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# Preputial gland activates olfactory receptor neurons in rat: Calcium imaging study using laser scanning confocal microscopy

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The rodent preputial gland is one of the major sources of odours and is reported to be involved in several behavioural activities. However, how the preputial gland initiates the olfactory response to manifest the effects is not known. Olfactory receptor neurons (ORNs) present in the olfactory epithelium are involved in the perception of odorant/pheromonal compounds. In the present study, the response of rat ORNs to preputial gland extract was evaluated by calcium imaging analysis. We found that some rat ORNs responded to the preputial gland extract by exhibiting an intracellular calcium response. By contrast, the ORNs did not respond at all to the foot pad extract (control). The results indicated that the substances contained in the preputial gland might interact with a type of receptor expressed in the female rat ORNs, suggested to manifest the behavioural responses, such as social and sexual interactions. This study provided the first evidence of activation of ORNs by the preputial gland extract.

**Keywords**: Olfactory receptor neurons, Main olfactory system, Calcium imaging, Preputial gland, Pheromone, Rat

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*Abbreviations*: MOS, main olfactory system; ORNs, olfactory receptor neurons; PMT, photo multipier tubes; VNO, vomeronasal organ.

Among mammals, pheromones send powerful messages with behaviour modulating effects that may be of considerable social importance. These chemosignals are present in body fluids like urine, faeces, mucous secretions of genitals and specialized exocrine glandular secretions[1,2]. Among the specialized exocrine glands, preputial gland is one of the major sources of pheromones[3].

The responses of pheromone require the fundamental operation of odour detection and discrimination. The main olfactory system plays a vital role for detecting air-borne pheromones. The destruction of main olfactory system (MOS) is studied by the intra-nasal application of $ZnSO_4$ in rats and mice, which causes necrosis of sensory cells in the olfactory epithelium, resulting in anosmia in treated animals[4]. The olfactory epithelium of MOS contains millions of olfactory receptor neurons (ORNs) and these neurons convert information contained in airborne odour molecules (odorants) into electrical membrane signals and neural space. They provide direct linkage between the external world and the brain[5].

ORNs are bipolar, extending apical dendrites to the surface of neuro-epithelium, sending unmyelinated axons through the basal lamina and erbiform plate (of ethmoid bone) to terminate in the brain on dentrites of mitral and tufted neurons in the glomeruli of olfactory bulb. The apical dendrites form dendritic knobs, from which arise specialized non-motile cilia, where initial events of olfactory transduction occur[4,6,7].

$Ca^{2+}$ plays a crucial role in regulating the sensitivity of olfactory system. From chemosensory transduction in distal tips of ORNs to the release of neurotransmitter at the first relay synapse in the glomeruli of olfactory bulb, $Ca^{2+}$ continuously regulates a multitude of cellular processes that influence the perception (smell)[5]. The depolarization of cell triggers a signal that is conducted along the olfactory cell axon to the main olfactory bulb (MOB)[8].

The pheromone signals, their effects, the bodily secreted odorants, manner of their emission or deposition in the environment and manner in which they are perceived by conspecifics altogether constitute the study of chemical communication[4].

So far, most of the research on pheromones has been focused on the identification of volatile compounds in the sources, such as urine, faeces, saliva and exocrine secretions with an assessment of the behavioural responses they elicit. However, work related to the role of ORNs in odour recognition has received less attention. Recent investigations have employed calcium imaging techniques to measure stimulus-elicited intracellular calcium changes in ORNs as an indicator of odorant sensitivity[5-7,9]. In the present study, we have investigated the activation of isolated female rat ORNs in response to preputial gland extract by calcium imaging for visualizing odour recognition.

## Materials and Methods

### Reagents

Normal Ringer's solution contained 138 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose and 1 mM Na-pyruvate supplemented with 0.1% (w/v) BSA (pH 7.4). For elevated extra-cellular $K^+$ solutions (60 µM), NaCl was replaced with an equimolar concentration of KCl. For divalent cation free isolation solution, $CaCl_2$ and $MgCl_2$ were omitted and replaced with 1 mM EDTA (pH 7.4). Farnesol was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other chemicals were obtained from Wako Pure Chemicals (Osaka, Japan).

### Animals

Eight to ten weeks-old Wistar adult male (weighing 175-200 g) and female rats (weighing 150-175 g), *Rattus norvegicus* were used in the study. The rats were procured from Clea Japan (Tokyo, Japan) and housed with food and water *ad libitum* under 12-h light/dark cycles at 22°C until use. The experiments were carried out at the Graduate School of Veterinary Medicine, Hokkaido University, Japan and conformed to the guidelines on the ethical use of animals set by the Institutional Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University. All efforts were made to minimize animal suffering and to reduce the number of animals used.

### Preparation of preputial gland and foot pad extracts

The preputial glands were dissected out from adult male rats and homogenized by adding ice-cold phosphate buffered saline (PBS) (100 mg tissue in 1 ml of PBS, pH 7.2). They were centrifuged at 10,000 rpm for 15 min and the clear supernatant was stored separately in fresh vials at -20°C until further use. Approximately 100 µl extract was thoroughly mixed with 10 µl of dimethyl sulfoxide (DMSO) and dissolved in 20 ml Ringer's solution (pH 7.4) for perfusion. The foot pad sample was prepared by the same protocol as that used for the preputial gland extract.

### ORN isolation

The female rats were euthanized by exposure to excess $CO_2$ and then decapitated. ORNs were dissociated from the olfactory epithelium[6]. The nasal septum and turbinates were excised and put in ice-cold divalent cation-free Ringer's solution. The olfactory epithelium was dissected out from the underlying septal and turbinate bone/cartilage, followed by mincing with dissecting scissors (1-2 $mm^3$) and digested with 1 unit per ml of papain in divalent cation-free Ringer's solution for 10 min at room temperature. The tissue was then transferred to normal Ringer's solution, washed several times and gently triturated with a disposable plastic Pasteur pipette to dissociate the cells. The cell suspension was filtered through a nylon mesh, centrifuged at 60 × *g* for 5 min and resuspended in 1-2 ml of normal Ringer's solution.

### Dye loading

Ten microlitres of 1 mM Fluo-4 acetoxymethyl ester (AM) (Molecular Probes, Eugene, OR, USA) in DMSO was mixed with normal Ringer's solution (990 µl) by sonication for 40 to 50 s. Then, 1 ml of cell suspension was mixed with the Fluo-4 solution to give a final concentration of 5 µM Fluo-4 AM and loading proceeded for 1 h at room temperature. The cell suspension was then centrifuged at 60 × *g* for 5 min, thoroughly washed and resuspended in 2 ml of normal Ringer's solution. To begin the experiment, 100 µl suspension of Fluo-4 loaded cells was transferred to the recording chamber, to the bottom of which Cell-Tak (Becton Dickinson Labware, Bedford, MA, USA) coated coverslips were attached. The chambers containing the cell suspension were left undisturbed for about 10 min. This method was sufficient to ensure firm attachment of dendritic knob and soma of isolated cells on to the cover slips of the chambers.

### Calcium imaging

The changes in intra-cellular $Ca^{2+}$ concentration $[Ca^{2+}]i$- was recorded using a confocal laser scanning

imaging system (Fluoview FV500, Olympus, Tokyo, Japan). This technique allowed the recording of odour-induced $Ca^{2+}$ transients at the region of interest (ROI). The recording chamber was set on the stage of inverted microscope with a perfusion system and cells were continuously perfused with normal Ringer's solution at a flow rate of 1 ml/min for 10-15 min prior to and throughout the experiments. A forced air-cooled argon ion laser beam (488 nm) with a total laser output of 10 mW and linear polarization was used and the emitted fluorescence (>505 nm) was guided through an x40 water immersion objective lens to a pinhole diaphragm.

Photodamage was minimized by operating the laser at its lowest power setting and by attenuating laser intensity by interposing a neutral density filter (1% transmission) into the illumination path. Isolated individual cells were imaged at X4-5 magnification with the electronic zoom setting of the imaging software. The resting fluorescence signal was adjusted to a minimum by changing the photo multiplier tubes (PMT) sensitivity. Images were acquired every 5 s in normal horizontal scanning mode (XYT). All the scan settings were kept constant over the series of experiments. To reduce photobleaching, scanning was paused during washings between two successive stimulus applications.

#### ORN selection

All the cells used in the study were clearly identifiable as ORNs by their characteristic morphology, having an oval to round soma with a slender dendrite that ended in a small knob-like swelling. Cilia were too thin to be seen clearly unless magnified above the normal experimental set-up. Only isolated ORNs maintaining morphological integrity were used in the experiments. The bath solution was superfused with the aid of a peristaltic pump and stimulus solutions were applied by switching the superfusion solution; this allowed a complete change of bath solution within approximately 30 s.

#### Experimental protocol for exposure of odorants to ORNs

ORNs were perfused with Ringer solution (pH 7.4) for the first two min and the odorant (preputial gland/foot pad extracts) was perfused for the next 7 min. At the beginning of 10 min, the high potassium ($K^+$) solution (30 mM) was given to the ORNs. The experiment was always terminated at 12 min.

#### Data analysis

Initially, time courses of changes in fluorescence intensities of ORNs were recorded in arbitrary units using Fluoview 4.2 with Tiempo (Olympus). The data were then converted to relative change in fluorescence intensity (% $\Delta F/F$), where $\Delta F$ and F refer to the stimulus-induced change in fluorescence and resting level fluorescence, respectively. The average fluorescence intensity during 20-30 s prior to stimulus application was taken as the resting level fluorescence. The magnitude of response was assessed by taking the peak % $\Delta F/F$ values during stimulation. The average results were expressed as means ± standard error of mean (S.E.M) of experiments ($n$ = 5), where $n$ refers to the number of ORNs.

## Results and Discussion

The rat ORNs responded well to the preputial gland extract. Amongst 18 ORNs perfused with preputial gland extract, 11 ORNs responded absolutely. Transmission images of an isolated rat ORN and pseudo-colour images of $[Ca^{2+}]_i$ response in the ORN during exposure of preputial gland and foot pad extracts are shown in Fig. 1. The preputial gland extract induced an increase in $[Ca^{2+}]_i$ in the ORN. However, relatively higher $[Ca^{2+}]_i$ was noticed in the ORN perfused with high $K^+$ (Figs 1A & **C**). In contrast, foot pad extract did not excite ORNs or generate any $[Ca^{2+}]_i$ response (Figs 1B & D). The present study confirmed that preputial gland extract evoked the responses in female rat ORN and was in agreement with earlier report on more number of preferences made by female rats towards the preputial gland than the control tissue (foot pad)[10].

The main olfactory system (MOS) contains ciliated ORNs which detect odorants and pheromones[5]. The role of the MOS can be established by studying the response through chemosensory neurons, in which the mammalian nose detects an array of odours and pheromones that carry essential information about the animals' environment[11]. Initially, the vomeronasal organ (VNO), an accessory olfactory organ was thought to be specialized in the perception of pheromones[12].

Experimental evidence indicates that perception of signaling pheromones may be mediated by the MOS[13,14] and physiological effects of most primer pheromones are initiated through the vomeronasal system[15,16]. The majority of ciliated ORNs in the

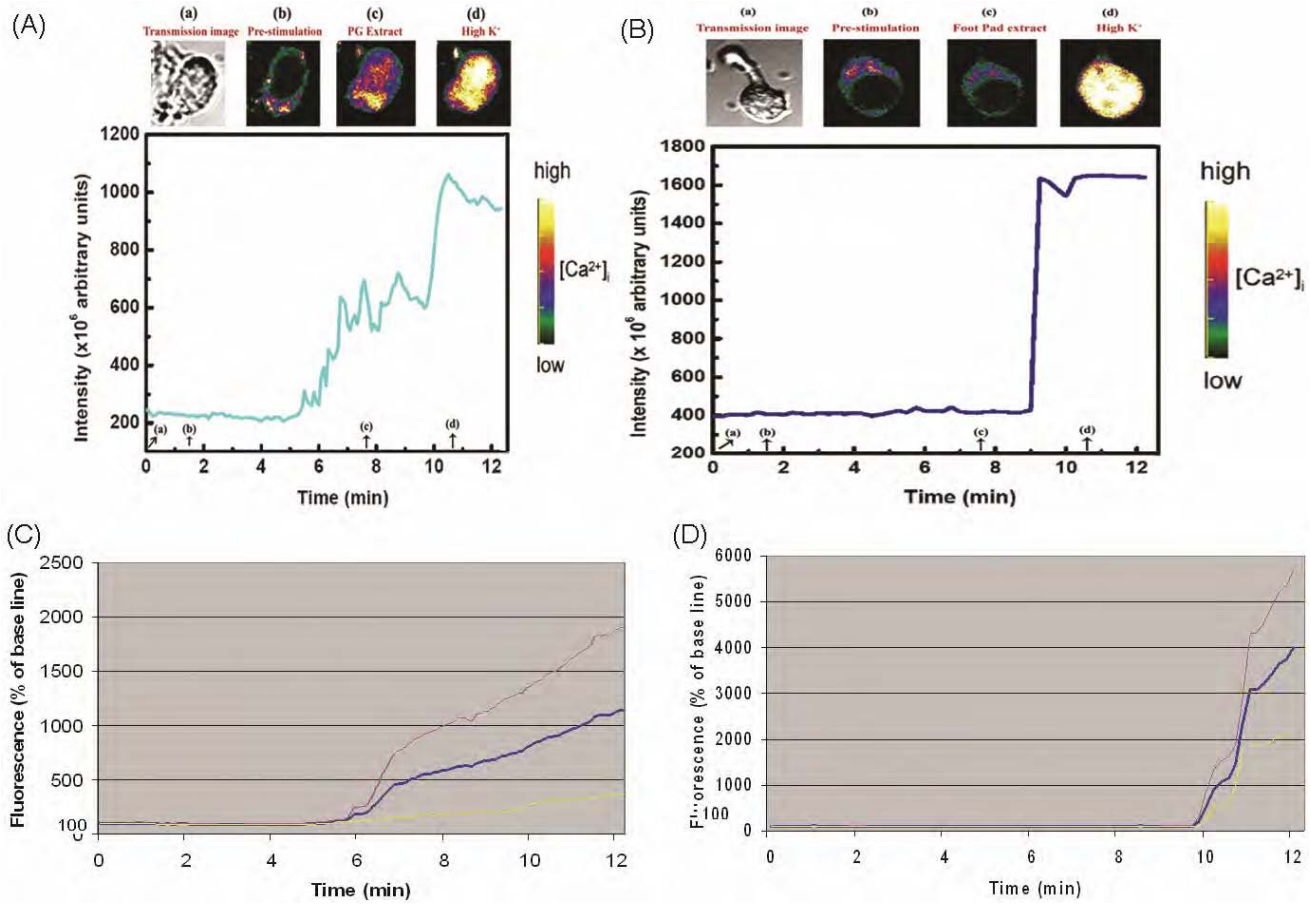


Fig. 1—$[Ca^{2+}]_i$ response to preputial gland and foot pad extracts. (**A & B**): Two min after starting image acquisition, an isolated female rat ORN was perfused with Ringer's solution containing preputial gland extract (A) and foot pad extract (B) for 7 min, followed by stimulation with 60 mM $K^+$. Changes of Fluo-4 fluorescence were expressed as a percentage of the basal level. Upper pictures (from left to right) show a translucent image (a) and pseudo-colour $[Ca^{2+}]_i$ images of pre-exposed phase (b), preputial gland extract-exposed phase (c) and high $K^+$-exposed phase (d); and (**C & D**): Preputial gland (C) and foot pad (D) stimulation caused fluorescence intensity increase in ORNs. The intensity was set to 100% and the average intensity +/- S.E. was calculated during 12 min exposure

MOS are reported to use the cAMP signaling cascade for transforming chemosensory information into electrical signals. Further, this pathway has been observed in olfactory signal transduction in the mouse septal organ and the signal transduction is reported to be similar in the septal and turbinate bones of the ORNs[17]. The results of the present study were consistent with the earlier reports on the activation of ORN towards the common odorants and pheromones in various animals, including the cat[18], salamander[19], hamster[20], newt[21], mice[17], rat[4] and pig[7].

Though preputial gland extract influenced to increase ($Ca^{2+}$) in ORN, remarkably higher $Ca^{2+}$ was noticed in the ORN by exposure to high $K^+$ (Figs 1A & C). Further, the foot pad extract failed to induce $Ca^+$ in ORNs, but high $K^+$ induced significant response in those ORNs (Figs 1B & D) as observed in the present study. Based on the response of ORNs by high $K^+$ followed the perfusion of foot pad extract, the results strongly indicated that isolated and tested ORNs were alive in condition. The high $K^+$ is generally used for depolarizing and confirming ORNs as alive by opening the cation channels[22]. As the preputial gland odour is believed to interact with a type of receptor expressed in the ORNs concerned with manifesting the behavioural responses, it suggested that the influx of calcium during exposure to preputial gland extract might be due to binding of the volatile compounds present in the glandular extract with the ORNs. Thus, the present study provided the first evidence that preputial gland pheromone evoked the calcium influx in the ORNs of the MOS. The results suggested that pheromones in the preputial gland might stimulate many different

types of receptors and thus stimulated a cAMP cascade, whereas foot pad might not contain such compounds, resulting in their inability to stimulate the receptors in ORNs.

The preputial gland is reported to produce powerful chemosignals involved in attraction as well as aggression in conspecific rodents[3]. Even though most of the actions of the primer pheromones are mediated by the vomeronasal organ, the signaling pheromones are transported through the MOS. Both olfactory systems are probably involved in the detection of biologically relevant odours[23] or more specifically they may act synergistically in the perception of conspecific odour[24]. Earlier study[25] has shown that aggression-promoting chemosignals from the preputial gland manifest effect through vomeronasal organ, indicating that signals are probably mediated in the V2R receptors of vomeronasal organ. However, the present study demonstrated that actions of sex attractive compounds present in the rat preputial gland were mediated through the MOS by presenting evidence of activation of ORNs. The study indicated that the response of female rat ORNs to the preputial gland extract might coordinate the process of behaviour and physiology of conspecifics.

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