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The Mouse Putative Pheromone Receptor Was Specifically Activated by Stimulation with Male Mouse Urine[1]

Kimiko Hagino-Yamagishi,*[,2] Masato Matsuoka,[†] Masumi Ichikawa,[‡] Yoshihiro Wakabayashi,*[,§] Yuji Mori,[§] and Kazumori Yazaki*

Department of Ultrastructural Research, Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8613; [†]Department of Neurobiology and Anatomy, Niigata University School of Medicine, 1 Asahi-machi, Niigata 951-8510; [‡]CREST (Core Research for Evolutional Science and Technology), Department of Developmental Morphology, Tokyo Metropolitan Institute for Neuroscience, Fuchu, Tokyo 183-8526; and [§]Laboratory of Veterinary Ethology, Veterinary Medical Science, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657

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To detect the biological activity of mammalian putative pheromone receptors (V1Rs and V2Rs), the mouse V1R gene was introduced into a primary culture of vomeronasal cells using the adenovirus expression system, and the response of these cells to mouse urine was analyzed by calcium imaging. These cells specifically responded to male but not female mouse urine. This response was attenuated by pertussis toxin, a specific inhibitor of G-protein $G_{i\alpha}/G_{o\alpha}$ coupling from receptors. Our findings indicate that a putative pheromone receptor was specifically activated by mouse urine, a major source of mouse pheromones, and suggest that G_i/G_o are functionally coupled with the receptor.

Key words: adenovirus expression system, G proteins, mammalian pheromone receptor, urine, vomeronasal organ.

Pheromones are chemical signals that play an important role in the sexual behavior and neuroendocrine responses of individuals of the same species (*1*). Although urine and other bodily secretions have been reported to have many different pheromone effects, only a few mammalian pheromones have been identified (*2*).

Genes of two families encoding seven-transmembrane proteins were isolated from mouse and rat vomeronasal organs (VNOs) (*3–6*). The two families (V1Rs and V2Rs, respectively) comprise about 35 and 150 genes, respectively, but each protein is only expressed in a small percentage of vomeronasal neurons. These families exhibit no sequence homology with each other. Although the presence of two types of receptors implies different classes of pheromones, the ligands for these receptors have not been identified yet. A functional expression system for these receptors would allow us to analyze the molecular and cellular mechanisms of pheromone sensing, and to identify the ligands for individual receptors.

To investigate the molecular mechanisms of pheromone sensing, we attempted to detect the biological activity of mouse putative pheromone receptors. We hypothesized that VNO neurons expressing a particular putative pheromone receptor would be activated upon stimulation with urine if the urine contained the corresponding pheromone molecule (*7*), and that this activation should be detected on calcium imaging, because the specific response of VNO cells to the putative pheromones was associated with intracellular $[Ca^{2+}]$ elevation (*8*). Thus, we introduced the cloned gene encoding the mouse putative pheromone receptor into VNO cells using the recombinant adenovirus expression system, and analyzed the response of these cells to urine by means of the calcium imaging technique.

As the first step, we isolated the gene encoding mouse V1Rs from mouse VNO cDNA based on the similarity to rat V1Rs motifs. The amino acid sequence of the newly isolated mouse receptor gene (clone 2–4) was 83% identical to that of the rat receptor, vno-1 (*3*) (Fig. 1). This gene (clone 2–4) was introduced into the adenovirus expression vector. We used this vector system because it could effectively transfer exogenous genes in differentiated neurons (*9*). In addition, expression of this receptor could be monitored by means of anti-hemagglutinin (HA) antibodies because the HA epitope was fused at the C-terminus of the receptor. The recombinant adenovirus generated was designated as Ad2-4HA (Fig. 1, legend).

Next, VNO cells were infected with Ad2-4HA. To produce measurable ligand activation, the receptor should be properly expressed in the cells and should couple with a second messenger system. We assumed that the VNO neurons contained all components required for the expression, targeting, and coupling of putative pheromone receptors. However, in adult rats, almost all VNO neurons extended axons to the accessory olfactory bulb (AOV). The VNO neurons would not survive if the axons of these neurons were excised. Thus we prepared VNO neurons from rat embryos on embryonic day 19, and infected a primary culture of rat VNO cells with Ad2-4HA. Two days after infection, the

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[2] To whom correspondence should be addressed. Tel: +81-3-3823-2101 (Ext. 5345), Fax: +81-3-3823-2965, E-mail: kyamagis@rinshoken.or.jp

Abbreviations: AOB, accessory olfactory bulb; G_i, G-protein subunit $G_{i\alpha}$; G_{i2}, G-protein subunit $G_{i2\alpha}$; G_o, G-protein subunit $G_{o\alpha}$; VNO, vomeronasal organ.

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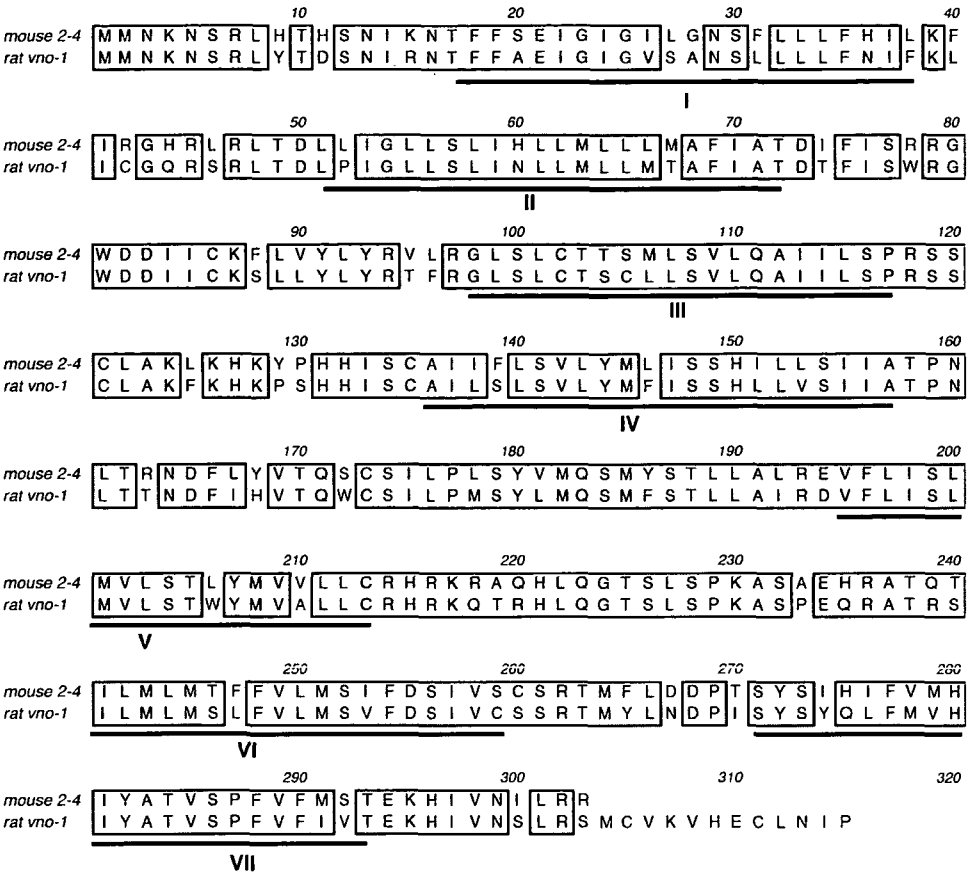

Fig. 1. **Deduced amino acid sequence of the newly isolated mouse putative pheromone receptor.** VNO cDNA was synthesized from RNA, which was prepared from VNOs of Crj:CD-1 (ICR) male and female mice. To obtain the mouse homologue sequences, PCR was performed with a pair of degenerated primers, which were synthesized from two conserved sequences of the rat putative pheromone receptor genes (V1Rs) (*3*) with the VNO cDNA as a template. The entire coding sequence of mouse V1R (clone 2–4) was obtained by 3′- and 5′-RACE. The sequence which corresponded to the HA epitope (YPYDVPDYA) was inserted at the 3′ end of the 2-4–coding sequence by PCR. The 2-4–HA fusion gene was inserted into the SwaI site of pAxCALALw (a generous gift from Dr. I. Saito, Laboratory of Molecular Genetics, Institute of Medical Science, University of Tokyo), and the resultant cosmid was used for constructing the recombinant adenovirus as described (*11*). The recombinant adenovirus generated was cloned and amplified by serial passages. In the final step of amplification of the virus, expression of the receptor-HA unit was turned on *via* site-directed recombination by Cre recombinase, which was supplied by coinfection with Cre-expressing adenovirus [AxCANCre (*14*)]. The resultant recombinant adenovirus was designated as Ad2-4HA. The sequence and the expression of the receptor-HA fusion gene present in the genome of Ad2-4HA were confirmed by sequencing and immunocytochemistry, respectively. Identical and conserved residues are indicated by shading. The seven putative transmembrane helices are also indicated (I–VII).

VNO neurons had extended axon-like fibers (Fig. 2A, arrow), and no toxic effects of adenovirus infection were observed (Fig. 2A). Under these conditions, 70 to 80% of the cells expressed the HA epitope, indicating that these cells also expressed the introduced receptor gene (Fig. 2B).

Since the G proteins, G_o and G_{i2}, have been suggested to play roles in the signal transduction of VNO neurons (*10*), the expression of G_o and G_{i2} in the VNO culture was examined using anti-G_o and anti-G_i antibodies, respectively. Almost all VNO neurons expressed Go (Fig. 2C). G_{i2} proteins were also expressed (Fig. 2D), but the number of cells expressing G_{i2} was lower than that of ones expressing G_o (data not shown). These results suggest, that under our experimental conditions, some VNO neurons coexpress G_{i2} and G_o simultaneously.

Then, virus-infected and non-infected cells were stimulated with urine, and then the responses of these cells were analyzed by calcium imaging. The male and female urine portions were passed through an Amberlite XAD-4 column. As catecholamines and their derivatives, which emit fluorescence by themselves, were adsorbed to this column, they were removed from the urine. The resultant eluate did not emit non-specific fluorescence when Rhod-2 was used as the fluorescent indicator.

In control experiments, VNO cells were stimulated with male or female mouse urine, but specific activation was not observed (in both experiments, 0 to 1 cell/30–60 KCl-responsive cells within the fields). Next, VNO cells were infected with the control adenovirus containing no receptor gene [Axlwl (*11*)], and then stimulated with male urine (Figs. 3b and 4, 4–6), but specific activation was not observed (0 to 1 activated cell/ 45 KCl-responsive cells within the field). A similar result was obtained for control adenovirus-infected cells stimulated with female urine (data not shown). These results indicate that almost no VNO cells respond to urine under our experimental conditions, and that adenovirus infection does not activate the VNO cells.

On the contrary, when Ad2-4HA–infected VNO cells were stimulated with male mouse urine, a transient increase in Rhod-2 fluorescence was detected (Figs. 3a and 4, 1–3) in 49% of the cells observed (21 activated cells/ 43 KCl-responsive cells within the field). Dose-dependent responses to male mouse urine were observed at 1/10 (final concentration; 1/100)– to 1 (final concentration; 1/10)-fold dilution (Fig. 3d). In contrast, when female mouse urine was used as a stimulator, the Ad2-4HA–infected cells did not respond (0 activated cell/36 KCl-responsive cells within the field) (Fig. 3c). These results show that the VNO cells only respond when the exogenous receptor gene is expressed (Figs. 3, a and b, and 4, 1–6), and suggest that this receptor is activated by male-specific urinary component(s). As urine is the major source of pheromones, it is conceivable that one

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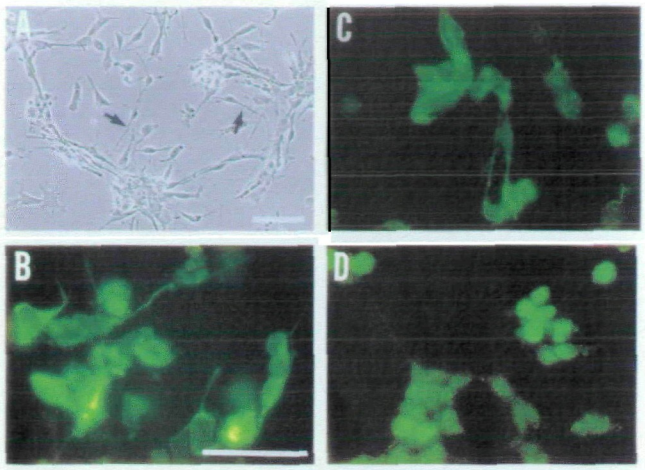

Fig. 2. **Immunohistochemistry of a rat VNO primary culture.** Embryos at embryonic day 19 of Sprague-Dawley rats were used to prepare VNOs. Day 0 of pregnancy was defined as the first day that a vaginal plug was observed. The procedures used for isolating the VNOs were those described by Osada *et al.* (1999) (*15*). The isolated VNOs were removed from the surrounding tissue under a dissecting microscope, treated with a trypsin solution (0.125% trypsin, 10 mM EDTA, and 20 mM glucose) in PBS for 30 min at 37°C, and then seeded onto a polyethylenimine (Sigma)-coated chamber slide (500 µl bath volume/well) (Nalgen Nunc International) in DMEM/F12 medium containing 5% fetal bovine serum (2×10^5 cells/well). These cells were infected with the adenoviruses at a multiplicity of infection (moi) of 9 or 10. Two days after infection, the infected cells were subjected to immunostaining as described (*16*). A rat anti-hemagglutinin protein (HA) monoclonal antibody (Boehringer Mannheim), rabbit polyclonal anti-G_i and anti-G_o antibodies (Wako Chemicals), and AlexaFluor™ 488 conjugated goat anti-rat and anti-rabbit IgG antibodies (Molecular Probes) were used. A: Light microscopy. B: Anti-HA, C: anti-G_o, and D: anti-G_i antibodies, respectively. Arrows indicate the axon-like structures in the cells. Bar, 0.05 mm.

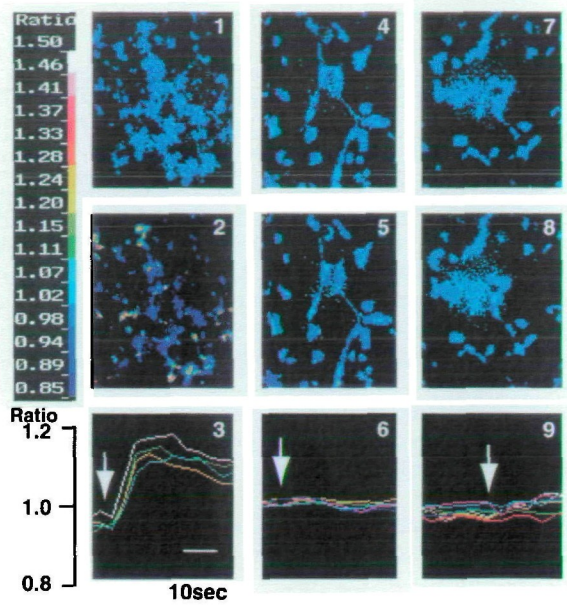

Fig. 4. **A putative mammalian pheromone receptor mediates $[Ca^{2+}]$ elevation in VNO cells caused by male mouse urine.** Panels 1, 2, 4, 5, 7, and 8 show pseudocolored images of the cells. VNO cells were infected with the recombinant adenovirus (1 and 2, and 7 and 8) or control adenovirus, Axlwl (4 and 5). In panels 7–9, the cells were treated with pertussis toxin. The urine from male mice was used as a stimulator. These cells were assayed for increases in $[Ca^{2+}]$ before (1, 4, and 7) and after (2, 5, and 8) the addition of urine. Line traces (3, 6, and 9) show the kinetics of $[Ca^{2+}]$ changes for representative cells in panels 2, 5, and 8. Arrows indicate the timing of stimulation with urine.

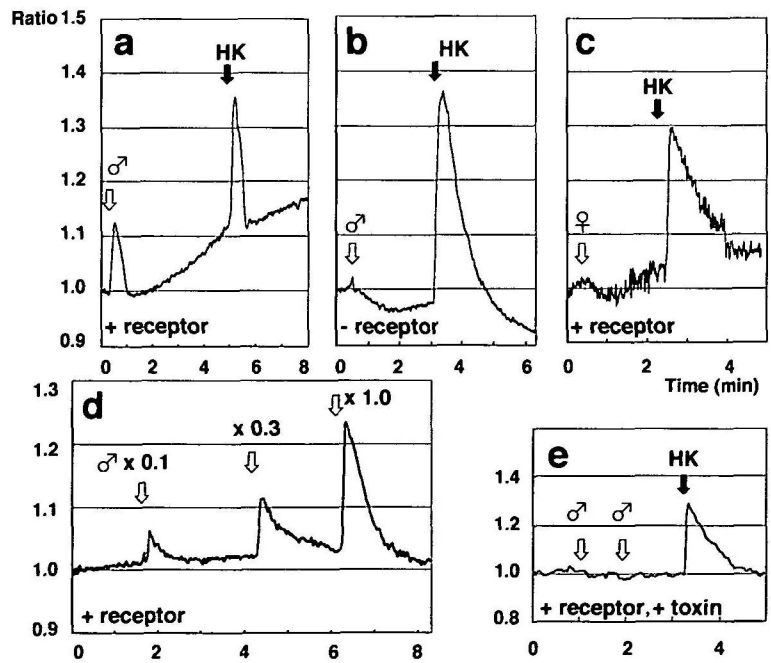

Fig. 3. **The response of a single VNO cell to mouse urine.** Urine from each of 10 male and female ICR mice was collected daily. The collected urine was centrifuged at 5,000 rpm for 10 min at 4°C, or passed through a 0.45 µm filter. The supernatant was passed through an AmberliteXAD-4 column (Organo), and the resultant eluate was collected and used for the calcium imaging assay. Two days after infection, the VNO cells were loaded with 5 µM Rhod-2/AM (Dojindo Lab) and 0.1% cremophore EL (Sigma) for 1 h, washed and then incubated in 250 µl of a BSS solution (130 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl, 5.5 mM glucose, and 20 mM HEPES-NaOH; pH 7.4) for 30 min. Twenty-five microliters of the partially purified urinary fractions, or 25 µl of a high K solution (0.8 M KCl in BSS) was manually applied to these cells, and then Rhod-2 fluorescence was monitored under an inverted fluorescent microscope (Olympus IMT-2) equipped with an Argus 50 calcium-imaging processing system (Hamamatsu Photonics). For single-cell calcium imaging, the cell was continuously washed out with the BSS solution. The flow rate was 0.4 ml/min. For treatment with pertussis toxin, Ad 2-4HA–infected cells were treated overnight with 50 ng/ml of the reagent, and then washed with the BSS solution immediately before the calcium-imaging assay. a: Recombinant adenovirus-infected VNO cells were activated by male mouse urine (white arrow). b: Axlwl-infected VNO cells were not activated by male mouse urine (white arrow). c: Recombinant adenovirus-infected VNO cells were not activated by female mouse urine (white arrow). d: Dose response of the putative pheromone receptor. Recombinant adenovirus-infected VNO cells were stimulated by male mouse urine of various concentrations. Mouse urine was diluted with 1× BSS solution. e: Treament with pertussis toxin attenuated the response of recombinant adenovirus-infected cells. White arrows indicate the timing of stimulation. HK: high KCl stimulation (black arrow).

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of the putative pheromone receptors, clone 2-4, recognizes male-specific pheromone(s).

G-protein–coupled signal transduction is suggested to play a role in mammalian vomeronasal neurons (*10*). The coexpression of G_{i2} with V1Rs and G_o with V2Rs implies that G_{i2} and G_o are functionally coupled with V1s and V2Rs, respectively, for the pheromone sensing pathway (*4*, *5*, *12*). To investigate the functional coupling of these receptors with these G proteins, Ad2-4–infected VNO cells were treated with pertussis toxin, a specific inhibitor of G_i/G_o coupling with receptors, and then the response to male mouse urine was analyzed. As shown in Figs. 3e and 4, 7–9, the activation of the infected cells by male mouse urine was attenuated (3 activated cells/31 KCl-responsive cells within the field), suggesting that pertussis toxin specifically attenuated the response of these cells, and that G_{i2} and/or G_o are functionally coupled with V1Rs.

An IP3-mediated pathway was proposed to play a role in the vomeronasal sensory system, although Leider-Zufall *et al.* (*8*) suggested that the response of the VNO neurons was triggered primarily by Ca^{2+} entry into the cells. The system described here will be useful for analyzing these transduction mechanisms.

In this study, we found that a putative pheromone was specifically activated by urine, and suggest that G_i and/or G_o are functionally coupled with the receptor for the signal transduction pathway. We believe that the system described above can be used for the assaying of other V1Rs and V2Rs of various mammals. Thus, our assay system can be used not only for analyzing the signal transduction of VNO neurons, but also for detecting and identifying putative pheromone molecules of various mammals.

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