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Encoding Gender and Individual Information in the Mouse Vomeronasal Organ

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Abstract

The mammalian vomeronasal organ detects complex chemical signals that convey information about gender, strain, and the social and reproductive status of an individual. How these signals are encoded is poorly understood. We developed transgenic mice expressing the calcium indicator G-CaMP2 and analyzed population responses of vomeronasal neurons to urine from individual animals. A substantial portion of cells was activated by either male or female urine, but only a small population of cells responded exclusively to gender-specific cues shared across strains and individuals. Female cues activated more cells and were subject to more complex hormonal regulations than male cues. In contrast to gender, strain and individual information was encoded by the combinatorial activation of neurons such that urine from different individuals activated distinctive cell populations.

Pheromones are a group of chemicals critical for social communication in many animal species (1,2). Information on sex, strain, social rank, reproductive status, and terrestrial ownership is represented in the complex pheromone components in urine and bodily secretions. In mice, detection of such complex chemical signals by the vomeronasal organ (VNO) and the olfactory epithelium plays an important role in triggering endocrine changes and eliciting innate territorial aggression and mating behaviors (3-5). The rodent VNO expresses more than 250 receptors that detect pheromones and transmit the signals to the brain (6-11). It is not well understood how these neurons encode information about gender and individuals. Urine contains hundreds or even thousands of substances, only a handful of which have been identified as putative pheromones (12-16). The complexity of natural pheromone signals poses a challenge to our understanding of what information is transmitted to the vomeronasal neurons (17,18).

Each vomeronasal neuron expresses only one of the ~250 estimated pheromone receptor genes (6-9,19,20), and the receptor's activation elevates intracellular calcium (21). To visualize pheromone-induced activity in a large population of neurons, we generated tetO-G-CaMP2 transgenic mouse lines (22-24). When crossed to animals carrying the OMP-IRES-tTA allele (25), G-CaMP2 expression was restricted to the neurons in the olfactory system (Fig. 1, A and B, and Movie S1). Electrophysiological properties of the G-CaMP2-expressing VNO neurons, as well as their response to pheromones, were indistinguishable from those of the controls (fig. S1). The projection patterns of the sensory neurons and the innate mating and aggressive behaviors of the G-CaMP2 mice were also indistinguishable from those of wild-type and littermate control animals (figs. S2 to S5).

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In VNO slices prepared from 2-to 6-month-old male or female animals, application of diluted urine elicited an increase in fluorescence in ~30 to 40% of G-CaMP2-positive neurons, some of which showed gender-specific responses (Fig. 1C and Movies S2 and S3). We did not observe significant differences between slices from male and female animals in detecting the gender-specific cues. Prolonged applications of urine elicited prolonged calcium increases (Fig. 1D). This non-adaptive nature of the responses was in agreement with electrophysiological recordings reported previously (17,26). In addition, the responses were dose dependent and were blocked by 2-APB and U71344, inhibitors of signaling pathways downstream of pheromone receptor activation (21,27) (Fig. 1, C and D, and fig. S7). Thus, the expression of G-CaMP2 provided us an easy and sensitive method to examine population responses of VNO neurons to multiple urine samples.

Initial analyses of VNO neuron response to male and female urine pooled from multiple individuals of the C57BL/6 strain showed that ~15% of GCaMP2-positive cells responded to both male and female urine. About 8% and 12% of the cells responded specifically to pooled male or female urine, respectively (Fig. 1C), suggesting that urine contained cues that were recognized by the VNO neurons to discriminate gender. To determine whether the gender-specific cues were shared among different individuals, we used individual urine from three strains (CBA, C57BL/6, and CD-1) to stimulate the same VNO slices. The activation patterns of the VNO were analyzed both visually (Fig. 1E) and using heat map plots (fig. S6 and Fig. 2A). In ~2100 G-CaMP2-positive cells (a total of eight slices from six animals), 5.0% ($n=106$) were activated by one or more male urine samples but not by female urine, whereas 9.5% ($n=200$) were activated by at least one female sample. However, most of these cells responded only to a subset of the sex-specific samples, suggesting that they could not contribute unequivocally to gender discrimination. Only a very small population of cells responded exclusively to urine samples from all individuals of the same sex but not the other (Fig. 2A). Cells that responded to all male samples, the male urine-specific cells (MUSCs), constituted less than 1% of the G-CaMP2-expressing neurons ($n=20$, 0.95%). The female urine-specific cells (FUSCs), which responded exclusively to all the female samples, constituted 2.6% ($n=54$) of the cells. We did not observe obvious differences in the percentage of MUSCs and FUSCs in male or female VNO slices (table S1). This was consistent with the previous studies of pheromone receptor genes, which showed little sexual dimorphism in their expression patterns (6-9). The MUSCs and FUSCs were found in both G_o and G_{12} layers in the VNO (table S2).

We applied cluster and principal components analysis (PCA) to identify the major variables that contributed to the differences in urine signals. Cluster analysis of the 134 responsive cells in Fig. 2 showed that the patterns of activation were grouped according to gender (Fig. 2D). Within each group, the samples were further grouped by strain. In PCA, the first principal component (PC1, ~35% of variance) separated the urine by gender. Interestingly, strains of the males were separated by the second principal component (PC2, 18% of variance) (Fig. 2B), whereas strains of the females were separated by PC3 (13%) (Fig. 2C). Analyses of multiple slices from both male and female animals produced similar results.

Each principal component is a linear summation of contribution by different cells in the group. Although MUSCs and FUSCs only represented <10% of all responding cells, they had the highest coefficient values and contributed a weighted average of >30% to PC1 (fig. S8). Further analyses by removing the MUSCs and FUSCs from the PCA pool showed that this “virtual knockout” compromised gender discrimination. Without MUSCs and FUSCs, urine samples were no longer segregated according to gender (fig. S9B). Removing equal numbers of cells that were activated only by subsets of sex-specific urine samples or removing equal numbers of random cells had little impact on segregation according to gender (fig. S9, C to F). Thus,

despite their small numbers, the MUSCs and FUSCs appeared essential for gender discrimination.

The MUSCs and FUSCs must recognize gender-specific cues emitted by individual mice. Altering the sexual characteristics of an animal should affect the expression of such cues. We thus analyzed the patterns of activation by urine from castrated C57BL/6 males. Castrate urine activated more cells than male urine (Fig. 3, A and B, and fig. S10), but it no longer activated the MUSCs (Fig. 3C). Concurrent with the loss of response from MUSCs, a number of cells that did not respond to any male urine were activated, some of which were FUSCs (Fig. 3F and fig. S10).

In contrast to MUSCs, female-specific cells recognized cues that were regulated by more complex hormonal states, such as estrus cycles. We induced ovulation and collected urine daily from the same female mice throughout their estrus cycles. Urine from different time points during the estrus cycle elicited distinct patterns of activation (Fig. 3, D to F). Estrus urine activated more cells (fig. S11), and some of the additional cues were shared across individuals of different strains. These cues thus activated additional FUSCs (Fig. 3F and fig. S10).

In addition to gender, urine also provides information that identifies individuals. Indeed, our experiments showed that no two urine samples elicited identical patterns of activation. The activation patterns in the VNO distinguished not only gender but also the strains of the animals (Fig. 2D). Furthermore, littermates from the same strain were distinguishable, and such distinction was significantly larger than variations among repeated applications of the same sample (fig. S12).

How are individuals distinguished? The principal components PC2 and PC3 were composed of a large population of cells. More than 50% of responding cells showed activation by multiple samples, suggesting that individual information could be encoded by the combinatorial activation of the neurons (Fig. 2A). A combinatorial code predicts that urine from similar animals activates more common cells and fewer unique cells and vice versa. We examined the responses to urine samples from littermate and non-littermate C57BL/6 males. For non-littermates, ~36% of the responsive cells were shared by all four male urine samples (Fig. 4A), whereas for littermate urine, this number increased to ~87.5% (Fig. 4B). Concurrent with the increase in shared cells, the number of cells responding to single urine samples decreased from 25% to ~8.3% (Fig. 4, A and B). These observations were consistent with the prediction of a combinatorial code for individual identities.

If individuals are identified by the combinatorial activation of VNO neurons, are there cells providing unique identifications for the strains? Recent evidence suggests that the MHC class I peptides may serve as strain-specific signals by directly activating the VNO neurons (16). Because each peptide is unique to a specific strain, one expects to find strain-specific cells that are activated by urine samples from different individuals of the same strain. Analyses of the activation patterns in our experiments, however, did not provide evidence for such strain-specific cells, even when the analyses were expanded to ~12,000 neurons (also see Fig. 2). We further compared responses elicited by strain-specific MHC class I peptides to those by urine of the same strain. Figure 4, C and D, shows one such experiment with AAPDNRETF, a MHC class I peptide identified in the C57BL/6 strain, and urine from male and female C57BL/6 mice. The peptides indeed elicited responses from a subset of VNO neurons, but the cells activated by urine and by the peptides did not overlap.

Our experiments demonstrate that the mouse VNO encodes information of gender and individual in urine pheromones with two distinct strategies. Gender is encoded by a small percentage of dedicated neurons, the MUSCs and FUSCs. Because the VNO expresses ~250

pheromone receptors, the MUSCs correspond to ~2 to 3 receptors. This result, together with the finding of a single male-specific peptide in the mouse exocrine gland (28), suggests that a small number of testosterone-dependent cues is required for male identification. FUSCs represent a larger percentage of cells (~3%). The number difference in gender-specific cells may reflect the more complex physiology and hormonal regulation in female animals.

Individual information, on the other hand, is encoded by the combinatorial activation of VNO neurons rather than dedicated cells that respond specifically to class I MHC peptides. Fragments of the MHC complexes were found in considerable amounts in urine (29), but it has never been demonstrated that MHC class I peptides exist in detectable free form in urine (29). It remains possible that the skin or body glands may contain these peptides as natural ligands instead. Pheromone signals in urine that identify strains may result from the expression of a set of pheromones or the carriers of pheromones, such as the major urinary proteins that are known to be genetically determined (30,31). Using a combinatorial code to represent individuals and strains is likely to be advantageous because 100 to 200 receptors can provide a virtually unlimited coding space. In contrast, information about gender and certain hormone-regulated states is perhaps better served by dedicated cells, because the information is largely shared by animals of different strains as a result of common physiology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Detection of urine-elicited responses in the VNO of G-CaMP2-expressing mice. (A) Expression of G-CaMP2 in the neurons of the mainolfactory epithelium (MOE), the vomeronasal organ (VNO), and their axonal projections to the olfactory bulb (OB). (B) Twophoton image of a VNO slice used in an imaging experiment. (C) VNO responses to pooled C57BL/6 female urine (green) or male urine (red). The VNO slice (from a 4-month-old male) was stimulated with female urine (F.U.) under control (c1), treatment with 50 μ M 2-APB (c2), and recovery (c3) conditions and male urine (c4, M.U.). Merge shows cells that respond to both male and female urine (c5). C6 shows the response traces of the three cells indicated in c1 to c5. (D) Fluorescence changes for a neuron responding to female urine applied for 10 (black), 20 (red), and 30 (blue) seconds, 10 s application following 2-APB treatment (black dot), and recovery (black dash), respectively. (E) The patterns of activation of a VNO slice by six different urine samples from different sex and strain animals are color-coded and shown in a merged picture.

Fig. 2.

VNO responses to individual male and female mouse urine. **(A)** Heat map of 134 VNO neurons from a single slice (from a 3-month-old male) that responded to male and female urine from C57BL/6 (B6), CBA, and CD-1 strains. **(B and C)** Principal components analysis of the data shown in two-dimensional plots for PC1 and PC2 in **(B)**, and PC1 and PC3 in **(C)**. Urine from males and females is labeled with black and red, respectively. **(D)** Hierarchical cluster analysis of responses shown in **(A)** is plotted as a dendrogram based on distance obtained from Pearson correlations between responses to different urine applications.

Fig. 3.

Hormone regulation of sex pheromones. (A) Responses to urine from two C57BL/6 males and castrated males in a VNO slice from a 2-month-old female. Two cells with differential responses are indicated. (B) Response traces of the cells indicated in (A). (C) A heat map showing all identified MUSCs, none of which responded to castrate urine. (D) Responses to female urine collected from a C57BL/6 mouse after injection of pregnant mare serum gonadotrophin in a VNO slice (from a 3-month-old male). Responses to urine collected on day 1 and day 4 are shown. Three cells with differential responses are indicated. (E) Response traces of the cells marked in (A). (F) A heat map showing the identified FUSCs (19 cells from two slices from a 3-month-old male and a 2-month-old female). Group A cells were activated by estrous urine from all three strains. Group B was activated by both estrous and diestrus urine, but not castrate urine. Group C responded to estrus, diestrus, and castrate urine. ♂, ♀, and ○ represent male, female, and castrate animals, respectively; E, day 1 (estrus); D, day 4 (diestrus).

Fig. 4.

Response of VNO neurons to different individuals and to MHC peptide. (**A** and **B**) Heat maps and pie charts of responses to urine from non-littermates (**A**) and littermates (**B**). The pie charts show percentages of cells activated by different numbers of urine samples. (**C**) Response patterns of a VNO slice to urine from a C57BL/6 male, a C57BL/6 female, and 10^{-9} M AAPDNRETF peptide, identified in the C57BL/6 strain. (**D**) A heat map for the responses summarizing the responses. The slice was from a 2-month-old female.