right). MV spread is therefore likely to be connected with apoptosis and a preprogrammed macrophagic response of neighboring cells to apoptotic bodies.

Vaccinia MVs use macrophagocytosis and apoptotic mimicry to enter host cells. There are several advantages of using this entry strategy. First, it permits endocytic internalization of particles too big for other viral endocytic mechanisms. Second, it allows the virus to enter many different cell types, because PS-mediated clearance of apoptotic material is common to most cells (19, 26). Finally, by mimicking an apoptotic body, MVs may avoid immune detection as they spread to surrounding cells, because macrophagocytosis of apoptotic debris suppresses the activation of innate immune responses (27). The lack of macrophage infiltration and T cell maturation during murine lung infection by vaccinia (27) may be explained by this “silent” mechanism of cell-to-cell spread.

References and Notes
6. Materials and methods are available as supporting material on Science Online.
28. We thank P. Traktman for providing viruses; H. Ewers for the production of liposomes; R. Sacher, B. Snijder, and L. Pelmans for assistance with siRNA screening; and the members of the Helenius lab for helpful discussion. Funding was obtained from ETH Zurich and the Roche Foundation.

Supporting Online Material
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Materials and Methods
Figs. S1 to S10
Table S1
References
Movies S1 to S6
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Encoding Gender and Individual Information in the Mouse Vomeronasal Organ
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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).

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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-IRE-sRTA 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).

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 G-CaMP2—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

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 main olfactory epithelium (MOE), the vomeronasal organ (VNO), and their axonal projections to the olfactory bulb (OB). (B) Two-photon 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 μM 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 334 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 dendogram based on distance obtained from Pearson correlations between responses to different urine applications.
studies of pheromone receptor genes, which showed little sexual dimorphism in their expression patterns (6–9). The MUSCs and FUSCs were found in both G6 and G12 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, Fig. 4.

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.
~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. 8B). 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 female 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 cells, because the information is largely shared by animals of different strains as a result of common physiology. 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.

References and Notes

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Materials and Methods
Fig. S1 to S12
Tables S1 and S2
References
Movies S1 to S3
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Encoding Gender and Individual Information in the Mouse Vomeronasal Organ

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This PDF file includes:

Materials and Methods
Figs. S1 to S12
Tables S1 and S2
References

Other Supporting Online Material for this manuscript includes the following:
(available at www.sciencemag.org/cgi/content/full/320/5875/535/DC1)

Movies S1 to S3
Materials and Methods

Animals

Conventional molecular techniques were used to generate the tetO-G-CaMP2 plasmid. The coding region of G-CaMP2 (S1, S2) was placed behind a tetracycline-dependent promoter (S3, S4) and followed by a polyA tail. The plasmid was linearized for pronuclei injection. Founders were crossed to OMP-IRES-tTA line (S4) to restrict expression of G-CaMP2 in the olfactory sensory neurons. Animals were maintained in Lab Animal Service Facility of Stowers Institute at 12:12 light cycle, and provided with food and water ad libitum. Experimental protocols were approved by the Institutional Animal Care and Use Committee at Stowers Institute and were in compliance with NIH Guide for Care and Use of Animals.

Pheromones and Chemicals

Urine samples were collected from animals using the free-catch method. The freshly collected urine samples were frozen at -80 °C until use for up to three months. Castration of male mice was performed on 3-4 week old animals and urine was collected 6 weeks after surgery. Ovulating females were prepared by injecting 4-week old mice with 5 IU pregnant mare serum gonadotropin (PMSG) to induce super-ovulation and synchronized estrus. The urine samples were collected daily after 48 hours of PMSG injection for up to five days. 2-APB was obtained from Alexis Biochemicals and U73122 was obtained from Sigma-Aldrich.

VNO Slice Preparation

2-6 months old mice of both sexes were decapitated following CO₂ euthanasia. The VNO was removed from the bone capsule and embedded in 4 % low melting agarose.
200 μm coronal slices were prepared in oxygenated mouse artificial cerebrospinal fluid (mASCF) at 4°C using Vibratome 3000 sectioning system. Slices were kept in oxygenated mACSF at room temperature for up to 6 hours. The composition of mACSF is (in mM): 130 NaCl, 5 KCl, 1 MgCl$_2$, 2.5 CaCl$_2$, 1.25 NaH$_2$PO$_4$, 25 NaHCO$_3$, 10 Glucose.

Confocal, 2-Photon and Calcium Imaging

Confocal images were obtained by a confocal system using 10x water immersion objective excited with a 488nm laser. Two-Photon images of the VNO slice were captured on the same system using a Chameleon Laser System at 900 nm wavelength.

Calcium imaging: VNO slices were continuously perfused with oxygenated mACSF at room temperature. Urine was delivered at 1:100 fold dilution via a BSP-8PG system using either 8 or 16-channel micromanifold. To minimize mechanical artifacts, a continuous flow of Ringer solution (in mM, 115 NaCl, 5 KCl, 2 CaCl$_2$, 2 MgCl$_2$, 25 NaHCO$_3$, 5 HEPES) was maintained during the experiment. Flow speed was controlled at 2-3 μl per second. Inhibitors were delivered through dedicated channels for 10-15 min before pheromone applications. The final concentrations of U73122 and 2-APB, were 5 and 50 μM, respectively.

For time-lapse recording, an EXFO X-Cite 120PC light source passed through a bandpass filter (450-490nm) was used to excite the samples. The epifluorescent images were acquired by a CCD camera with 2x2 or 4x4 binning depending on the expression levels of different G-CaMP2 lines. At the end of some experiments, confocal images were taken to ensure no cells overlapped in the regions of interest. For most experiments, repetitive applications and reverse order applications were performed to ensure reproducibility of the responses.

Data analysis

Image processing was performed using ImageJ v1.37c software (http://rsb.info.nih.gov/ij/, NIH, Bethesda, MD). Briefly, a background image sequence was generated by applying Gaussian filter (radius 50 pixels) from the raw image sequence. A scattering-corrected image (S5) was obtained by subtracting the background
from the raw image sequence. Subsequently, the scattering-corrected image sequence was divided into pre- and post- pheromone application substacks. Responding cells emerged from the background after subtracting the pre- from the post-application stack. To obtain information on response amplitude and dynamics, individual responsive cells were identified manually using the Multi-Measure Plug-In in ImageJ. Response curves were plotted as ΔF/F.

The response heatmaps were produced using a custom written program in Bioconductor R package (S6) (http://www.R-project.org). The maps were based on the value according to the ΔF/F of each individual responses using Heatmap 2 function in R2.4.1 (color key designation: 0, ΔF/F < 0.1; 1, 0.1< ΔF/F<0.2; 2, 0.2< ΔF/F<0.3; 3, 0.3< ΔF/F<0.4; 4, ΔF/F > 0.4). Only the responses that could be repeated were plotted.

**PCA and cluster analysis:** To perform PCA and cluster analysis, a response matrix was first generated by assigning 1 to cells with ΔF/F> =0.1 and 0 to cells with ΔF/F<0.1. For hierarchical clustering, the R hclust() function was applied to the response matrix to group the samples. Pairwise Pearson correlation values were calculated between samples and the distance was defined as 1-correlation. The similarity between different samples was plotted as a dendrogram, which grew heuristically by merging pairs of most similar samples into clusters.

Principal components analysis was performed with the Mulvar Principal Components function in PAST, an open source statistical analysis software (PAlaeontological Statistics, ver.1.71; http://folk.uio.no/ohammer/past). Variance-covariance matrix was used.

**Electro-vomeronasogram (EVG) Recording**

In EVG experiments, local field potentials were recorded from the microvillous layer of intact VNO sensory epithelia as described (S7, S8). Briefly, the VNO epithelium was exposed and perfused with oxygenated mACSF. Field potential was recorded using glass pipettes (10 micron diameter) connected to an AI 401 pre-amplifier. The signals were further amplified by a signal conditioner, digitized at 1 kHz and low-pass filtered at
20 Hz. The data was further analyzed using Clampfit 10.0. Urine was delivered through another glass pipette controlled by Pressure System 2.

**Patch Clamp**

Quartz electrode (OD 1.2 mm, ID 0.9 mm) was fabricated and polished to have resistance ranging from 3 MΩ to 7 MΩ with P-2000 micropipette puller. Whole cell recordings were performed after forming giga-Ω seal. The pipette solution contained (in mM): 130 KCl, 1 KOH, 1 MgCl₂, 1 EGTA, 10 HEPES, 0.5 GTP Na-salt, 2 ATP Mg-salt (pH 7.1, 290 mOsm). Signals were amplified and recorded with MultiClamp 700A amplifier and digitized with Digidata 1440A. Spontaneous action potentials and resting membrane potentials were measured under current clamp mode (I = 0) with 10-kHz sampling rate and filtered at 0.5 kHz. Data were acquired and analyzed with Clampex 10.1 and Clampfit 10.1 software.

**X-gal Staining**

X-Gal staining was performed as described in Mombaerts et al. (S9).

**Single Cell RT-PCR**

Cells identified in imaging experiments were isolated individually using a modified patch clamp electrode with an opening of 3-5 μm. RT-PCR reactions were performed as described in Dulac and Axel (S10) to generate single cell cDNA library. Additional steps of PCR were performed to detect G₁₂ and G₉ expression using primer pairs: G₁₂ forward 5’-CACGGTGTGCAAGCCTGCTT-3’ and reverse 5’-GGGAAACAGATGGTCAGGGAGC-3’; G₉ forward 5’-ACAAGGCTACCTGCTCAATTCTGCC-3’ and reverse 5’-CAGCCCTGCTTGTACCTTGCTT-3’.

**Resident-intruder Aggression and Mating Assay**
Aggression and mating assays were performed as described previously (S8).

Briefly, G-CaMP2 positive mice and control mice were single-housed right after weaning for 4-5 weeks before testing. For intruder assay, each test lasted 15 min and started with the introduction of a group-housed adult C57BL/6 male into the home cage of the test mouse. The cage beddings of testing animals were maintained for at least one week. Latency to first attack, cumulative attack duration and number of attacks were scored with the help of the Observer 5.0 software by experienced personnel. Two days after the completion of the resident-intruder assay, the mice were used in mating assays. Mating tests lasted 30 min and started with putting a C57BL/6 estrous female into the home cage of the test mouse. Latency to first mount, total number of mounts and cumulative mount duration were scored as above.

One week after the first intruder assay, animals were subject to a second round of tests following the same schedule. These animals were considered sexually experienced.

Reference:

Fig. S1. EVG Recordings of G-CaMP2 and Control Mice

A. Representative traces of EVG responses to 100-fold diluted mouse urine from the VNO of control and G-CaMP2 expressing animals. Bars above the traces indicate duration of urine application. B. Bar graph of EVG amplitude recorded from 7 control and 4 G-CaMP2 animals. Error bar shows standard deviation. C. Spontaneous firing for control and G-CaMP2 expressing VNO neurons. D. Resting membrane potentials (RMP) for control and G-CaMP2 cells. The number of cells recorded from each group is indicated.

Fig. S2. Expressions of VN2 and VN12 Receptors in G-CaMP2 and Control Mice

X-gal staining of VNO sections showing the VN2 (A and B) and VN12 (C and D) positive cells in control and G-CaMP2 expressing animals. Genotypes of the animals are indicated.

Fig. S3. Projection Patterns of VN2 and VN12 Neurons in G-CaMP2 and Control Animals

A. Whole mount X-gal staining of bisected heads showing the axonal projections of VN2 neurons in control (a-c) and G-CaMP2 (d-f) animals. B. Staining shows the projections of VN12 cells for control (g-i) and G-CaMP2 (j-l) animals. Genotypes of the animals are indicated.

Fig. S4. Aggression Assay of the G-CaMP2 and Control Animals
Bar graphs for average latency to first attack (A), number of attacks (B) and cumulative attack duration (C) for control (OMP-IRES-tTA; black bars) and G-CaMP2 (OMP-IRES-tTA/tetO-G-CaMP2; red bars) animals.

**Fig. S5. Mating Assay of the G-CAMP2 and Control Animals**

Bar graphs for average latency to first mount (A), number of mounts (B) and cumulative mount duration (C) for control (OMP-IRES-tTA; black bars) and G-CaMP2 (OMP-IRES-tTA/tetO-G-CaMP2; red bars) animals.

**Fig. S6. Illustration of Image Processing Paradigm**

Time-lapse image sequences were separated into pre- and post-application substacks. Each frame was Gaussian filtered and subtracted from the original raw image. The pre-application stack (Background) was subtracted from the post-application stack (Response). Each responsive cell was identified and the trace was plotted. The amplitude of the responses from different cells to different applications was color-coded to generate a heatmap.

**Fig. S7. U73122 Inhibits Pheromone-Induced Responses**

A-D. Responses elicited by 1:100 dilution of female urine (F.U.) under normal (A), DMSO (carrier for U73122) treated (B), U73122- treated conditions (C) and following 20 minutes recovery (D). E. Fluorescent signal traces of one of the responding cells shown in A-D.
Fig. S8. Coefficient Values for Principal Component 1

The coefficient values for the 134 cells in PC1 from Fig. 2 are plotted as a bar graph in descending order. MUSCs, FUSCs and the cells removed randomly for PCA analyses shown in Fig. S9 are colored red, green and magenta, respectively.

Fig. S9. Comparison of Principal Components Analyses of Different Subsets of Cells

A. PC1 vs. PC2 plot for PCA using all cells that responded to urine (identical to Fig. 2B) shows segregation of urine samples according to the sex. B. The same plot with MUSCs and FUSCs removed (total 13 neurons) from the analyses. C and D. PC1 vs. PC2 plots with 13 cells randomly removed from the data set. The cells randomly removed for the two sets were marked in Fig. S8.

Fig. S10. Responses to Castrate Urine

A. The total number of responsive cells to individual male and castrate urine. B. Response profile to different urine applications for a subset of cells that responded to male but not castrate urine. The heatmap is produced based on a total of 138 responsive cells in 3 slices from 2 animals. The cells in the group A respond to male urine specifically, not to castrated male and female urine. C. Response profile for a subset of cells that responded to castrate urine. The heatmap is produced based on a total of 207 responsive cells in 2 slices from 2 animals. The cells in the group A are only activated by castrate urine. The cells in the group B are activated by both castrate and female urine, but not male urine. Cells in group C are activated by both castrate and male urine.
Symbols and abbreviations: ♂, ♀, ○ represent male, female and castrate animals, respectively; E: Day 1 (estrous); D: Day 4 (diestrous).

**Fig. S11. Responses to Estrus and Diestrus Urine**

A. VNO responses to female urine collected from a C57BL/6 mouse after PMSG injection. Collection starts at 48 hours after injection (Day 1). Three cells that show differential responses are indicated (same as Fig. 3D). B. Total number of responsive cells to the female urine collected from Day 1 to Day 5.

**Fig. S12. Comparison of responses to littermate urine samples and to repetitive applications**

Hierarchical cluster analysis was plotted as a dendrogram to compare responses to urine samples from two C57BL/6 littermates and repeated application of the same urine. The images showed similar patterns of activation of the two urine applications.
Supplemental Movies:

Movie S1. Three-dimensional reconstruction of 2-Photon images of a coronal VNO slice used in experiments.

Movie S2. Responses of a VNO slice to application of male urine without image processing. A circle on the upper left corner indicated the duration of urine application.

Movie S3. Processed images shown in Movie S2.
Fig. S3

A. VN2-IRE5-ctacZ

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B. VN12-IRE5-ctacZ

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<td></td>
<td></td>
</tr>
<tr>
<td>k</td>
<td></td>
<td></td>
</tr>
<tr>
<td>l</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. S4

Fig. S5

[A] Latency to First Attack (sec)
[B] Number of Attacks
[C] Cumulative Attack Duration (sec)

[A] Latency to First Mount (sec)
[B] Number of Mounts
[C] Cumulative Mount Duration (sec)
Fig. S8

Fig. S9.

A. All Cells  
B. MUSCs and FUSCs removed  
C. Random Cells Removed (1)  
D. Random Cells Removed (2)  
E. Sex-biased cells  
F. Sex-biased Cells Removed
Fig. S10

A. 

B. 

C. 

Color Key

Fig. S11

A. 

Day 1  Day 2  Day 3  Day 4  Day 5  

Estrous  →  Diestrous

B. 

Number of Responsive Cells

Day
Fig. S12
Table S1. Distribution of MUSCs and FUSCs in VNO slices obtained from male and female animals

<table>
<thead>
<tr>
<th>VNO slices</th>
<th>MUSCs</th>
<th>FUSCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Number of Slices</td>
<td>Total MUSCs</td>
</tr>
<tr>
<td>Male</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>Female</td>
<td>5</td>
<td>13</td>
</tr>
</tbody>
</table>

*: Expressed as Mean and SD. At the 0.05 level, One-Way ANOVA shows the population means are not significantly different.

**: Expressed as Mean and SD. At the 0.05 level, One-Way ANOVA shows the population means are not significantly different.

Table S2. Identification of $G_{i2}$ and $G_{o}$ signal in MUSCs and FUSCs

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>$G_{i2}$</th>
<th>$G_{o}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUSCs</td>
<td>9</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>FUSCs</td>
<td>12</td>
<td>8</td>
<td>4</td>
</tr>
</tbody>
</table>