Molecular characterization of vomeronasal sensory neurons responding to a male-specific peptide in tear fluid: Sexual communication in mice*

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Abstract: Pheromonal signals received by the vomeronasal organ (VNO) have been shown to elicit various behavioral and physiological responses that are typically stereotyped and preprogrammed. Recently, we found a novel male-specific peptide, named exocrine gland-secreting peptide 1 (ESP1), that is secreted in tear fluid and stimulates the VNO in mice. Excreted ESP1 appears to be transferred to the female VNO, where it induces c-Fos expression and elicits an electrical response in a small subset of vomeronasal sensory neurons (VSNs). We report here the identification of molecular components expressed in ESP1-stimulated VSNs by double-staining with c-Fos. We found that the c-Fos-induced cells were localized amongst the $G\alpha o$-expressing VSNs. Furthermore, the ESP1 signal was received by VSNs expressing a single type of vomeronasal receptor type 2 (V2Rp5). Finally, double in situ hybridization of the V2Rp5 and various members of the M1 and M10 families of major histocompatibility complex (MHC) class Ib molecules revealed that V2Rp5-expressing VSNs can express multiple MHC molecules. These results suggest that a V2R rather than MHC molecule is mainly responsible for the recognition of ESP1. The identification of the putative sex-pheromone ESP1 and its cognate receptor therefore will help clarify the molecular mechanisms underlying pheromone recognition in the mouse vomeronasal system.

Keywords: vomeronasal organ; pheromone; mouse; ESP; V2R; extraorbital lacrimal gland.

INTRODUCTION

Many mammals utilize chemical signals, commonly termed pheromones, for intraspecies social and sexual communication [1–3]. In mice, pheromone signals are received by two anatomically distinct olfactory systems: the vomeronasal organ (VNO) and the main olfactory epithelium (MOE) [4,5]. Signals received by the VNO have been shown to elicit various behavioral and physiological responses that are typically stereotyped and preprogrammed, including inter-male aggression, male sexual preference, maternal aggression, acceleration of puberty (Vandenbergh effect), and pregnancy block (Bruce effect) [1–3]. Although these pheromonal effects have long been known in mice, many of the molecular features of pheromonal communication have remained elusive.

To elucidate the molecular mechanisms underlying pheromonal communication mediated by the VNO, we attempted to identify pheromones that specifically act on the VNO and their receptors. In this report, we describe the discovery of a novel male-specific pheromonal peptide that stimulates the mouse VNO. We then describe the identification of molecular components that are expressed in the vomeronasal sensory neurons (VSNs) responding to the peptide and therefore that may be involved in pheromone reception.

METHODS

Homogenate of the extraorbital lacrimal gland (ELG) from BALB/c adult male mice was separated by three-step column chromatography as described previously [6]. All column fractions were assayed for c-Fos-inducing activity using 10-week-old individually housed sexually naïve female mice as described previously [6]. Briefly, the column fractions were lyophilized, resuspended in 20 mM tris-HCl (pH 7.5), absorbed by cotton swabs (~35 mg), and dried in a Speed Vac. Female mice were exposed to the cotton swabs for 1.5 h, after which immunohistochemistry for c-Fos and double-staining for c-Fos and vomeronasal receptor type 2 (V2R) were performed using anti-c-Fos antibody (Calbiochem, #PC38) as previously described [7].

For double in situ hybridization, the nucleotide sequences of the N-terminal extracellular regions of the M10 family of major histocompatibility complex (MHC) proteins were used as probes. For double-labeling with digoxigenin-labeled MHC and fluorescein–isothiocyanate-labeled V2R probes, hybridization was performed at 68 °C, followed by successive washing with 2x saline sodium citrate (SSC) containing 50 % formamide, 2x SSC, and 0.2x SSC at 65 °C. After washing, detection of double-hybridization signals was performed using a TSAplus fluorescence system (PerkinElmer) according to the manufacturer's protocol. Finally, the signals were analyzed by a confocal microscopy.

RESULTS

Based on the observation that soiled bedding of adult BALB/c male elicits c-Fos expression in the VSNs of female BALB/c mice under behaving conditions [8–10], we reasoned that adult male mice excrete male-specific cues that are taken up by the female VNO. Subsequent investigation of the source of the c-Fos-inducing activity led to a surprising finding: the activity was not found in urine but in the ELG [6]. Three-step column purification of active fractions from the male ELG resulted in the resolution of the activity into two peaks (Fig. 1). N-terminal sequence analysis and mass spectrometry (MS) were then performed for the peptides in these two fractions. The basic local alignment search tool (BLAST) search against the mouse genome revealed an open reading frame on chromosome 17 corresponding to the N-terminal sequence and to the molecular masses of the peptides (Fig. 1). We named this novel peptide ESP1, for exocrine gland-secreting peptide 1. The expression of the ESP1 gene in the ELG was male-specific and was observed after four weeks of age [6], suggesting that it functions as a sex pheromone. ESP1 was detected in male tear fluid by Western blot analysis using an anti-ESP1 antibody [6], indicating that the male-specific peptide ESP1 is secreted in the tears by the male ELG and then transferred to the female VNO through physical contact upon investigation of the facial areas.
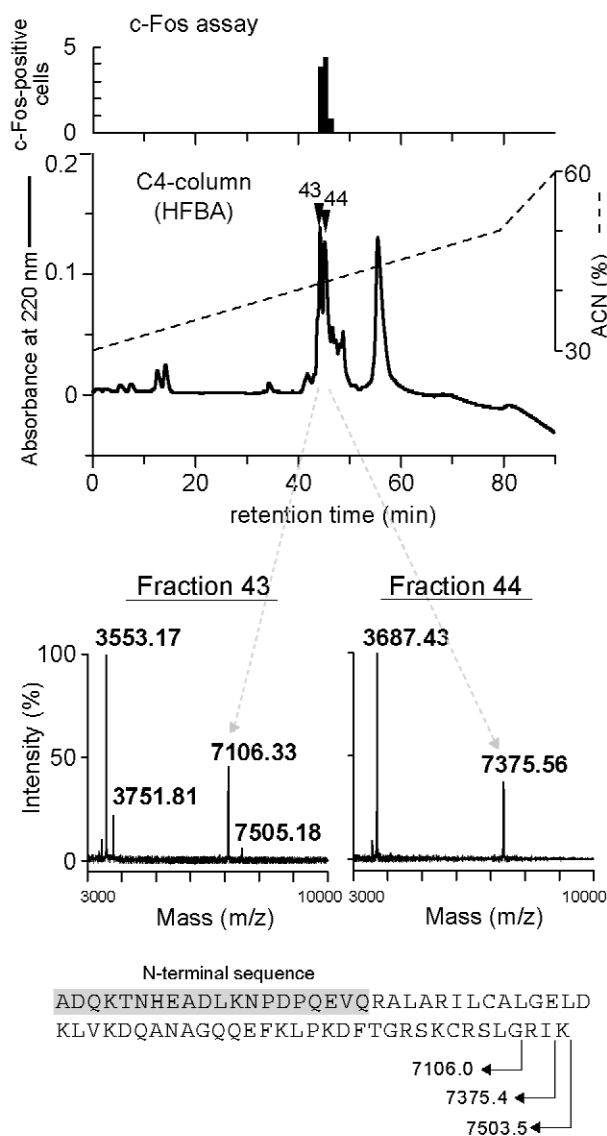
Fig. 1 Purification and characterization of peptides that induce c-Fos activity from male ELGs. Briefly, homogenate of ELGs from BALB/c adult male mice was loaded onto an anion-exchange high-performance liquid chromatography (HPLC) column (TSK-GEL DEAE-5PW; Tosoh). The flow-through, which contained the activity, was then loaded onto a reverse-phase (RP) HPLC column (PEGASIL-300 C4P; Senshu) in the presence of 0.1 % trifluoroacetic acid. All fractions were analyzed using the c-Fos assay. The active fractions were further separated using the same column but in the presence of heptafluorobutyric acid. The peaks observed at 220 nm (*second panel*) were collected, and each fraction was assayed for c-Fos-inducing activity (*top panel*). Fractions 43 and 44 were analyzed by MS and peptide sequencing. Fraction 43 (*third panel, left*) showed a major peak with a molecular mass of 7106 Da and a minor peak of 7505 Da. Fraction 44 (*third panel, right*) showed a main peak with a molecular mass of 7376 Da. The N-terminal amino acid sequences obtained from the peptides in fractions 43 and 44 are shown highlighted in gray (*bottom panel*). The molecular weights determined by MS corresponded to three different C-terminal truncations of the peptide. The numbers show the theoretical molecular weights for each peptide.

We next characterized the molecular features of the VSNs in which c-Fos expression is induced by ESP1. The vomeronasal epithelium can be divided into two functionally distinct layers characterized by the expression of putative pheromone receptors and G proteins: the apical layer, which expresses the vomeronasal receptor type 1 (V1R) class of receptors and heterotrimeric G protein α subunit type i_2 ($G\alpha i_2$); and the basal layer, which expresses the V2R class of receptors and heterotrimeric G protein α subunit type o ($G\alpha o$) [1–3] (Fig. 2A). Double-staining of c-Fos and $G\alpha o$ demonstrated that c-Fos positive neurons responding to ESP1 were localized in the basal layer (Fig. 2B).
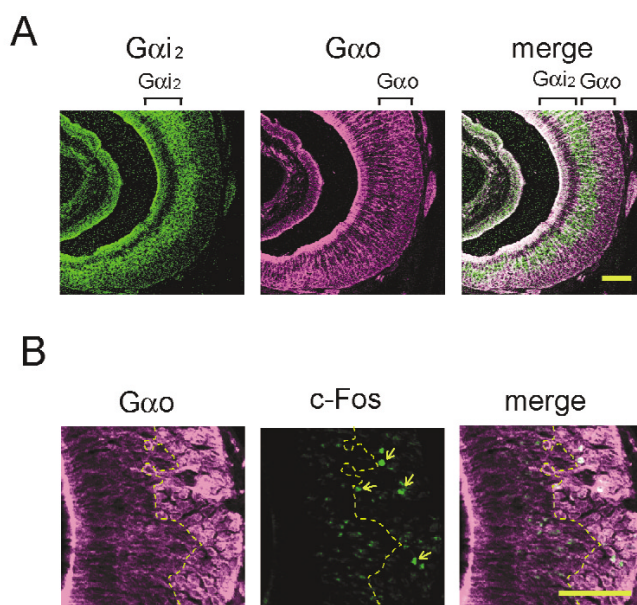

Fig. 2 c-Fos-expression observed in $G\alpha o$-neurons in the VNO. (A) Two-color fluorescent staining for $G\alpha i_2$ (*left column, green*) and $G\alpha o$ (*middle column, magenta*) in VNO. The $G\alpha i_2$ and $G\alpha o$ signals were visualized with 2-hydroxy-3-naphthoic acid-2'-phenylanilide phosphate (HNPP)/Fast Red and Alexa488, respectively. (B) Double-immunofluorescence staining for $G\alpha o$ (*left column, magenta*) and c-Fos (*middle column, green*) in VSNs. The c-Fos-positive VSNs were a subset of the $G\alpha o$-expressing VSNs. The $G\alpha o$ and c-Fos signals were visualized with Alexa546 and Alexa488, respectively. Scale bars indicate 50 µm.

We next investigated which V2R was expressed in c-Fos-induced cells by in situ hybridization. We designed 12 cRNA probes recognizing a total of 36 V2R genes that are expressed in the VNO. Each probe appeared to specifically recognize one to six annotated V2R genes and thus labeled different populations of VSNs. All c-Fos-positive neurons activated by ESP1 were labeled by the V2Rp probe that potentially hybridized with six homologous V2R genes, and they were not labeled with the other probes (Fig. 3A). Approximately 10 % of the V2Rp-labeled neurons were c-Fos-positive, indicating that ESP1 is likely received by one of the six homologous V2Rs. Indeed, most of c-Fos-positive neurons were labeled by a probe that specifically recognized a single V2R (V2Rp5) (Figs. 3B,C), but not labeled by the other five V2Rp probes.
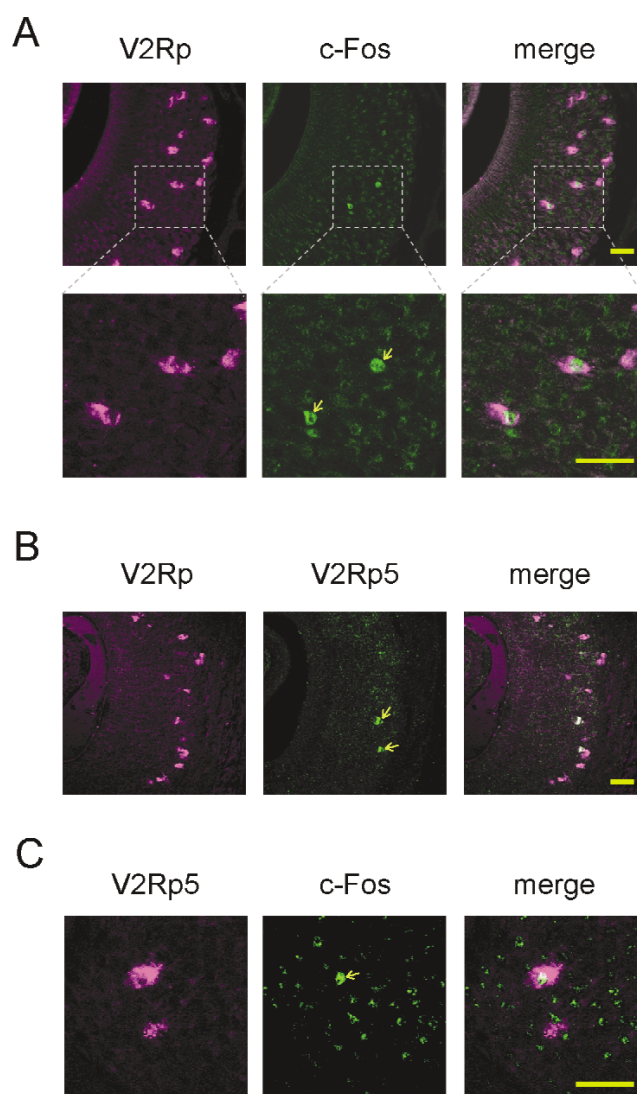
Fig. 3 c-Fos-expression observed in V2R-neurons in the VNO. (A) Two-color fluorescent localization of in situ hybridization for V2Rp (*left column*) and c-Fos immunoreactivity (*middle column*) in VSNs. All c-Fos-positive VSNs expressed V2Rp as shown in the merged view (*right column*). The V2Rp and c-Fos signals were visualized with HNPP/Fast Red and Alexa488, respectively. The *bottom panels* show enlarged views of the areas indicated by dashed rectangles. (B) Double in situ hybridization for Cy3-labeled V2Rp (*left column*) and Cy-5 labeled V2Rp5 (*middle column*) in VSNs. About 10 % of the V2Rp-labeled neurons were colabeled by V2Rp5 probe. (C) Two-color fluorescent localization of in situ hybridization for V2Rp5 (*left column*) and c-Fos immunoreactivity (*middle column*) in VSNs. c-Fos-positive VSNs expressed V2Rp5 as shown in the merged view (*right column*). Scale bars indicate 20 µm.

Basal V2R/Gαo-expressing VSNs are known to express members of the M1 and M10 families of MHC class Ib [11,12]. The M1 and M10 families comprise three (M1, M9, and M11) and six genes (M10.1–M10.6), respectively. It has been suggested that MHC genes are coexpressed with some V2Rs in specific combinations and that MHC molecules, together with a specific V2R, function as multimeric units for pheromone detection. Thus, we used double in situ hybridization to examine whether specific

MHC genes are expressed in the V2Rp5-labeled VSNs. The probes against various members of the M10 family colocalized with V2Rp5 (Fig. 4). Of the V2Rp5-positive VSNs, 11 % (10/95) were M10.1-positive, 66 % (61/92) were M10.2-positive, 100 % (72/72) were M10.3-positive, 58 % (66/114) were M10.4-positive, 61 % (69/113) were M10.5-positive, and 13 % (14/108) were M10.6-positive. Furthermore, VSNs expressing M10 family members could coexpress various V2Rs in addition to V2Rp5. For example, although all V2Rp5-positive VSNs express M10.3, approximately 90 % of M10.3-positive VSNs express V2Rs other than V2Rp5. Finally, the probes against the three genes of the M1 family did not colocalize with the V2Rp5 probe (data not shown). Thus, V2Rp5-positive neurons do not appear to express a specific type of MHC gene, suggesting that MHCs are not directly involved in the reception of ESP1.
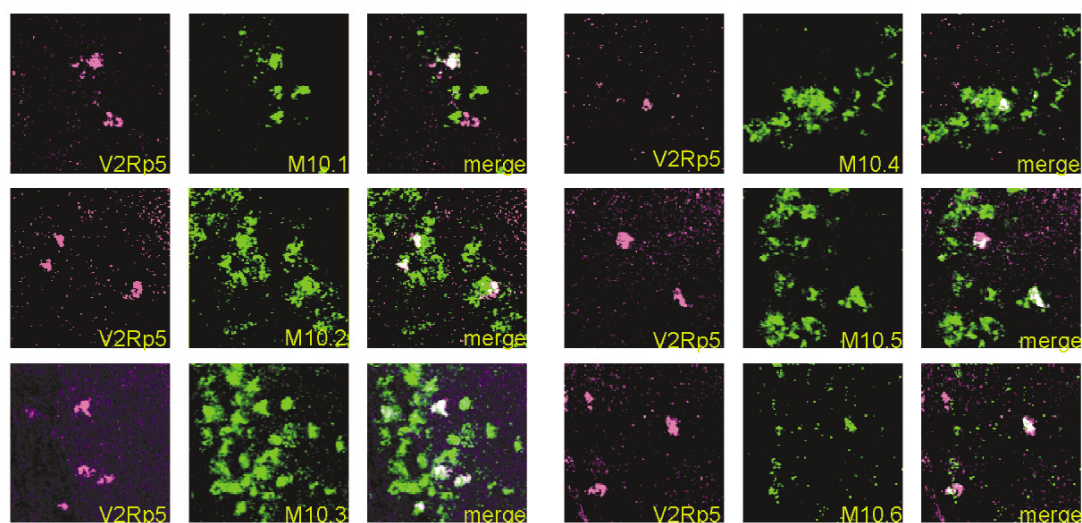

Fig. 4 Double in situ hybridization for V2Rp5 and members of the M10 family of MHCs in VSNs. Cy5-labeled V2Rp5 probes (*left column*) colocalized with various Cy3-labeled M10 family MHC probes (*middle column*).

DISCUSSION

In the present study, we describe the identification of a novel VNO-stimulating peptide, ESP1, and molecular characterization of VSNs that respond to it. ESP1 is secreted in male tear fluid and stimulates the female VNO, inducing c-Fos expression in some VSNs. Double-staining of G-protein subtypes and c-Fos revealed that ESP1 stimulates Gαo-expressing VSNs. Further, double-staining for various types of V2Rs and c-Fos suggested that ESP1 activates VSNs that express only V2Rp5. All of the c-Fos-positive VSNs express V2Rp5, whereas various types of MHC molecules are expressed in c-Fos-positive VSNs. These results suggest that V2Rs but not MHCs are mainly responsible for the reception of ESP1, and further demonstrate that pheromone detection in the VNO is narrowly tuned.

Many reports have shown that urine contains volatile pheromonal substances. For example, volatile urinary compounds, such as 3,4-dehydro-*exo*-brevicomin [13–15], 2-*sec*-butyl-4,5-dihydrothiazole [13–15], α-farnesene, β-farnesene [13,16], 6-hydroxy-6-methyl-3-heptanone [17], 2-heptanone [18,19], *n*-pentyl acetate [18,19], and 2,5-dimethylpyrazine [18,19] elicit behavioral and endocrinological changes in receptive individuals (Fig. 5B). Recently, (methylthio)methanethiol (MTMT), which is a male-specific compound in urine, was suggested to enhance the attractiveness of male urine to female mice via the main olfactory system [20] (Fig. 5).
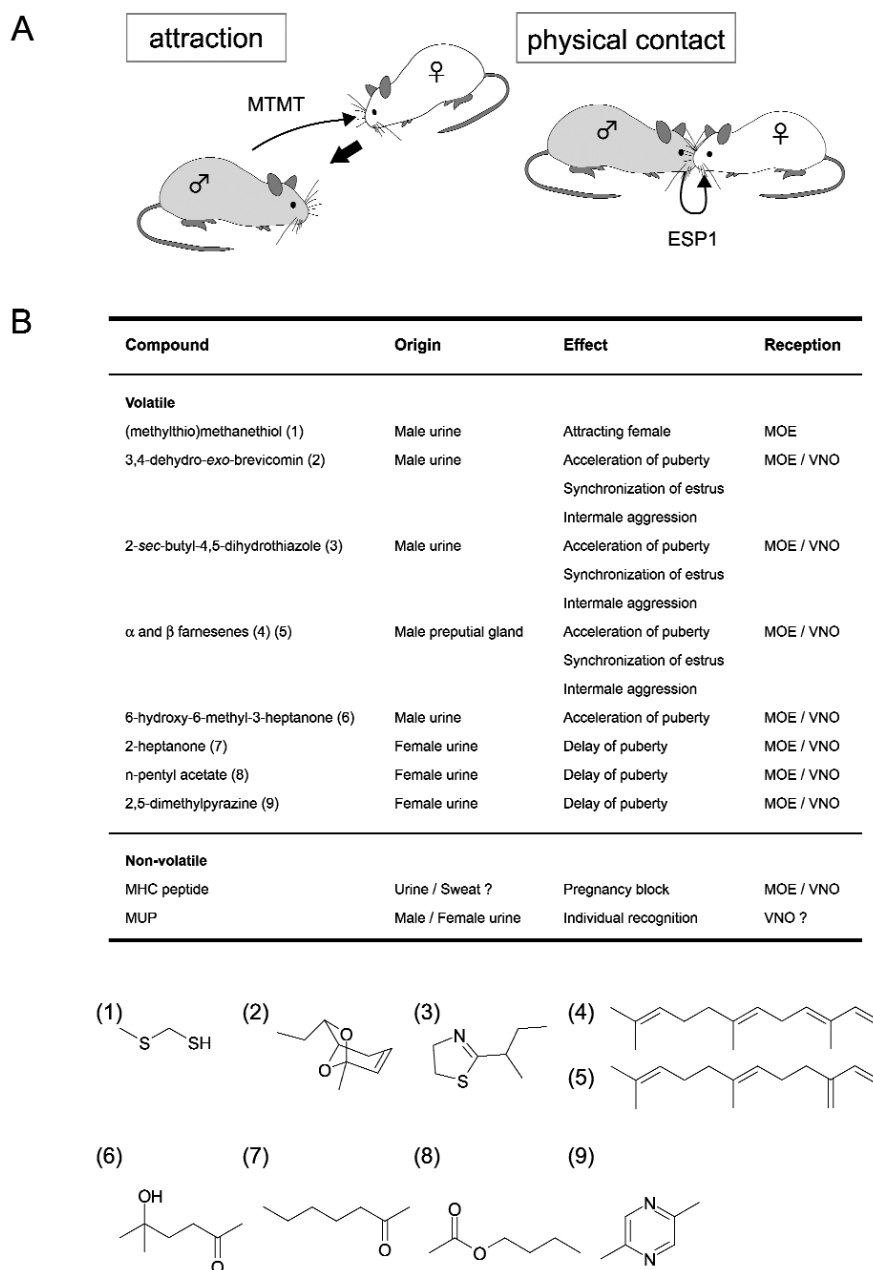
Fig. 5 Putative pheromones and their functions in mice. (A) A model of the sexual recognition process in mice. A female mouse is attracted to a male mouse releasing MTMT. Once the female reaches the male, she makes a direct physical contact and recognizes ESP1. (B) The structure, source, and possible effect in female mice of the pheromonal compounds.

In addition, nonvolatile urinary molecules, for example, the major urinary proteins (MUPs), trigger ovulation [21], acceleration of puberty [22], and participate in the recognition of individuals [23] (Fig. 5B). The MHC peptides, which are ligands for class I MHC molecules, induce a pregnancy block when they were added in urine [24] (Fig. 5B). In contrast to the volatile compounds, these nonvolatile

cues are thought to be taken up by the VNO via an active pumping mechanism upon physical contact [25]. Previous studies using calcium imaging and electrophysiological recording suggest that V1R/Gαi$_2$-expressing VSNs respond to volatile pheromones [26], whereas V2R/Gαo-expressing VSNs respond to nonvolatile compounds such as MHC peptides [24]. Biochemical experiments using rat VNO membrane preparations also show that V2R/Gαo-expressing VSNs are stimulated by MUPs [27]. Therefore, it has been thought that V1Rs are receptors for volatile chemicals and V2Rs for nonvolatile molecules. Our finding that ESP1 stimulates VSNs expressing a single type of V2R strengthens this notion.

In the current working hypothesis for sexual communication in mice, a female mouse is first attracted from a distance to a male mouse by volatile cues such as MTMT. Physical contact of the female mice with the facial areas of the male mouse covered with nonvolatile ESP1 then leads to full sexual recognition (Fig. 5A). Mice lacking cyclic nucleotide-gated channel α2, which is required for odorant signaling in the MOE, fail in sexual behaviors [28], suggesting that the MOE and the VNO are activated sequentially during sexual recognition: attractant volatile compounds from male mice detected by the MOE may provoke chemoinvestigation involving physical contact, permitting the VNO to access both volatile and nonvolatile cues.

Identification of the specific V2R expressed in ESP1-responding VSNs enables us to propose a model for ESP1-mediated signal transduction (Fig. 6). ESP1 appears to be recognized by a single V2R that likely couples to Gαo, resulting in stimulation of the phospholipase C (PLC) cascade. Subsequent generation of downstream products such as diacylglycerol (DAG), inositol-1,4,5-trisphosphate, and arachidonic acid (AA) lead to activation of the transduction channel [29,30] such as transient receptor potential cation channel subfamily C type 2 (TRPC2), which is one of the primary transduction channels in VSNs [31–33]. Our results suggest that MHC genes of the M10 family are not directly involved in the recognition of ESP1, but they may play a role in transporting V2Rp5 to the plasma membrane in the VSNs.
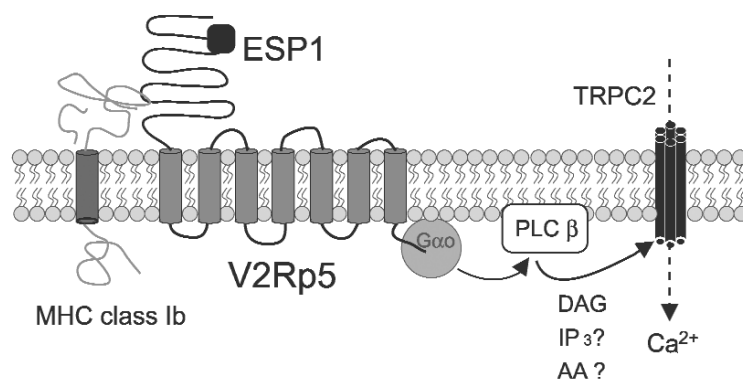

Fig. 6 A model of ESP1-mediated signal transduction in VSNs. Binding of ESP1 to V2Rp5 stimulates heterotrimeric G proteins (Gαo), which in turn triggers the PLC-β signaling cascade, enhancing the production of DAG, inositol-1,4,5-trisphosphate, and AA. This eventually leads to the opening of the TRPC2 cation channel. TRPC2, transient receptor potential cation channel, subfamily C, member 2.

In conclusion, we identified a novel male-specific peptide ESP1 that is secreted in male tear fluid, and is taken up by the female VNO, wherein it activates VSNs. Although behavioral and endocrinological effects of ESP1 remain to be elucidated, we speculate that it acts as a male signal. We also found that ESP1 stimulated VSNs expressing a single specific type of V2R. Our finding of ESP1 and its cognate receptor will help clarify the molecular mechanisms underlying pheromone recognition in the VNO system.

ACKNOWLEDGMENTS

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