detergent did, indeed, solubilize the pheromone. Since NP-40 has been widely utilized in other studies (e.g., Chu *et al.*, 1992), we used this detergent.

We initially demonstrated that garter snake sexual attractiveness pheromone activated neurons in the VN sensory epithelium by incubating the pheromone and Harderian gland homogenate with VN sensory epithelium homogenate and assaying for IP<sub>3</sub> production. As indicated in Table 1, female snake pheromone increased IP<sub>3</sub> generation by more than 200%.

To clearly visualize bipolar neurons and their dendrites in the slice preparation, dead cells were removed using cleaning pipettes (8- to 10-μm diameter) filled with Ringer's solution (Edwards *et al.*, 1989). Whole-cell currents were recorded from 139 receptor neurons of male VNOs. The resting membrane potential and the input resistance measured 30–60 s after rupture of the patch membrane were  $-67.2 \pm 7.3$  mV ( $n = 98$ ) and  $3.7 \pm 0.6$  GΩ ( $n = 98$ ), respectively. Nine cells exhibited spontaneous spike discharges in the absence of external stimulation. The resting membrane potential of these spontaneously active cells was measured when the cells were, on occasion, silent and was recorded at  $45.6 \pm 8.9$  mV ( $n = 5$ ).

Application of female pheromone with Harderian gland homogenate for 4–8 s induced an inward current at a holding potential of  $-70$  mV in 39 of 139 cells tested. To exclude the effect of Harderian gland on pheromone-induced current, we applied Harderian gland homogenate before application of female pheromone. Harderian gland homogenate did not produce an inward current, although these cells did respond to the pheromone ( $n = 7$ ). Increasing the amount of pheromone caused an increase in the observed currents (Figure 1A). The pheromone-induced current was activated with a latency of  $5.7 \pm 3.3$  s and slowly returned to control levels within  $51.2 \pm 9.1$  s. The amplitude of the pheromone-induced current was dose dependent from 10 to 40 μl (Figure 1B). The dose-response pheromone-induced inward currents were fit to the Hill equation, giving a Hill coefficient of 3.21, suggesting that multiple transduction molecules, for example,

receptor proteins, are involved in the pheromone signal transduction pathway.

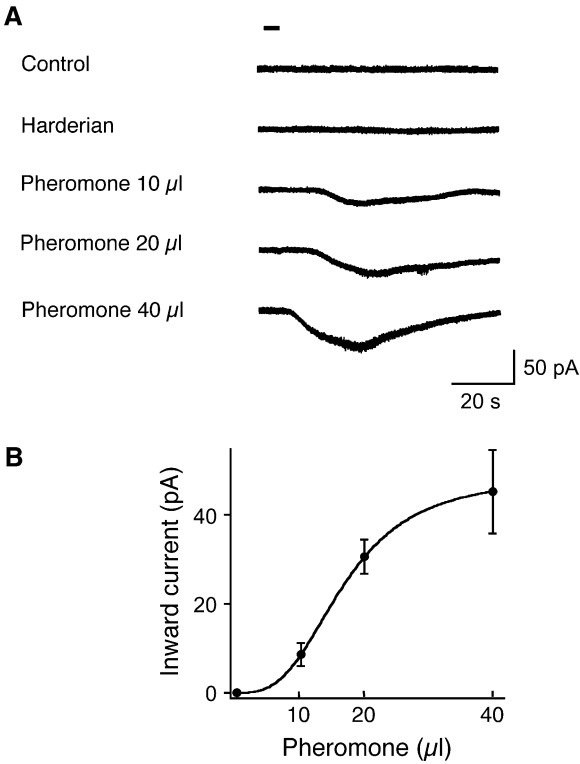
Under current-clamp conditions, of the 20 cells tested, four cells responded to pheromone stimulation with membrane depolarization. The membrane depolarization in response to pheromone was significantly different from that observed to control ( $57.0 \pm 3.4$  mV vs.  $69.3 \pm 1.4$  mV,  $t = 3.4$ ;  $P < 0.05$ ,  $n = 4$ ). Pheromone alone did not significantly increase action potential firing. During injection of a 2-pA current step to the same cell, pheromone application increased the number of action potentials from  $2.0 \pm 0.4$  to  $3.7 \pm 0.3$  ( $t = 2.9$ ;  $P < 0.05$ ,  $n = 4$ ). Action potential changes before, during, and after application of pheromone are illustrated in Figure 2.

We evaluated the current peak voltage relationship for stimulation with female snake sex pheromone by varying the holding potential between  $-70$  and  $+70$  mV. In four of 21 cells recorded from 12 male snakes, the time course of the rising phase of the responses at different voltages did not change. In Figure 3, a typical reversal potential is illustrated. The mean reversal potential was  $-10.6 \pm 2.8$  mV

**Table 1** The effect of garter snake sexual attractiveness pheromone on the yield of IP<sub>3</sub> in VN sensory epithelial homogenate of male garter snake

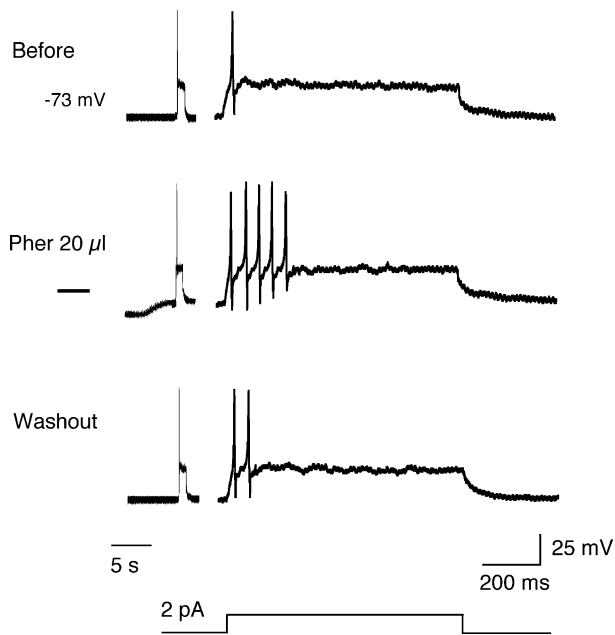
Treatment	Amount of IP <sub>3</sub> generated (pmol/mg protein)
Control (Harderian gland homogenate)	70.8 ± 0.9
Snake sex pheromone (and Harderian gland homogenate)	176.4 ± 12.7

VN homogenate (12 mg of protein) was incubated with snake pheromone in Harderian homogenate in a reaction solution (50 ml) of 25 mM Tris acetate, pH 7.5, 5 mM MgAc<sub>2</sub>, 0.5 mM ATP, 1 mM dithiothreitol, 0.01 mM GTP, 0.1 mM CaCl<sub>2</sub>, and 0.1 mg/ml bovine serum albumin. Harderian homogenate was used as a control. The incubation was carried out for 2 min at 37°C. The data are means of three sets of separate experiments and each treatment with duplicate samples.

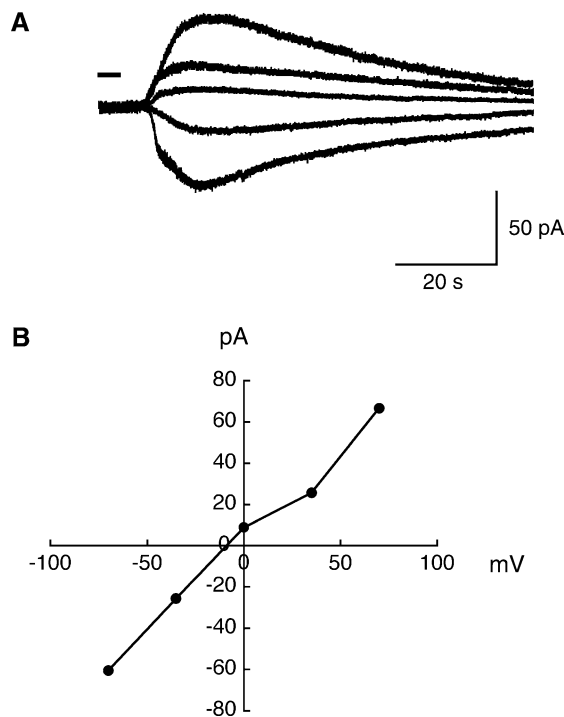


**Figure 1** Female sexual attractiveness pheromone evoked inward currents in male VNO neurons. **(A)** Representative currents induced by various concentrations of pheromone. The bar at the top indicates the timing of application. Pheromone was applied for 4–8 s at a holding potential of  $-70$  mV. Pheromone-induced current was not observed with buffer solution or Harderian gland homogenate without pheromone. **(B)** Dose-response curve for pheromone induced inward currents. Each point shows the mean  $\pm$  SE of responses of several different neurons ( $n = 4-7$ ). The smooth line was the best fit of the Hill equation,  $I = I_{\max} \times P^n / (P^n + K_{1/2}^n)$ , where  $I$  is the current,  $P$  is the volume of pheromone,  $I_{\max} = 45.8$  pA, and  $n = 3.21$ .





**Figure 2** Under current-clamp condition, application of pheromone produced a depolarizing membrane potential leading to increased number of action potentials in response to a current pulse of 2 pA.



**Figure 3** Currents induced by repeated application of pheromone (20  $\mu$ l) for 4 s at different holding potentials. **(A)** Thirty-five-millivolt steps were applied to cells held between  $-70$  and  $+70$  mV. **(B)** Current-voltage relationships of pheromone induced currents plotted from A at peak current. The reversal potential of the current induced was estimated to be  $-10.4$  mV.

( $n = 4$ ). The linear I–V plot indicates that the conductance was voltage dependent.

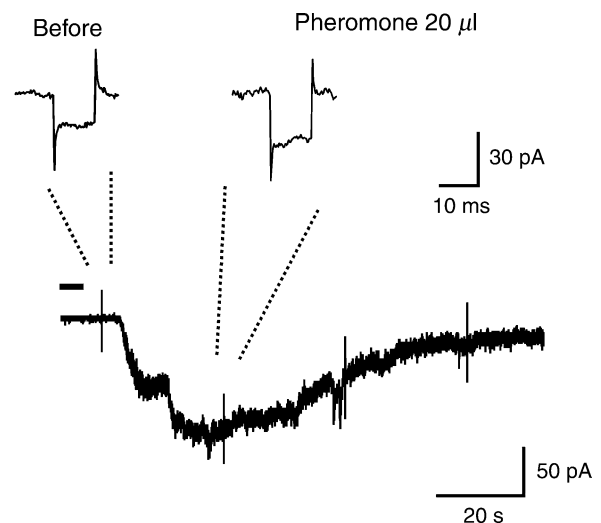
We examined changes in membrane conductance during application of female snake sex pheromone under voltage-clamp conditions by applying a series of 10 mV depolarizing voltage pulses (10 ms, 0.03 Hz) before and during the response to 20  $\mu$ l pheromone. In four of 15 cells, pheromone application increased the amplitude of voltage step-induced response from  $16.5 \pm 3.2$  to  $28.7 \pm 2.8$  pA, and conductances were increased from  $1.6 \pm 0.3$  to  $2.9 \pm 0.3$  nS ( $t = 3.4$ ;  $P < 0.05$ ,  $n = 4$ ). The remaining 11 cells did not respond to pheromone and did not change membrane conductance. A typical response is illustrated in Figure 4.

Finally, we examined whether female pheromone also had an effect on female VNO neurons. Under the same conditions as in Figure 1, applying pheromone to female snake VNO neurons did not induce an inward current ( $n = 11$ ). To confirm that the VNO neurons had intact and functional dendritic processes, we applied ESS to these same VNO neurons since binding of ESS to receptor neurons of the VN epithelium generates inward current in the VNO (Jiang *et al.*, 1990; Taniguchi *et al.*, 2000). Application of ESS induced inward currents in three of 11 neurons from female VNOs (Figure 5).

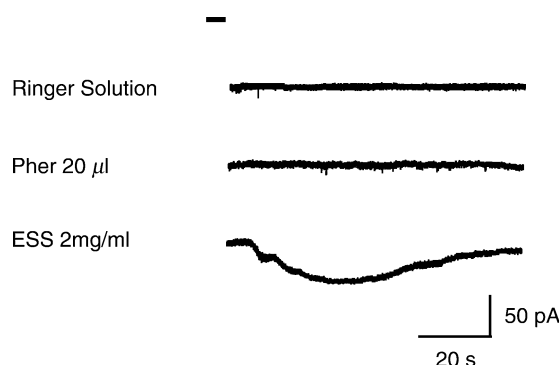
## Discussion

### Response to pheromone

In the present work, we describe for the first time the physiological responses in snake VN neurons to purified female sexual attractiveness pheromone. Stimulation with female



**Figure 4** Membrane conductance was increased by the application of pheromone. Under voltage-clamp conditions, a series of 10-mV depolarizing voltage pulses (0.03 Hz) was applied before and during the application of pheromone. In the presence of pheromone, the current pulse amplitude increased from 16 to 28 pA, resistance decreased from 625 to 357 M $\Omega$ , and membrane conductance increased from 1.6 to 2.8 nS.



**Figure 5** Female pheromone did not evoke inward currents in female of VN organ neurons. None of the female VN organ sensory neurons responded to the pheromone, whereas three of 11 responded to ESS with induced inward current.

pheromone produced inward currents in patch-clamped neurons of male snake VNO slices in a dose-dependent manner. This inward current was accompanied by an increase in membrane conductance. These results suggest that the effect of female pheromone on male VNO neurons is to open ion channels causing membrane depolarization and initiation of action potentials. Inward currents in VN sensory neurons in response to urine have been reported in female rats (Inamura and Kashiwayanagi, 2000). Similarly, ESS evoked inward currents in garter snake VNO neurons (Taniguchi *et al.*, 2000). However, the response latency observed in the present study was significantly longer than that observed in rat VNO neurons (Inamura and Kashiwayanagi, 2000) but similar to that reported for snake VNO neurons (2–3 s, Taniguchi *et al.*, 2000).

Dissociated mouse receptor cells from female mouse VNOs respond to dehydro-exo-brevicomin (DHB), a pheromone present in male mouse urine, with outward current at negative holding potentials. In current-clamp mode, DHB causes a hyperpolarization of the neurons (Moss *et al.*, 1997). Note that this finding contrasts with the studies referenced above and by Leinders-Zufall *et al.* (2000), which report inward, depolarizing currents in response to VNO stimulants.

#### Reversal potential for the pheromone effect

Female snake pheromone-induced current was inward at negative holding potentials and outward at positive holding potentials. The average reversal potential was  $-10.6 \pm 2.8$  mV, which suggests that the pheromone is activating nonselective cation conductances. This reversal potential is similar to that for the response to chemoattractant ( $-21 \pm 4$  mV) and  $IP_3$  in the snake VNO ( $-14 \pm 5$  mV, Taniguchi *et al.*, 2000) and  $IP_3$  in rat VNO neurons ( $-7.2 \pm 3.8$  mV, Inamura *et al.*, 1997). These results indicate that the pheromone-induced membrane response is mediated via  $IP_3$ . However, in addition, diacylglycerol (DAG) may also be a critical member of the signal transduction pathway for VNO neurons response to female pheromone. The response of VNO neurons

to chemoattractants and pheromones is known to involve the phospholipase C second messenger signaling cascade resulting in an increase in intracellular  $IP_3$  and DAG (Luo *et al.*, 1994; Kroner *et al.*, 1996; Wekesa and Anholt 1997; Sasaki *et al.*, 1999; Cinelli *et al.*, 2002). The present results, together with prior observations in snakes and mammals, support the idea that the female snake pheromone effect on male snake VNO neurons is via the  $IP_3$  second messenger system.

#### Dose-response relationship

The Hill coefficient is a central parameter in the study of ligand–protein interactions, which measures the degree of cooperativity between subunits that bind the ligand in multi-subunit proteins. The pheromone dose-response relationship of the transduction current yielded a Hill coefficient of 3.21, suggesting that there are multiple pheromone receptors on VNO neurons and that binding of pheromone to its binding site facilitates further binding. Our finding of an increase in evoked inward current as a function of pheromone dose is similar to that reported for rat urine (Inamura and Kashiwayanagi, 2000).

#### Action potentials

We also found female snake pheromone increased action potential firing by membrane depolarization in male snake VNO neurons. Voltage-activated ion channels are critical to the generation of action potentials that transmit olfactory information to the brain (Trotier and Døving, 1996). Opening of transduction channels result in a graded membrane depolarization that triggers self-regenerative action potentials that transmit odorant information to the olfactory bulb (Getchell, 1977; Trotier and MacLeod, 1983). Our results are similar to those reported for mouse urine, in which VNO neuron firing increased as a function of urine concentration (Holy *et al.*, 2000), but differ from that reported for DHB which decreased the action potential firings in female mouse VNO neurons (Moss *et al.*, 1997).

#### Behavioral and physiological responses to pheromones

Although this is the first demonstration of physiological alteration in VNO neurons by female sex pheromone in snakes, it is not a surprising finding. Male garter snake courtship behavior is released by this female sexual attractiveness pheromone (Kubie *et al.*, 1978; Mason *et al.*, 1989, 1990; LeMaster and Mason, 2001; Shine and Mason, 2001), and detection of this pheromone requires a functional VNO in garter snakes (Kubie *et al.*, 1978; Halpern and Kubie, 1983). Similarly in the adder, *Vipera berus* (Andren, 1982), detection of the female sex pheromone requires a functional VNO. Furthermore, many, but not all, sexual pheromones in mammals are detected by the VN system (see reviews by Halpern, 1987; Wysocki and Meredith, 1987; Meredith, 1998; Johnston, 2000; McClintock, 2002; Halpern and

Martinez-Marcos, 2003; Baxi *et al.*, 2006). Only a few studies have used electrophysiological techniques to investigate the effects of pheromones or fluids containing pheromones on VN neurons. These include responses to ESS in garter snakes (Inouchi *et al.*, 1993; Taniguchi *et al.*, 2000), urine in turtle (Fadool *et al.*, 2001) and mouse (Leypold *et al.*, 2002; Stowers *et al.*, 2002), and purified pheromones in mouse (Moss *et al.*, 1997, 1998; Leinders-Zufall *et al.*, 2000).

### Sexual dimorphism

We have found that the neurons of male, but not female, VNOs respond to the female sex pheromone. This finding strongly suggests that discriminated response to this pheromone originates at the periphery, that is, in the VNO and not further centrally in the central nervous system. VN stimulants bind to G-protein-coupled receptors on snake VN neurons (Luo *et al.*, 1994), and sexually dimorphic expression and localization of the  $G_{\alpha 1-3}$  subunit of GTP-binding proteins has been reported in the musk turtle *Sternotherus odoratus* (Murphy *et al.*, 2001). Differences in the signal transduction machinery could account for differential behavioral responses to sex pheromones. Female snakes have never been observed to respond behaviorally to female sexual attractiveness pheromone (Mason, 1992) and do not follow trails left by other females (Noble and Clausen, 1936; Noble, 1937; Ford and O'Bleness, 1986; LeMaster *et al.*, 2001). Here we have provided a physiological basis for differential responding by male and female garter snakes to the female sex pheromone.

It should be noted, however, that male and female mouse VN neurons respond to the urine of both sexes (Holy *et al.*, 2000). Thus, although individual mouse neurons respond with greater sensitivity to male or female urine, within the VNO of each sex are neurons that respond to urine from one or the other sex. This finding is consistent with the observation that all mammalian VN receptors so far studied are expressed in both sexes (Dulac and Axel, 1995; Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997). Urine is a complex stimulus that contains components other than sex pheromones, which may account for a lack of sexually dimorphic specificity. Similarly, in axolotls (*Ambystoma mexicanum*), the olfactory and VN epithelium of both males and females respond to whole-body odorants from both sexes, although in all cases, the response to odorants from the opposite sex are stronger than responses to odorants from the same sex (Park *et al.*, 2004). To date, no one has identified a VN receptor in a reptile, so it is not possible to determine if there is sexual dimorphism in reptilian VN receptor expression or at some other stage in the signal transduction pathway.

### Harderian gland

We found, in preliminary experiments, that it was not possible to solubilize the female sex pheromone in aqueous

solution or in mild detergents. However, when mixed with Harderian gland homogenate, the pheromone easily solubilized. The Harderian gland, a large retro-orbital gland in squamate reptiles, produces secretions that enter the oral cavity through the VN duct (Rehorek, 1997). These secretions also reach the VNO (Rehorek *et al.*, 2000). As the tongue passes adjacent to the opening of the VN duct, it is coated with these secretions, it is therefore more than likely that as the tongue of male snakes pick up molecules of the female snake pheromone, there is an interaction between the Harderian gland secretion and the pheromone. Since the pheromone is insoluble in water, it is possible that the Harderian gland secretion contains a protein (or proteins) that facilitates solubilization or transport of the pheromone. Determination of the precise interaction between the Harderian gland secretion and the pheromone awaits further research.

This study provides the first evidence of electrophysiological response to a purified pheromone in a reptile and one of the few in any vertebrate species. Furthermore, it demonstrates that the response to that pheromone is sexually dimorphic. An interesting finding, as well, is that the pheromone, insoluble in aqueous solutions or with mild detergents, became soluble with the addition of Harderian gland homogenate. This latter observation suggests that normally Harderian gland secretions facilitate delivery of the pheromone and perhaps other nonpolar biologically active molecules to the VNO.

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