Supporting Information

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SI Materials and Methods

Animals. All experiments were approved by the Animal Care and Use Committee of the Faculty of Agriculture of the University of Tokyo and were based on guidelines that were adapted from the Consensus Recommendations on Effective Institutional Animal Care and Use Committees by the Scientists Center for Animal Welfare (1).

Experimentally naïve male Wistar rats were purchased at 7 wk of age (Clea) and housed in pairs in wire-topped transparent cages (400 × 250 × 180 mm) with wood shavings for bedding. For experiments assessing Fos expression (see below), experimentally naïve male Wistar rats were purchased aged 8 wk and housed individually in the same wire-topped transparent cage. The animals were provided with water and food ad libitum and kept on a 12-h light–dark cycle (lights turned off at 2000 hours). The vivarium was maintained at a constant temperature (24 ± 1 °C) and humidity (40–45%).

Collection and Analysis of Released Volatiles from the Perianal Region of Donor Rats. Eight donor rats were used for each experimental analysis. We anesthetized each male adult donor rat with sodium pentobarbital (10–16 wk of age; 50 mg/kg Somnopentyl; Schering–Plough Animal Health) and electrically stimulated the perianal region (10 V for 1 s, 20 s intervals, 10 min) via two intradermal needle electrodes (27 G). The electrical stimulation of the perianal region in this manner induced the release of the stress-related odor (2). During electrical stimulation, the released odor was sucked into a glass tube (100 × 6 mm, 1 mm thickness) packed with 0.25 g of adsorbent Tenax (TENAX-TA; Buchem B.V.) with a simple glass funnel, which was connected to the donor side of the tube, using a suction pump (300 mL/min; MP-2N, Sibata Scientific Technology). For control samples, the odor released from skin on the neck was adsorbed on Tenax by the same method. All procedures were performed in a cylindrical glass desiccator filled with activated charcoal-filtered air (Fig. S2).

Frac. 1, 2, and 3 were prepared by using an Agilent 7890A gas chromatograph equipped with an on-column injector and a TC-WAX column (0.53-mm i.d. × 30 m; GL Sciences Co.). Volatiles trapped in Tenax were extracted with purified ether. Each ether-extracted sample was injected in a split mode (50:1) at a constant temperature of 250 °C. The column temperature was kept at 40 °C for 3 min and then increased to 230 °C at a rate of 3 °C/min. Helium was used as the carrier gas at a flow rate of 10 mL/min.

After being dissolved in purified ether, Frac. 1 was analyzed or was further fractionated by using an Agilent 7890 gas chromatograph combined with a 5973 mass selective detector and a flame ionization detector (FID; 250 °C) equipped with a TC-WAX capillary column (0.25-mm i.d. × 60 m). The effluent of the column at the end of the capillary was divided into two branches and routed by deactivated fused silica capillaries to the mass detector and FID. The oven temperature was maintained at 40 °C for 3 min and then increased to 230 °C at a rate of 5 °C per min, with a constant flow of helium as the carrier gas at 1.8 mL/min.

At the final phase of molecule identification, 50 male donor rats were used for the analyses. The perianal region of each donor rat was electrically stimulated by using the same method as described above, and the released odor was sucked by suction pump into a thermal desorption system (TDS) tube (Gerstel KK) packed with Tenax using a newly developed glass funnel connected to the donor side of the tube. These procedures were performed in a glovebox under positive pressure with clean synthetic air (Fig. S6). Blank adsorption inside the glovebox was performed by using the TDS tube to obtain a background control sample. The TDS tube was transported to, and subsequently desorbed in, the TDS (TDS 3; Gerstel KK). The TDS was programmed from 20 °C (held for 0.1 min) to 260 °C (held for 1 min) at 280 °C per min with 50 mL/min desorption flow. Desorbed compounds were kept at −150 °C on a Tenax-packed liner in the cooled injection system (CIS; CIS 4, Gerstel KK) inlet. After desorption, the CIS was programmed from −150 °C (held for 0.1 min) to 250 °C (held for 1 min) at 12 °C per min to inject trapped compounds into the analytical column. The injection was performed by using the split option controlled by the pneumatic box of the TDS system. Then, GC and MS analyses were performed by using the same method as described above.

Sample Preparation for Bioassays. Each fractionated sample (Frac. 1, 2, and 3 in Fig. 1*A*, and Frac. 1-1 and Frac. 1-2 in Fig. 1*B*) was dissolved in 3 mL of purified ether. All control samples were made in the same manner. Twenty compounds (Fig. S4) were dissolved in purified ether in the same ratios as detected in Frac. 1 at a concentration of 10^{-5} M. Purified ether was used as a control sample. Then, 0.15 mL of ether solution was dropped onto a sheet of clean filter paper (50 × 50 mm), which was dried with nitrogen gas, treated with activated charcoal, and presented to each subject.

Selected candidate compounds (4-methylpentanal, hexanal, and binary mixture) were dissolved in purified water. Purified water was used as a control sample. Each of these samples (0.75 mL) was dropped onto a sheet of clean filter paper and presented to each subject (3). Commercial compounds were used in this study, except 4-methylpentanal, which was produced by the oxidation of commercial 4-methyl-1-pentanol. The purity of each compound was confirmed by using GC. The ratio of 4-methypentanal and hexanal in the binary mixture (13:87) was calculated from the ratio obtained from the gas chromatograms.

ASR Test. When the subjects were 9 wk old, we conducted the ASR test as described in our previous study (3), by using startle apparatus and software (Startle Reflex System 2004; O'Hara & Co.). Three and 2 d before the first test day, each rat was transported to the experimental room (temperature 22 °C, humidity: 50–55%) and handled for 5 min. The rats were also acclimatized to an animal holder for 5 min in the experimental room. The holder consisted of an acrylic cylinder (length, 200 mm; diameter, 56 mm), an acrylic plate with 42 perforations (diameter, 2 mm) as the front stopper, an acrylic plate as the rear stopper, and an acrylic bottom plate to support the cylinder. During the acclimation, the rat was kept inside the cylinder by inserting front and rear stoppers into the slits on the cylinder. One day before the first test day, rats were acclimatized to the entire ASR test procedure. In the experimental room, each rat was placed inside an animal holder, and the holder was attached to the platform in a dark soundproof chamber (480 × 350 × 370 mm) with background noise (65 dB wideband). Following this, the ASR test, consisting of a baseline trial, sample presentation, and a test trial, was initiated. During the baseline trial, the rat was exposed to 30 auditory stimuli (105 dB, 100 ms, white noise) at an interstimulus interval of 30 s, after an initial 5-min acclimation period. The sample presentation took place immediately after the baseline trial. The door of the soundproof chamber was opened and a folded filter paper (50 × 50 mm) was inserted into a slit on the cylinder so that the filter paper was placed 10 mm away from the rat's nose. The perforated front stopper enabled the rat to perceive the volatile odor of the samples. Because this

was the acclimation procedure for the ASR test, filter paper containing the vehicle of the test sample was inserted. Then, the door was closed, and the test trial was conducted in the same manner as the baseline trial.

On the first and second test days, rats underwent the ASR test with filter paper containing either the test sample or vehicle of the test sample during the sample presentation, in a counterbalanced order. During the trials, rat movements within the holder resulted in displacement of an accelerometer affixed to the bottom of the platform. The voltage output of the accelerometer was digitized and recorded. The startle amplitude was defined as the maximal peak-to-peak voltage that occurred during the first 200 ms after the onset of the startle-eliciting auditory stimulus.

All experimental procedures were conducted between 1000 and 1500 hours. For data analysis, we defined individual baseline data as the mean amplitude of the last 20 responses in the baseline trial. The test data were defined as the mean amplitude of all responses in the test trial. The increase in amplitude between the test data (T) and the baseline data (B) was calculated as T-B for each subject. Data on the first and second test days were statistically compared within each experimental group using a paired t test. The criterion for statistical significance was $P < 0.05$ for all comparisons.

Drugs. The following drugs, each of which was dissolved in a vehicle (saline containing 0.5% tragacanth gum powder), were prepared and used in the experiments: diazepam (0.2 and 1.0 mg/kg; Wako Pure Chemical Industries) and buspirone (5.0 mg/kg; Sigma Chemical). The doses for each drug were determined in reference to our previous studies (4, 5). Drugs were administered intraperitoneally 45 min before the ASR test.

Modified Open-Field Test. Modified open-field test was performed when the subjects were 9 wk of age, as described in a previous study (6). Two and 1 d before test day, each rat was transported to the experimental room (temperature: 24 °C, humidity: 50–55%) and kept there for 5 min. On the test day, we conducted the modified open-field test in the experimental room. We attached two sheets of filter paper containing either the binary mixture or vehicle in one corner of a plastic open field (440 × 660 × 400 mm) (Fig. 3*A*). Subsequently, we placed a rat in the center of the arena and allowed it to move freely for 5 min to acclimatize. After acclimation, a small polycarbonate box (175 × 245 × 125 mm), called the hiding box, was placed in the corner opposite to the samples. The behavior of the rat during the subsequent 10 min was recorded using a camera (DCR-SR300; Sony). The hiding box had a small round hole (diameter, 7.5 cm) in the center of a wall that allowed only enough space for the rat to enter. The rats had been habituated to the hiding box in their home cage for ∼20 h on the day before the test.

All experimental procedures were conducted between 1000 and 1500 hours. The duration of head out, concealment, and outside were recorded and analyzed during the 10-min test periods. A researcher who was blind to the experimental conditions analyzed the behavior of the rats. Outside was defined as the time spent in the open arena; head out was defined as the rat poking its head, or head and shoulders, out of the hiding box entrance with their hind paws remaining inside the box; and "concealment" was defined as the rat's head, body, and paws being entirely inside the hiding box. Statistical analyses were performed by using the Student t test. The criterion for statistical significance was $P < 0.05$ for all comparisons.

VNX Surgery. The surgery was performed 1 or 2 d after purchase as described in previous studies (7, 8). The rat was placed in a head holder under anesthesia with sodium pentobarbital, and its mouth was opened. After making a midline incision in the palate from the incisor and retracting the tissue to access the vomeronasal organ (VNO), the wound was closed in the sham surgery. In the VNX surgery, the rostral end of the VNO was exposed by drilling the incisive bone, and the caudal end of the vomer was cut. Then, the VNO was removed bilaterally using forceps. Bleeding was controlled by using cotton swabs and the wound was closed. Postoperatively, the rats were housed individually in standard polycarbonate rat cages in a colony room. The habituation/dishabituation test (see below) and ASR test were performed 6 or 7 d and 9 or 10 d after surgery, respectively.

Verification of the VNX Surgery. To assess whether VNX surgery preserved MOS function, the habituation/dishabituation test was conducted in the experimental room 6 or 7 d after surgery (3 d before the ASR test), as described in previous studies (8, 9). Two and 1 d before the test, each rat was handled in the vivarium for 5 min. On the day of the test, each animal was transported to the experimental room and kept in its home cage for at least 60 min. Each experiment was conducted in the home cage of the subject rat. Before initiation of experimental procedures, the home cage was moved to an experimental area in the same experimental room, and the food pellets and water bottle on a stainless steel cage top were removed. After a 3-min acclimation period, each rat was exposed to four consecutive stimuli. First, a sheet of filter paper (50 × 50 mm) soaked with purified water (100 μL) was indirectly presented to the rat 10 mm away from the wire ceiling of its home cage using a stainless mesh (55 × 55 mm, 480 perforations of 1.5-mm diameter) for 2 min. The rat received two additional consecutive 2-min presentations of purified water (100 μL each), followed by a 2-min presentation of a 5% (wt/wt) cocoa solution (100 μL) at 30-s intervals, each using a new sheet of filter paper and the same stainless mesh. The behavior of each subject rat was recorded by a video camera (DCR-SR300; Sony) for later analyses. A researcher blind to the experimental conditions measured the investigation time (time spent sniffing toward the stimulus and poking the nose into the ceiling) of each stimulus. The difference between the investigation time in the third water presentation and the cocoa solution presentation was analyzed by using a paired t test. The criterion for statistical significance was $P < 0.05$.

To verify the complete removal of the VNO, we assessed the glomerular layer of the AOB, which is known to disappear after a successful lesion (7, 8). After the ASR test, rats were housed two to four animals per cage for ∼3 wk. Each rat was then deeply anesthetized with sodium pentobarbital and perfused intracardially with 0.9% saline, followed by 4% (wt/wt) paraformaldehyde in 0.1 M phosphate buffer. The brain was removed, immersed in the same fixative overnight, and then placed in 30% (wt/wt) sucrose/phosphate buffer for cryoprotection. The olfactory bulb on both the sides was cut into 30-μm-thick sagittal sections, and all of the sections containing the AOB were mounted on glass slides. After being washed in PBS for 10 min, the sections were incubated with soybean agglutinin–fluorescein isothiocyanate conjugate (20 μg/mL; Vector Laboratories) in PBS for 20 min and rinsed with PBS for 10 min. A coverslip was then placed over each section, and they were observed under a fluorescence microscope (BX51; Olympus) equipped with a digital camera (DP30BW; Olympus).

Analyses of Fos Expression in Response to the Candidate Molecules. We prepared the following samples; control sample, stress-related odor sample, 4-methylpentanal sample (1.3×10^{-6} M), hexanal sample (8.7×10^{-6} M), and the binary mixture sample (4-methylpentanal 1.3×10^{-6} M + hexanal 8.7×10^{-6}M, total 10^{-5} M).

The 4-methylpentanal, hexanal, and binary mixture samples were prepared as described above. The stress-related odor sample was prepared according to an established method (10). Briefly, we anesthetized an adult male Wistar rat (12 wk old) as a donor and attached two intradermal needles (27 G) at the perianal region for electrical stimulation through these needles. After spraying

purified water (5 mL) on the ceiling of an acrylic box (20 × 20 × 10 cm), we placed the anesthetized donor rat in the box and gave 15 electrical stimuli (10 V for 1 s) over 5 min at 20-s intervals. Then, the donor was removed, and the water droplets on the ceiling of the box were collected.

Subjects were kept individually in their home cages 4 or 5 d before sample presentation. Each sample (600 μL) was loaded into one end of a glass tube (360 mm in length, 6-mm outer diameter, and 3-mm i.d.) and presented in the home cage of each subject for 5 s by an experimenter holding the other end of the glass tube, during which time the subject sniffed the sample. The distance between the tip of the glass tube and the subject's nose was maintained at 10 mm.

Sixty minutes after presentation, each subject was deeply anesthetized with sodium pentobarbital and perfused intracardially with 0.9% saline, followed by 4% (wt/wt) paraformaldehyde in 0.1 M phosphate buffer. The brain was removed, immersed overnight in the same fixative, and then placed in 30% (wt/wt) sucrose/phosphate buffer for cryoprotection. The avidin–biotin–peroxidase method was used for immunohistochemical detection of target antigens, as described (11). Briefly, the left and right olfactory bulb were cut into 25-μm-thick sagittal sections, and the rest of the brain was cut into 25-μm-thick coronal sections. All sections that contained the AOB and six successive sections that contained the BNST (Bregma −0.12 mm) or PVN (Bregma −1.80 mm) were collected for free-floating immunohistochemistry. Sections were incubated with primary antibody to Fos protein (PC38; Merck Millipore) for one night, followed by biotinylated anti-rabbit secondary antibody (BA-1000; Vector Laboratories) for 2 h. Sections were then processed with the ABC kit (Vector Laboratories) and developed by using a diaminobenzidine solution with nickel intensification. Sections containing the AOB were further incubated with primary antibody for G protein Giα-2 (MAB3077; Merck Millipore) for 1 night and then incubated with biotinylated anti-mouse secondary antibody (BA-2000; Vector Laboratories) for 2 h, processed with the elite ABC kit (Vector Laboratories), and developed using a diaminobenzidine solution.

To analyze Fos expression in the AOB, the mitral/tufted cell layer was divided into rostral and caudal regions based on Giα-2 expression in the glomerular layer. The number of Fos-immunoreactive cells in each region was counted bilaterally in all sections by an experimenter blinded to the experimental groups. The total number for each subject was calculated and analyzed by ANOVA, followed by Dunnett's post hoc test. The criterion for statistical significance was $P < 0.05$ for all comparisons.

To analyze Fos expression in the BNST and PVN, we randomly chose three sections to analyze the nuclei in the left hemisphere and three sections to analyze the nuclei in the right hemisphere (12). Sections of each region were captured by using a microscope equipped with a digital camera (DP30BW; Olympus). Then, Fos-immunoreactive cells were counted within a 0.5-mm square for each region by an experimenter blinded to the experimental groups. When the designated region was smaller than the boundaries of a 0.5-mm square, only the cells in the region of interest were counted. The mean number for each subject was calculated and analyzed by ANOVA, followed by Dunnett's post hoc test. The criterion for statistical significance was $P < 0.05$ for all comparisons.

1. Scientists Center for Animal Welfare (1987) Consensus recommendations on effective institutional Animal Care and Use Committees. *Lab Anim Sci* 37(Special issue):11–13.
2. Kiyokawa Y, Kikusui T, Takeuchi Y, Mori Y (2004) Alarm pheromones with different functions are released from different regions of the body surface of male rats. *Chem Senses* 29(1):35–40.
3. Inagaki H, et al. (2009) The volatility of an alarm pheromone in male rats. *Physiol Behav* 96(4–5):749–752.
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6. Kiyokawa Y, Shimozuru M, Kikusui T, Takeuchi Y, Mori Y (2006) Alarm pheromone increases defensive and risk assessment behaviors in male rats. *Physiol Behav* 87(2): 383–387.
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12. Kiyokawa Y, Honda A, Takeuchi Y, Mori Y (2014) A familiar conspecific is more effective than an unfamiliar conspecific for social buffering of conditioned fear responses in male rats. *Behav Brain Res* 267:189–193.

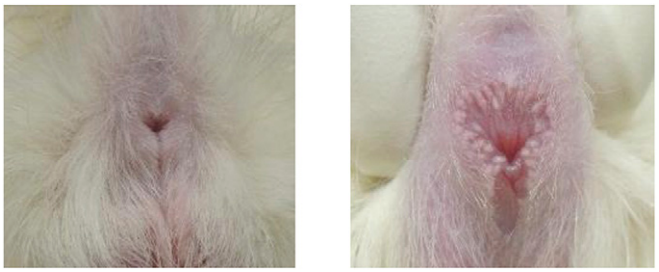

Fig. S1. The anal glands in rats. (*Left*) Glands located just inside the anal verge are not visible. (*Right*) The glands are visible when the anus is opened.

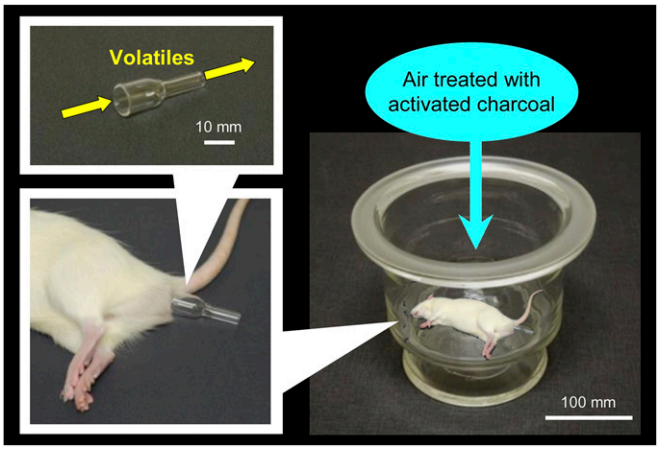

Fig. S2. First version of the apparatus. An open cylindrical glass desiccator (*Left*) and a small glass funnel (*Right*) to collect volatiles released from the perianal region of a donor rat.

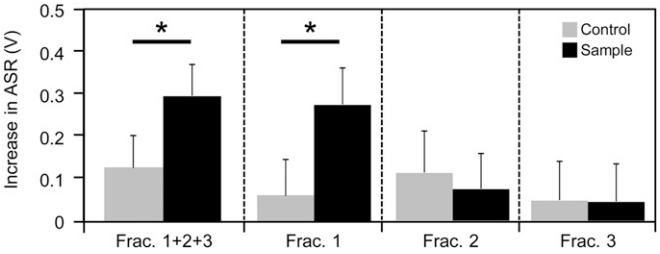

Fig. S3. Results of the bioassay of Frac. 1, 2, and 3. Only Frac. 1 enhanced the ASR ($n = 14$ for all fractions). Each bar represents the mean + SEM. *$P < 0.05$ vs. control (paired t test).

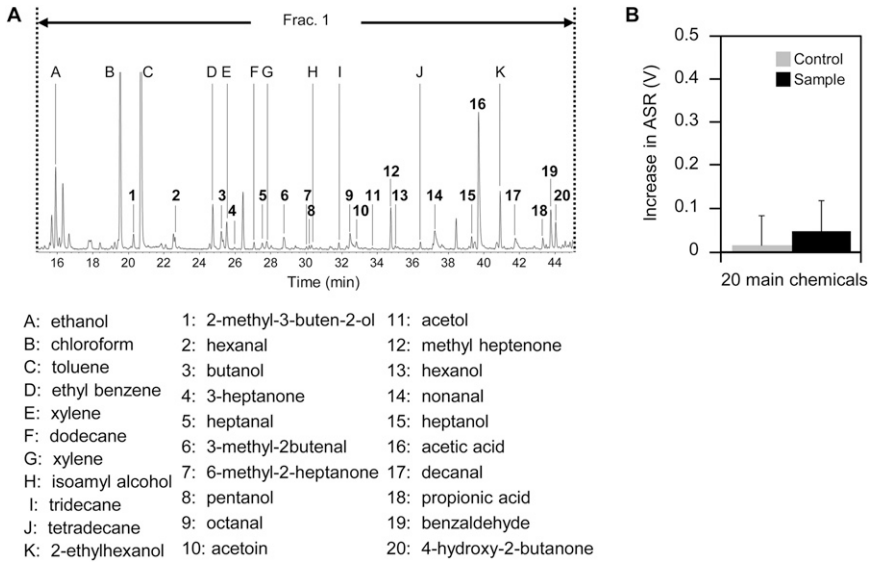

Fig. S4. The main peaks found in Frac. 1. (*A*) Total ion chromatogram of Frac. 1. Thirty-one chemicals (A–K and 1–20) were identified as main peaks. (*B*) Results of the bioassay of 20 chemicals ($n = 8$). Eleven chemicals (A–K) appeared to be contaminants from the environment; therefore, we focused on the remaining 20 chemicals (1–20) and performed bioassays. We mixed the 20 chemicals in the same ratio as those found in the gas chromatogram (total concentration, 10^{-3} M). These 20 chemicals did not enhance the ASR ($P = 0.79$, paired t test). Each bar represents the mean + SEM.

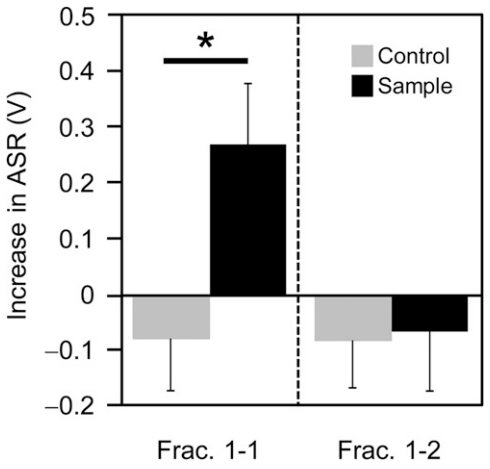

Fig. S5. Results of the bioassay of Frac. 1-1 and 1-2. Frac. 1-1 enhanced the ASR ($n = 8$ for both fractions). Each bar represents the mean + SEM. *$P < 0.05$ vs. the vehicle control (paired t test).

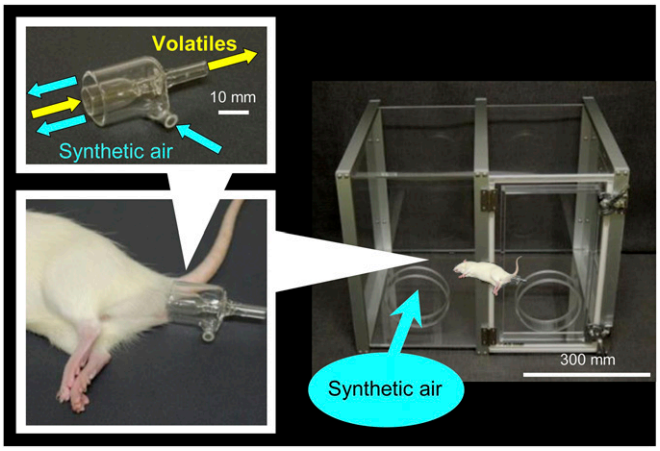

Fig. S6. Refined system for collecting the stress-related odor from 50 donor rats. We collected the stress-related odor in a glovebox filled with synthetic air using a specific glass funnel. With this funnel, we collected stress-related odor (yellow arrows) while dispelling the other molecules in the environment by blowing synthetic air through the outer space in the funnel (blue arrows).

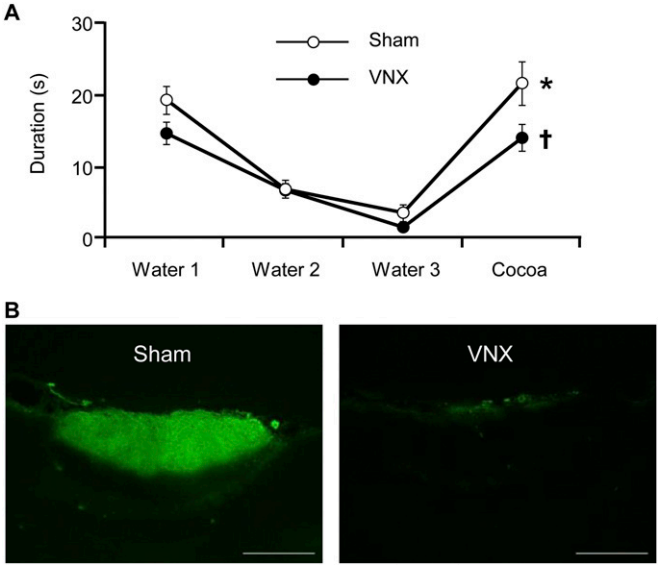

Fig. S7. Verification of the VNX surgery. (*A*) Rats that underwent either VNX (n = 17) or sham (n = 15) surgeries showed an equivalent duration of investigation time on each sample in the habituation/dishabituation test. $^{*,\dagger}P < 0.05$ vs. the third water sample presentation of the same group (paired t test). (*B*) Photomicrographs of soybean agglutinin binding in the AOB of the rats that underwent either VNX (*Left*) or sham (*Right*) surgeries. The lack of the binding indicates the complete removal of the VNO by VNX surgery. The rostral portion is shown on the left. (Scale bar: 500 μm.)

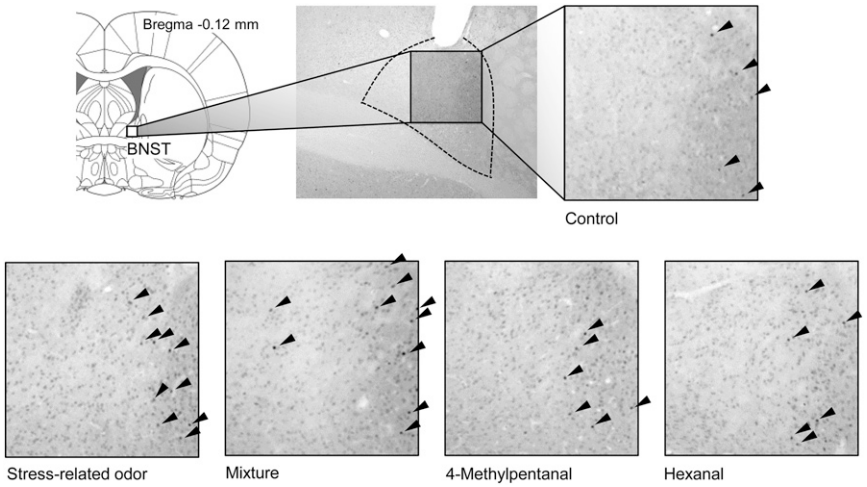

Fig. S8. Analyses of Fos expression in the BNST. Schematic diagram of the location of the brain regions (open square) in which Fos-immunoreactive cells were counted and representative photomicrographs showing Fos expression in the BNST. For simplification, the location is shown only in one side.

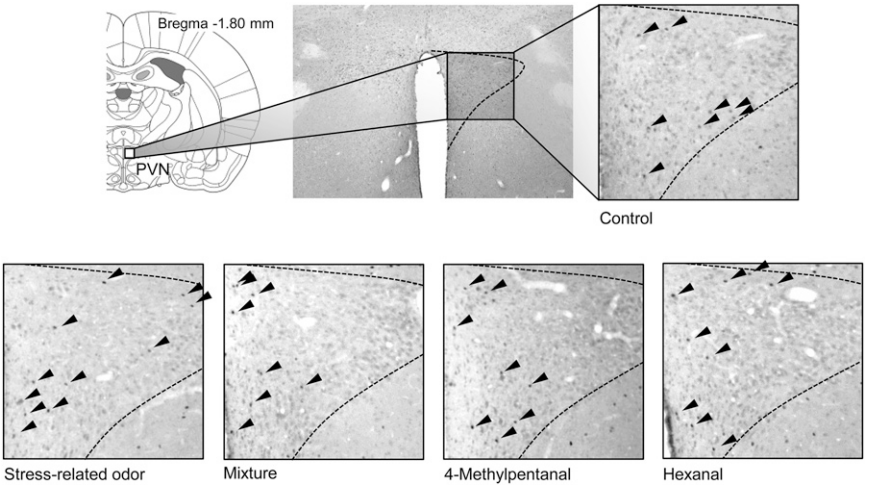

Fig. S9. Analyses of Fos expression in the PVN. Schematic diagram of the location of the brain regions (open square) in which Fos-immunoreactive cells were counted and representative photomicrographs showing Fos expression in the PVN. For simplification, the location is shown only in one side.