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The Olfactory Organ is Activated by a Repelling Pheromone in the Red-spotted Newt *Notophthalmus viridescens*

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The pheromonal repelling response occurs when a combination of female and male pheromones is found to be less attractive to courting males than are female pheromones alone. This repelling response may act to conserve a courting males' reproductive fitness by minimizing male-male competition within a courting group. Recently, a pheromonal repelling response was first reported for vertebrates in the red-spotted newt, *Notophthalmus viridescens*. A male cloacal pheromone, a ~33 kDa protein, was identified as a repelling pheromone. In this study, to determine whether both the main olfactory epithelium (MOE) and/or the vomeronasal organ (VNO) are activated by the repelling pheromone, we recorded electrical field potentials from both olfactory epithelia while applying the repelling pheromone. The repelling pheromone induced electrical responses from both olfactory organs, and the magnitude of the response was greater in the VNO than in the MOE. Our results suggest that both the VNO and MOE may be involved in the pheromonal repelling response.

To increase mating success when the operational sex ratio (OSR; the ratio of fertilizable females to sexually active males; Clutton-Brock and Vincent, 1991), is male-biased, a courting male should utilize strategies to avoid increasing male-male competition within a courting group. For example, a male approaching a courting group should assess whether his chance of successful reproduction will be higher by competing for a female who is already being courted or by searching for an, as yet, uncourted female. Many species use chemical signals during this critical period of mating (Johnston et al., 1999), and they may use these signals to access their potential for a mating opportunity.

In several arthropod species, pheromones that repel conspecific males have been documented. Pheromones from male and females placed together decrease a conspecific male's sexual behavior (Bijpost et al., 1985; Lecomte et al., 1998). It has been suggested that the repelling response could increase mating success of males releasing the pheromone by minimizing male-male competition within the courting area. Recently, a similar response in vertebrates was documented in red-spotted newt, *Notophthalmus viridescens* (Park and Propper, 2001): test males significantly prefer female pheromones compared to combined pheromones from females and males. An approximately 33 kDa male cloacal pheromone was identified and partially purified as a repelling pheromone (Park, 2001).

The olfactory system in amphibians, reptiles, and mammals comprises two distinct peripheral organs, the main olfactory epithelium (MOE) and the vomeronasal organ (VNO) (Halpern, 1987; Keverne, 1999). The two systems use different receptor families, different signal transduction pathways, and separate neural projections to separate olfactory bulbs (Keverne, 1999; Johnston, 2000). For example, olfactory information detected by the MOE is sent to the main olfactory bulb (MOB) and to the cortical and neocortical centers of the brain where cognitive and emotional responses to smell occur. While, VNO neurons send fibers to the accessory olfactory bulb (AOB) the neurons of which, in turn, projects to the amygdala and the ventromedial hypothalamus that are involved in reproductive and aggressive responses (Dulac, 2000). One hypothesis to explain the existence of the two systems is that the MOE functions in general odorant detection and the VNO functions exclusively in pheromone detection. However, recent studies reveal functional overlap between the two systems (i.e., for mammals, Sam et al., 2001; Swann et al., 2001; for salamanders, Dawley and Bass, 1989; Todyoda et al., 1999). In the red-spotted newt, electrical field potentials from the VNO

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have not been previously studied during the application of pheromones as odorants.

In this study, to determine which olfactory organ is activated by the repelling pheromone, we recorded electrical field potentials from both the MOE and VNO while applying partially purified repelling pheromone.

Materials and Methods

Pheromone extraction

Red-spotted newts in breeding condition were purchased from a local supplier (Sullivan) and kept as previously reported (Verrell, 1982; Park and Propper, 2001). Extracts of crude pheromones from male cloacal glands were prepared as described previously (Park, 2001). Briefly, males were anesthetized in 4% ether, and the glands were excised and incubated in 1 mL of 0.8 mM acetylcholine chloride (AchCl; pH 8.4) in distilled water for 30 min (Pool and Dent, 1977; Rollman et al., 1999), which induces pheromone secretion into the solution (Pool and Dent, 1977). The resulting supernatants were centrifuged at 10,000 ×g for 10 min, frozen at -80℃, and thawed before use. In a control study, AchCl did not affect olfactory preferences of test males.

Partial purification of the repelling pheromone

To partially purify repelling pheromone, 2.2 mg of lyophilized crude repelling pheromone extracts (approximately 12 individuals) were separated using a column chromatography with Bio-Gel P60M polyacrylamide matrix (Bio-Rad) preequilibrated with 0.15 M NH_4HCO_3 (pH 8.0) (Kikuyama et al., 1995). A flow rate of 0.02 mL/min was maintained by an EP-1 Econo peristaltic pump (Bio-Rad), and 0.5 mL fractions were collected using a FC 203B fraction collector (Gilson). The absorbance at 280 nm for each fraction was measured with a Shimadzu UV 160U dual beam spectrophotometer (Shimadzu Scientific Instruments). The column was standardized with the proteins Ferritin (440 kDa), Chicken Albumin (40 kDa), Carbonic anhydrase (29 kDa), and Cytochrome C (2.5 kDa), producing the following regression equation: Elution volume (mL) = 506.11 mL-211.67 × log [molecular weight (kDa) of sample]. This equation was used to estimate the molecular weight of peak proteins and to separate the fractions into four different molecular weight groups, > 36.2 kDa (A), 36.2-30.6 kDa (B), 30.6-14.3 kDa (C), and 14.3-0.46 kDa (D). The repelling activity of the purified pheromone was confirmed using the behavioral tests described in Park and Propper (2001).

Testing repelling activity of purified pheromone

The pheromonal repelling activity of these four fraction groups was determined using Y-maze tests as described in Park and Propper (2001). A single reservoir containing 300 mL of aged tap water was the source of continuous flow (60-mL/min) entering each side arm of the Y-maze, which remained laminar until exiting the Y-maze (4.5 × 22 × 5 cm). All test males were arbitrarily selected from a pool of 50 males, and were held behind a start gate for 3 min prior to the gate being slowly raised. No male was tested more than once a day and trials occurred during daylight (1000-1430 h). We recorded arm preference, defined as the arm in which the test newt traveled more than half its length. After each trial, the Y-maze was washed using aged tap water.

For preparing stimulus samples for the Y-maze trial, we boiled each fraction group for 5 min to remove some of the NH_4HCO_3 (a boiled 0.15 M solution of NH_4HCO_3 did not repel males), created aliquots of 1/3 the total fraction volume for each group (11, 19, 40, 55 mL for A, B, C, and D, respectively), and then brought aliquots up to 300 mL with distilled water. During Y-maze trials, we placed three females into the reservoir as male attractants and delivered the fraction solution dissolved into one side arm of the Y-maze at a flow rate of 1.35 mL/min. Thus, test males had a choice between female pheromones alone and female pheromones plus fraction solutions. We determined that a fraction group has pheromonal repelling activity if test males significantly prefer to enter the side arm that contains female pheromones alone against the other side arm containing female pheromones plus fraction solutions. As found previously (Park, 2001; Rohr et al., submitted), only the second fraction group, 36.2-30.6 kDa, which contains approximately 33 kDa male cloacal pheromone, induced the pheromonal repelling response.

To increase the concentration of the repelling pheromone, we mixed 1.8 mg (approximately 8 individuals) of lyophilized crude cloacal pheromone extracts into the second molecular weight group that showed pheromonal repelling activity, and then ran the sample again on the same Bio-Gel P60M column with the same buffer. Molecular weight groups were pooled as previously, and again were tested for pheromonal repelling activity using the behavioral assay. The second molecular weight group, 36.2-30.6 kDa, again exhibited repelling activity, and was lyophilized and dissolved into 4 mL of distilled water (Park, 2001). This final product was used in the electrophysiological studies described below.

Recording electrical field potentials from the olfactory organ

To measure electrical field potentials from the main olfactory epithelium (MOE) and vomeronasal organ (VNO), subject males were anaesthetized by subcutaneous injection of ketamine hydrochloride (100 mg/Kg body weight; Park et al., 2001), and then double pitched. Electro-olfactograms (EOG) and electro-vomero

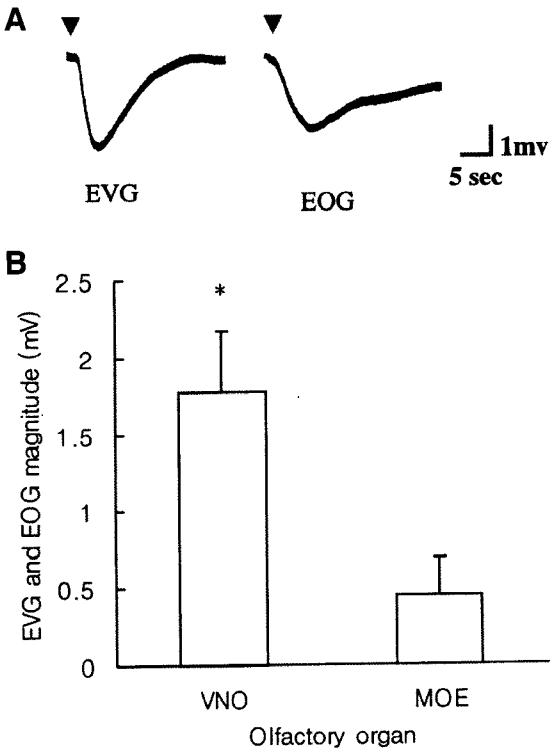

Fig. 1. Electro-vomeronasogram (EVG) and electro-olfactogram (EOG) recordings from the vomeronasal organ (VNO) and main olfactory epithelium (MOE) while applying partially purified repelling pheromone. A, A representative EVG (left) and EOG (right) in response to the repelling pheromone. Each arrowhead indicates the starting point of the electrical response following exposure to the repelling pheromone. B, Partially purified repelling pheromone induced a greater electrical response from the VNO than from the MOE. Vertical bars are mean ±SE. Asterisk denotes that the value of one bar is different from the other at $P < 0.005$.

nasograms (EVG) were recorded from the MOE and VNO respectively while applying partially purified repelling pheromone.

Control EOG and EVG recordings were conducted on three males by applying the buffer solution (0.15 M NH_4HCO_3) used in the column-chromatography. Four mL of 0.15 M NH_4HCO_3 solution was boiled for 5 min, lyophilized, and then dissolved in 1 mL of distilled water. Control stimulus solution consisted of 50 µL of the prepared buffer solution, 50 µL of distilled water, and 2 µL of 6% bromophenol blue. We added the bromophenol blue to confirm delivery of our stimuli (Park et al., 2001). Stimulus solution was delivered by a glass pipette (tip diameter 10-20 µm) connected to a Picospritzer (General Valve). We set the Picospritzer at 0.5-3 psi pressure for injection and 30- 45 msec for delivery duration. To compare EOGs and EVGs in each individual, we did not change any parameters of the Picospritzer during recording a paired EOG and EVG. The delivery pipette was not directed at recording area to avoid possible recording error by pressure changes on the olfactory epithelium. We measured EOGs and EVGs from 2-3 different areas of the MOE and VNO respectively and averaged values to produce an individual response. We continuously delivered aged tap water into the olfactory chamber of subject males at a flow rate of 0.1 mL/min using an EP-1 Econo peristaltic pump (Bio-Rad).

The experimental stimulus solution for EOG and EVG recordings contained 50 µL of partially purified repelling pheromone prepared as described above and mixed with 50 µL distilled water and 2 µL of 6% bromophenol blue. Paired EOGs and EVGs were recorded from six subject males.

To record electrical field potentials, a glass capillary electrode (50-100 µm tip diameter) was filled with Ringer's solution in 1% agar. The electrode was bridged to a chloride-coated silver wire. An Ag-AgCl reference electrode was placed under the head skin (Park et al., 2001). Electrodes were coupled to a Grass P-18 AC/DC pre-amplifier (Grass) and the signals were displayed on a V-552 Hitachi oscilloscope (Tokyo, Japan) and recorded simultaneously on a Vetter 420-M instrumentation tape recorder (Reversburg). Response magnitudes were defined as a relative peak phasic displacement measured from the baseline in mV (Fig. 1A). The magnitude of EOGs and EVGs were analyzed using pCLAMP 6.0 software (Axon Instruments) and compared using paired t-test (Sokal and Rohlf, 1981).

Results

In the control test, the buffer solution used in the column induced some field potentials, but differences between EOG and EVG magnitudes were not significant (mean EOG ±SE = 0.64±0.10, mean EVG ±SE = 0.44±0.07, paired t-test, df = 2, t = 1.225, $P = 0.345$). During control experiments, the control solutions induced small field potentials from both MOE and VNO. The electrical responses may be caused by the chemicals that we used in the column chromatography to partially purify the pheromones. However, a similar magnitude of EOG and EVG responses implies that the control solutions in which the repelling pheromone was dissolved did not cause the difference in EOG and EVG responses to the repelling pheromones.

Application of partially purified repelling pheromone induced field potentials from both olfactory organs. To calculate the magnitude of field potentials generated by the repelling pheromone, we subtracted control EOG and EVG values from each averaged experimental EOG and EVG values of six subject males and then compared them. The magnitude of EVGs was significantly greater than that of EOGs (Fig. 1B, mean EOG ±SE = 0.45±0.24, mean EVG ±SE = 1.77±0.40, paired t-test, df = 5, t = 4.728, $P = 0.005$).

Discussion

To determine which olfactory organ is activated by the

repelling pheromone, we recorded electrical field potentials from both the MOE and VNO of male red-spotted newts while applying partially purified repelling pheromone. In the result, a partially purified repelling pheromone induced field potentials from both the VNO and MOE. The magnitude of the EVG was greater than the EOG. These results suggest that both olfactory organs may play a role in transmitting pheromonal information involved in the repelling response.

The result that the MOE responded to the purified repelling pheromone suggests potential roles of the MOE in the pheromonal repelling response. Several studies from several different taxa demonstrate involvement of the MOE in pheromone-mediated behaviors. Female ewes whose vomeronasal nerves were sectioned still exhibited neuroendocrine responses when exposed to male odors (Cohen-Tannoudji et al., 1989). To induce androstenone-mediated sexual behaviors, female domestic pigs (*Sus scrofa*) in estrous do not need an intact VNO (Dorries et al., 1997). In Urodeles, female red-spotted newt's odorants induced EOG responses from the male's MOE (Park et al., 2001) and male sex attractants of the red-bellied newt, *Cynops pyrrhogaster*, evoked small electrical potentials in the female's MOE (Toyoda et al., 1999; Toyoda and Kikuyama, 2000). Thus, it is possible that the MOE may be partially involved in the pheromonal repelling response.

Our finding that repelling pheromone application to the VNO induced a greater electrical response than that seen from the MOE suggests that the VNO may play a major role in mediating the pheromonal repelling response. Main functions of the VNO in pheromone-mediated behaviors such as mating, territorial defense, and neuroendocrine changes have been reported in many species (Halpern, 1987; Keverne, 1999). Male guinea pigs (Beauchamp et al., 1982) and male lesser mouse lemur (*Microcebus murinus*, Aujard, 1997) lacking the VNO exhibited less sniffing display when investigating female's urine than did intact males. In female prairie voles, *Microtus ochrogaster*, VNO lesions prevented pheromone-mediated estrus induction and impaired pair bonding (Curtis et al., 2001). Mouse vomeronasal neurons exclusively responded to putative pheromones (Leinders-Zufall et al., 2000). Involvement of the VNO in pheromone-mediated behavior has also been suggested in several salamanders. Odorants taken up by nose-tapping behavior that is used to detect pheromones in a terrestrial salamander, *Plethodon cinereus*, was exclusively delivered into the VNO (Dawley and Bass, 1989). Female red-bellied newts exposed to sex attractants showed much greater electrical responses in the VNO than in MOE (Toyoda et al., 1999; Toyoda and Kikuyama, 2000). These results imply that the VNO may mainly function in pheromone-mediated behaviors of salamanders possibly including the pheromonal repelling responses, although currently, we cannot rule out the partial involvement of the MOE in the response.

Because pheromone exposure usually involves mixtures of compounds in the field, it has been thought that individuals should employ mechanisms to integrate information from multiple pheromones (Vickers et al., 1998a, b). Although knowledge of the physiological mechanisms underlying multi-pheromonal interactions should contribute to our understanding of information coding involved in pheromone-mediated behaviors, to date no model system has been developed in vertebrates. Considering that under natural conditions, animals need to encode information from multiple and simultaneous pheromonal stimuli such as occurs in the mix of male and female pheromones that leads to the newt repelling response (Park and Propper, 2001), red-spotted newts could be developed as a model system for the study of neurophysiological mechanisms underlying information integration of pheromones in vertebrates.

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