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Identification of a Natural Source for the OR37B Ligand

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Abstract

In search for biological sources of the long-chain fatty aldehydes (penta-, hexa-, and heptadecanal), which we recently identified as ligands for members of the mouse odorant receptor subfamily OR37, the headspace of secretions and excretions from mice was analyzed by gas chromatography and mass spectrometry. In urine, skin swabs, and saliva, these components were not detectable. However, in fecal pellets, a substantial amount of hexadecanal, the OR37B ligand, was found. Accordingly, exposure of mice to feces induced an activation of the OR37B glomerulus, whereas the OR37A and the OR37C glomerulus were not responsive. The amount of hexadecanal deposited with feces varied significantly; however, it was independent of the amount of feed. In many species, feces is covered with secretion from anal glands. Due to the size and the inaccessibility of these glands in mice, the headspace of anal gland secretion from dog was analyzed by gas chromatography–mass spectrometry, which resulted in a prominent peak for hexadecanal. Exposure of mice to anal gland secretion from dog activated the OR37B glomerulus. Altogether, these data suggest that hexadecanal, a ligand for the receptor OR37B, is produced in anal glands and deposited with feces into the environment.

Key words: mouse, natural ligand source, odorant receptor, olfaction

Introduction

Olfactory cues trigger numerous behaviors that are not only crucial for the survival of an individual, such as finding food sources or avoiding predators, but also for the subsistence of an entire species, for example, recognizing mating partners or own offspring to care for. Mammalian species have developed a number of structurally and functionally distinct olfactory subsystems (for reviews, see Breer et al. 2006; Munger et al. 2009; Ma 2010) that contribute to perform these complex tasks. However, the precise functional role that each of them plays under the various circumstances is not entirely understood, yet. One of these subsystems is formed by olfactory sensory neurons (OSNs) within the main olfactory epithelium, which express the odorant receptors (ORs) of the so-called OR37 subfamily (Strotmann et al. 1992, 1994). These receptors are structurally unique due to an insertion of 6 amino acids in their third extracellular domain (Kubick et al. 1997). Comparative studies have demonstrated that this OR type exists exclusively in mammalian species (Hoppe et al. 2006), and in contrast to ORs in general, the various OR37 subtypes are highly conserved across species from opossum to human (Hoppe et al. 2006). OSNs that express them target only single glomeruli in the main olfactory bulb (MOB), which are grouped together in the ventral domain (Strotmann et al. 2000); interestingly, this region of the MOB has been discussed to play a role in processing socially relevant olfactory cues (Schaefer et al. 2001, 2002; Lin et al. 2007). Our most recent data provided evidence that also the connectivity of the projection neurons from an OR37 glomerulus into higher brain centers is organized in an unusual manner: the corresponding mitral/tufted (M/T) cells do not send their axons to the typical olfactory cortical areas, but instead to the medial amygdala (Me) (Bader et al. 2012). This is of particular interest because the Me receives input primarily from the accessory olfactory system, which is specialized for the detection of socially relevant chemical cues (Kevetter and Winans 1981; Halpern and Martinez-Marcos 2003). Thus, several pieces of evidence support the idea that the OR37 subsystem may be tuned to the detection of compounds, which could be involved in social communication. We have recently shown that OSNs

that express distinct OR37 receptors are activated by long-chain aldehydes with 15–17 C-atoms, so-called fatty aldehydes (Bautze et al. 2012). Each of the three analyzed OR37 subtypes was found to respond preferentially to an aldehyde with a particular chain length (penta-, hexa-, or heptadecanal, respectively), and the related alcohols or acids had only little or no effects, indicating that the OR37 receptors are specifically tuned to one of the aldehyde compounds.

Toward an understanding of the functional role of the OR37 subsystem, an important step is the identification of biological sources for the compounds, which are ligands for these receptors. Because we think that the OR37 receptors might be involved in recognizing socially relevant signals, we focused this study on odor sources that are derived from conspecifics. Different secretions and excretions of mice were analyzed specifically for the presence of the long-chain fatty aldehydes with 15–17 C-atoms. Activity-based assays were subsequently employed to study whether such odor sources were capable to selectively activate defined OR37 subtypes.

Materials and methods

Experimental animals

In the present study, adult wild-type mice (C57BL6) and OR37-transgenic animals that carry targeted mutations of IRES-taulacZ or IRES-tauGFP at the mOR37A, mOR37B, or mOR37C locus (Strotmann et al. 2000) were employed. Mice were housed in groups or individually at the Central Unit for Animal Research at the University of Hohenheim. Animals were killed by cervical dislocation and subsequent decapitation. The institutional and national guidelines for the care and use of laboratory animals according to the Society of Laboratory Animals (GV-SOLAS) were followed and the institutional internal review committee has approved the work (T42/10 Phy).

Sample preparation and headspace solid-phase microextraction procedure

Pentadecanal, hexadecanal, and heptadecanal utilized as reference compounds for analyses by gas chromatography–mass spectrometry (GC/MS) were purchased from TCI Europe; the solvent toluene SupraSolv was purchased from Merck. All biological samples were freshly collected and transferred to 20-mL crimp neck vials (Machery-Nagel); the samples were covered with a few drops of liquid nitrogen, and the vials were closed with an aluminum-coated silicone septum (Machery-Nagel). Fecal samples were collected from animals, which were placed in a closed plastic chamber for 20 min (approximately 11.5 cm height/14 cm width/29 cm length); pellets were transferred to the test vials using forceps. Fecal samples from the intestine were collected from killed mice by carefully opening their abdominal cavity. The entire gut was removed and feces was collected from the ileum and colon by gently squeezing the tissue with forceps. The amount of feed consumed during the 24-h period prior to the collection of feces was determined by weighing the feed pellets from individually housed mice. Saliva samples were pooled by using a cotton bud inserted into the mouth of mice immediately after killing (5 females and males). Cotton swabs were also used to wipe the skin at different positions on the back and abdomen. Urine from mice was collected in Eppendorf vials by gently pressing their abdomen; 100 µL from each gender was transferred to the test vial for GC/MS analysis. Fecal pellets from rats were sampled from Lister Hooded rats kept at the animal facility of the Institute of Parasitology (University of Hohenheim). Anal gland secretion (AGS) from a dog was collected during a routine examination by a Veterinarian at the animal clinic of the University of Hohenheim. All samples were kept in the test vials for 1 h at room temperature before further processing. Headspace volatiles for GC/MS analyses were enriched by a polydimethylsiloxane-coated (100 µm) solid-phase microextraction (SPME) fiber assembly fitted to a manual holder (Sigma–Aldrich), which was inserted into the vial and exposed to the headspace for 60 min at 100 °C. Subsequently, the fiber was desorbed inside the GC injector for 1 min. Before each sampling, the fiber was activated at 270 °C for 10 min. The amount of hexadecanal in single fecal pellets was referred to the dry weight of the pellet. Therefore, each pellet was dried after SPME sampling by heating to 110 °C for 2 h.

Gas chromatography–mass spectrometry

GC/MS analyses were performed on a Varian 3800/1200 system. Chromatographic separation was obtained by a 60-m DB-5MS column (0.25 mm id, 0.25 µm film thickness) (J&W Scientific, Agilent). The GC oven program started at 60 °C, held for 2 min, followed by a temperature ramp of 10 °C/min to 260 °C, held for 8 min. The carrier gas was helium at a constant flow rate of 1.2 mL/min. The injector was set to 250 °C (splitless), the transfer line to 280 °C, and the ion source to 200 °C. The electron energy was 70 eV. Full scan mass spectra were recorded from m/z 50 to 600; for selected ion monitoring (SIM), fragments of highest abundance (m/z 29, 41, 86, 96) were monitored. For the rat and dog samples, a TRACE GC Ultra/Polaris Q system was used (Thermo Scientific), while the same column and the same GC and MS parameters were applied. To evaluate retention times and mass spectra of the long-chain aldehydes, the respective standards were individually dissolved in toluene (0.2 mg/mL); the injection volume was 1 µL. For quantification, a stock solution of hexadecanal in toluene (5 mg/10 mL) was prepared and diluted with toluene to obtain working standards of 50, 100, 150, 200, and 250 µg/mL. To prepare the calibration samples, headspace vials were thoroughly purged by nitrogen. Using 1-µL microcaps (Hirschmann), the working standards were transferred to the headspace vials, together with the microcap. The vials were immediately capped and subjected to SPME–GC/MS.

Odor exposure

Adult OR37-transgenic mice were placed individually into a closed plastic box (approximately 11.5 cm height/14 cm width/29 cm length). The floor of this box was covered with material to adsorb secretions and excretions of the test animal (cat litter, EAN 4311501304792). Prior to the exposure to the odor sample, animals were allowed to acclimate to the box for 120 min and to decrease the c-Fos level due to cage and self-odors. Odor samples in small glass vials were placed into a second box of the same size, to which the test animals were transferred for 90 min. As odor samples, about 10 mouse fecal pellets, 2–3 rat fecal pellets, and about 150 µL of AGSs of the dogs were used. After exposure to these samples, the mice were sacrificed and prepared for immunohistochemistry.

Immunohistochemistry

All bones surrounding the olfactory bulb and the nasal turbinates were excised. To remove the air from the nasal cavity, the specimens were immersed in fixative (4% paraformaldehyde in 150 mM phosphate buffer, pH 7.4, 4 °C) and a light vacuum was applied for 5 min; fixation was continued for 10 min on ice. Subsequently, the tissue was cryoprotected by incubation in 25% sucrose in phosphate-buffered saline (PBS) (0.85% NaCl, 1.4 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.4) overnight at 4 °C. Finally, the tissue was embedded in "Tissue Freezing Medium" (Leica Microsystems) and frozen on dry ice. Twelve micrometer sections were generated using a CM3050S cryostat (Leica Microsystems) and mounted onto microscope slides (Superfrost slides, Menzel). Sections were air dried for 30 min and rinsed in PBS for 10 min at room temperature. Mouse anti-β-galactosidase (Promega) (1:1500) and rabbit anti-c-Fos (Santa Cruz Biotechnology) (1:600) were diluted in PBS/0.3% Triton X-100 containing 10% normal goat serum (NGS) (Dianova) and incubated over night at 4 °C. After 3 rinses for 5 min in PBS, the bound primary antibodies were visualized by incubating appropriate secondary antibodies conjugated to Alexa 488 or Alexa 568 (Invitrogen) diluted in PBS/0.3% Triton X-100 containing 10% NGS for 2 h at room temperature. After washing for 3 times for 5 min, the sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; 1 µg/mL, Sigma–Aldrich) for 3 min at room temperature, rinsed with H_2O, and mounted in MOWIOL (33% glycerin, 13% polyvinylalcohol 4-88 [Sigma–Aldrich] in 0.13 M Tris pH 8.5).

Microscopy and photography

Sections were analyzed using a Zeiss Axiophot microscope (Carl Zeiss MicroImaging). Fluorescent images were captured using a "Sensi-Cam" CCD camera (PCO imaging).

Quantitative analyses

Quantitative analyses were performed according to Oliva et al. (2008) and Bautze et al. (2012). For cell counts, the sections were examined with a ×40 objective. An OR37 glomerulus was defined as a region of β-galactosidase immunoreactive neuropil delimited by DAPI-stained juxtaglomerular cells (JCs); c-Fos immunoreactive and DAPI-stained cells that were immediately adjacent to the OR37 glomerulus (maximal 4 nuclei widths from the outer boundary of the axon fibers within the glomerulus) were counted on serial sections. c-Fos signals were counted when their signal intensity was above background and the signal was colocalized with a DAPI-stained nucleus. The percentage of c-Fos immunoreactive cells from the number of DAPI-stained cells surrounding the glomeruli was determined and given as means ± standard deviation (SD). Statistical significance was determined by using the unpaired *t*-test with Graphpad Software (GraphPad Software, Inc.). Statistical significance was set at $P < 0.05$.

Results

In the search for biological sources containing those long-chain fatty aldehydes (penta-, hexa-, and heptadecanal), which were recently identified as ligands for distinct members of the mouse OR37 subfamily (Bautze et al. 2012), we have screened the headspace of various secretions and excretions from mice using GC/MS. For an efficient separation of homologous long-chain hydrophobic molecules, an apolar 60-m capillary column was employed. For the enrichment of volatiles from the headspace, a SPME procedure was optimized in terms of extraction temperature and time; 100 °C and 60 min turned out to be most efficient. Because fatty aldehydes are highly sensitive to oxidation, a few drops of liquid nitrogen were given into the headspace vials before crimping. Samples of urine, skin swabs, saliva, and feces from several male and female individuals were analyzed first by GC/MS in the full scan mode. The chromatograms for the urine (shown as a representative trace in Figure 1A), skin swab, and saliva samples gave rather small peaks. Based on the retention times of standard substances as reference and mass spectra comparison (Supplementary Figure S1), none of the peaks corresponded to penta-, hexa-, or heptadecanal. Analyzing samples of pooled feces resulted in a number of significantly higher peaks (Figure 1B). In fact, by comparing the MS spectra (Supplementary Figure S1), pentadecanal (arrow in Figure 1B) and hexadecanal (arrowhead) were identified as constituents; heptadecanal was hardly detectable. Interestingly, hexadecanal represented the largest peak in the chromatogram. The results of GC/MS analyses thus indicate that feces from mice contained small amounts of pentadecanal and quite substantial amounts of hexadecanal.

Based on the experimental evidence that hexadecanal was well detectable in mouse feces, it was hypothesized that exposure of mice to feces may lead to an activation of OR37 neurons and the OR37B glomerulus. To scrutinize this view, we employed the previously established activity-measure paradigm, monitoring c-Fos upregulation in JCs at

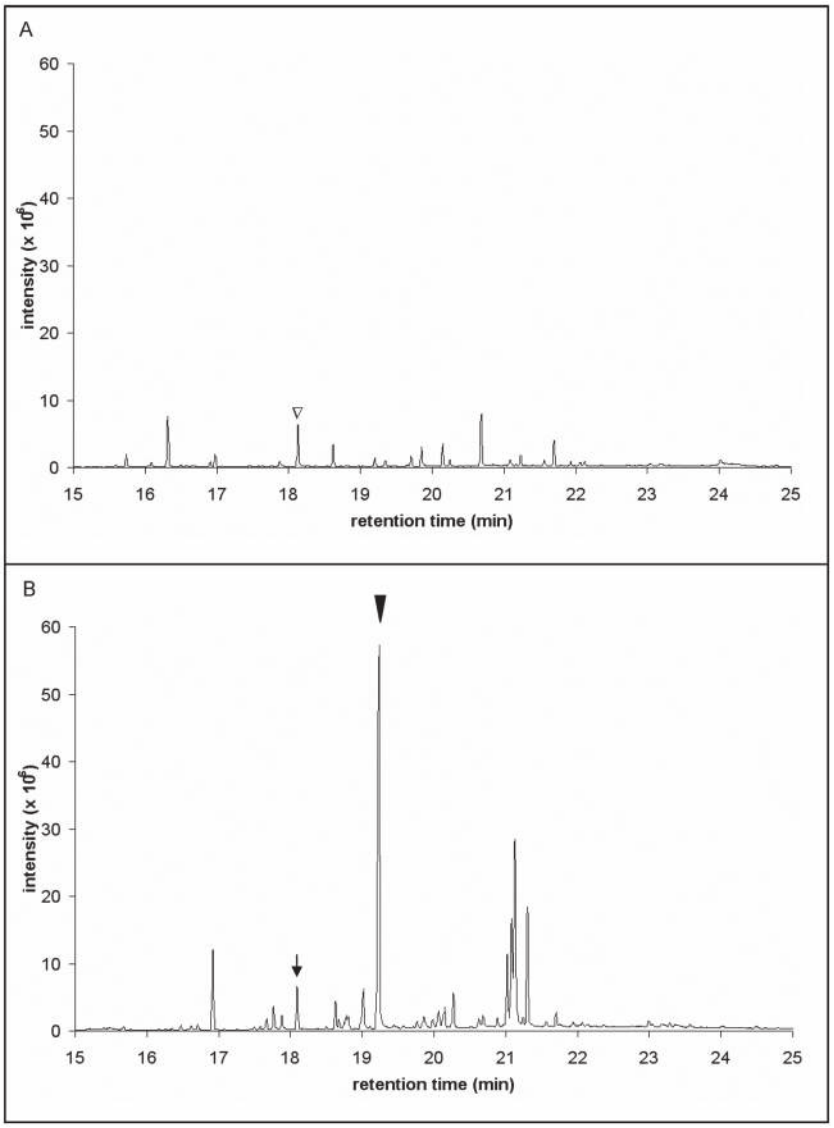

Figure 1 Hexadecanal is a constituent of mouse feces. (**A**) Total ion current GC/MS chromatogram of volatiles from mouse urine pooled from both sexes. Penta-, hexa-, or heptadecanal could not be identified by GC/MS. The peak marked by the triangle appears at the same retention time as pentadecanal but was shown by MS not to be that compound (see Supplementary Figure S1). (**B**) Total ion current GC/MS chromatogram of volatiles from mouse feces pooled from both sexes. The arrow marks the peak for pentadecanal, and the peak for hexadecanal is indicated by the arrowhead; both substances were confirmed by MS (see Supplementary Figure S1).

receptor-specific glomeruli (Bautze et al. 2012). After exposure of transgenic mice (Strotmann et al. 2000) to such samples, it was found that c-Fos upregulation was indeed induced in cells surrounding the OR37B glomerulus (Figure 2B); in contrast, only very few c-Fos positive cells were detectable around the OR37A and the OR37C glomerulus (data not shown). The notion that the OR37B glomerulus was significantly activated upon the exposure of mice to fecal probes was substantiated by quantitative analyses (Figure 2C) (feces: 18 ± 8%, n = 45; air: 3.7 ± 3.3%, n = 7; P = 0.0001). No significant increase in the number of c-Fos positive cells was found at the OR37A (feces: 9.5 ± 4.9%, n = 6; air: 7.5 ± 0.9%, n = 3; P = 0.7132) and OR37C glomerulus (feces: 7.1 ± 4.1%, n = 12; air: 5.2 ± 1.5%, n = 5; P = 0.3736), respectively (Figure 2C).

Examination of the sections that contained the OR37 glomerulus at lower magnification (as exemplarily shown in Figure 2E–G) demonstrates that on these sections, typically the OR37B glomerulus was surrounded by numerous c-Fos positive cells. Interestingly, at this magnification, it became obvious that also in the deeper layer of the OB (mainly the granule cell layer) close to the OR37B glomerulus, strongly c-Fos positive cells were detectable. This observation is consistent with previous data that showed a columnar organization of the OB that extends across all layers from the glomerulus to the deep granular cell layer (Willhite

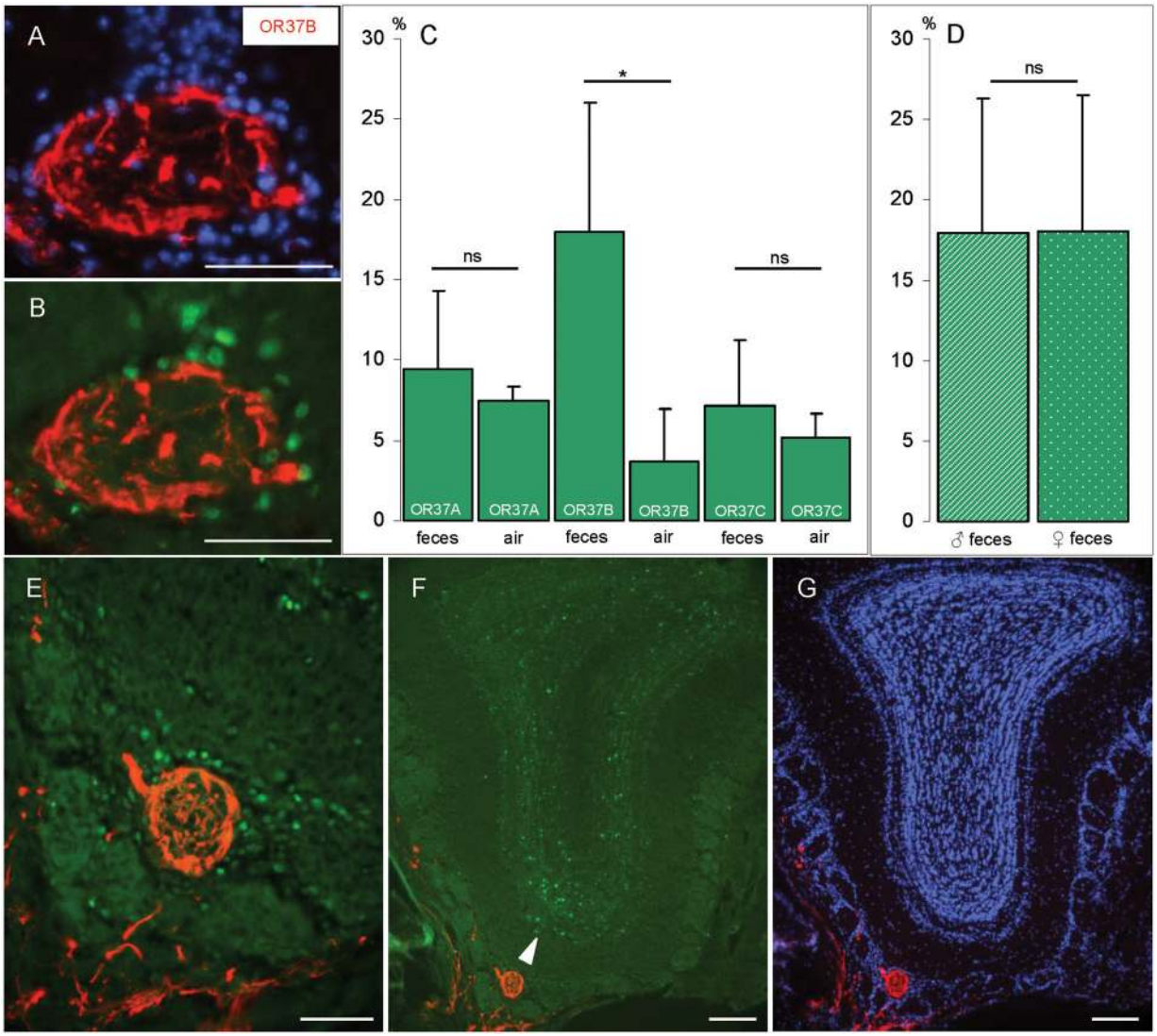

Figure 2 Exposure of mice to mouse feces activates the OR37B glomerulus. (**A**) Cross-section through an OR37B glomerulus visualized by anti-β-galactosidase staining and counterstaining with DAPI. Scale bar: 50 μm. (**B**) After exposure of the mouse to feces, c-Fos positive cells are visible at the OR37B glomerulus. Scale bar: 50 μm. (**C**) Percentage of c-Fos positive JCs at OR37-specific glomeruli induced by mouse feces. ns, statistically not significant; *, statistically significant; data are given as means ± SD. (**D**) Percentage of c-Fos positive JCs at the OR37B glomerulus induced by fecal pellets from male (n = 25, striped bar) or female mice (n = 20, dotted bar). ns, statistically not significant; data are given as means ± SD. (**E**) Cross-section through the activated OR37B glomerulus seen in (**F**) visualized by anti-β-galactosidase staining; c-Fos positive cells are visible at the OR37B glomerulus. Scale bar: 50 μm. (F) Cross-section in lower magnification through the OB. The OR37B glomerulus is visualized by anti-β-galactosidase staining; many c-Fos positive cells are visible at the OR37B glomerulus and in deeper layer of the OB close to the OR37B glomerulus (marked by an arrowhead). Scale bar: 20 μm. (**G**) Corresponding cross-section in lower magnification through the OB. The OR37B glomerulus is visualized by anti-β-galactosidase staining and counterstaining with DAPI. Scale bar: 20 μm.

et al. 2006). Such a labeling of deeper cells was not visible, when mice were exposed to clean air and the OR37B glomerulus was not activated (Supplementary Figure 2A–C). Close examination of sections in other regions of the OB in animals that were exposed to feces revealed that the OR37B glomerulus was surrounded by c-Fos positive cells and several other glomeruli were labeled (Supplementary Figure 2D–L), suggesting that they also responded to compounds present in the fecal sample.

During the study, it was noticed that the number of cells at the OR37B glomerulus, which were activated by fecal samples varied to quite a large extent (±8%). Because the fecal samples were collected from both sexes, it was analyzed whether gender differences may play a role. It was found that exposure of mice to samples either from males or females (Figure 2D) increased the number of c-Fos positive JC around the mOR37B glomerulus to a similar level (♂: 17.9 ± 7.7%, n = 25; ♀: 18.1 ± 8.4%, n = 20; statistically not significant P = 0.9532). Subsequently, it was analyzed whether the hexadecanal content of individual fecal pellets differs. Analyses of single pellets by GC/MS using the SIM mode to improve the sensitivity resulted in readily detectable

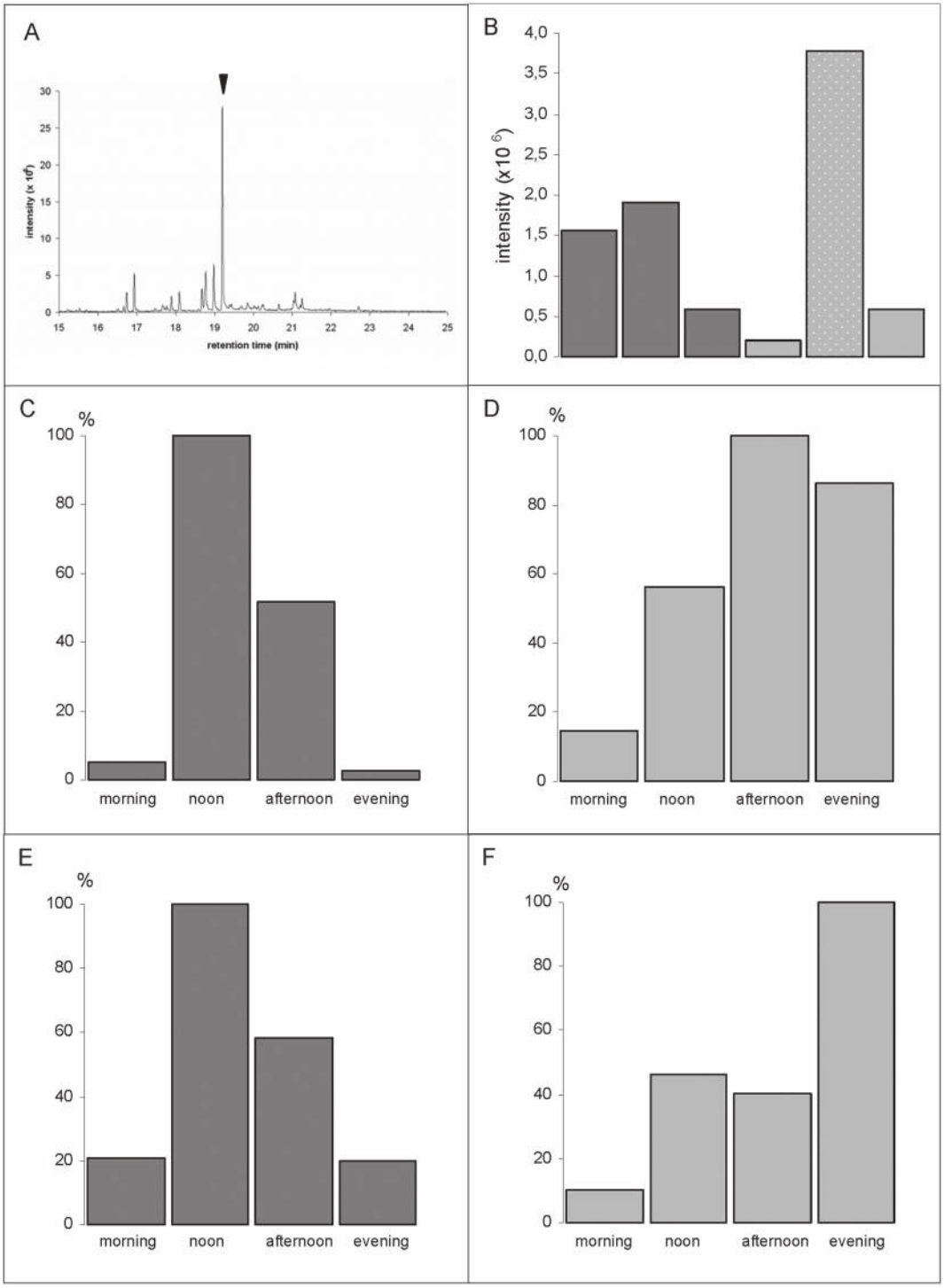

Figure 3 Fecal pellets contain different amounts of hexadecanal. (**A**) GC/MS SIM ion trace (*m/z* 41) of a single fecal pellet from a mouse. The arrowhead marks the peak for hexadecanal. The chromatogram corresponds to the dotted bar in (**B**). (B) The amount of hexadecanal in single fecal pellets collected randomly throughout the day from 3 different males (dark gray bars) and females (light gray bars) determined by GC/MS SIM (*m/z* 41). The amount is given as peak areas. The dotted bar corresponds to the chromatogram shown in (A). (**C**) Amount of hexadecanal in single fecal pellets of a male mouse at 4 different times of a day (morning, noon, afternoon, and evening) determined by GC/MS SIM (*m/z* 41). The largest amount was set as 100%. (**D**) Amount of hexadecanal in single fecal pellets of a female mouse at 4 different times of a day (morning, noon, afternoon, and evening) determined by GC/MS SIM (*m/z* 41). The largest amount was set as 100%. (**E**) Amount of hexadecanal in single fecal pellets of a second male mouse at 4 different times of a day (morning, noon, afternoon, and evening) determined by GC/MS SIM (*m/z* 41). The largest amount was set as 100%. (**F**) Amount of hexadecanal in single fecal pellets of a second female mouse at 4 different times of a day (morning, noon, afternoon, and evening) determined by GC/MS SIM (*m/z* 41). The largest amount was set as 100%.

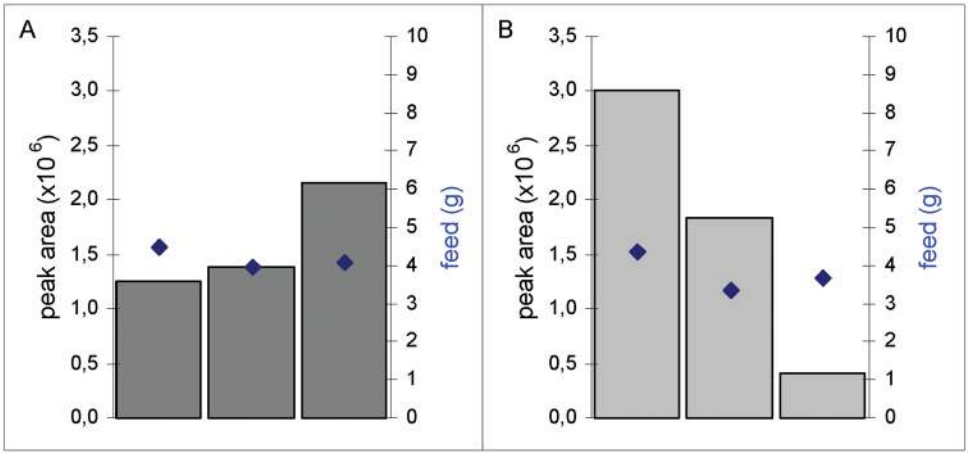

Figure 4 Gender-specific variations in hexadecanal amounts on consecutive days are independent of the amount of consumed feed. (**A**) The amount of hexadecanal in single fecal pellets of a male mouse measured at the same time on 3 consecutive days by GC/MS SIM (*m/z* 41) represented by peak areas on the left *y* axis. The amount of feed (right *y* axis) consumed by the animal 24 h before the analysis is indicated by the diamonds. (**B**) The amount of hexadecanal in single fecal pellets of a female mouse measured at the same time on 3 consecutive days by GC/MS SIM (*m/z* 41) represented by peak areas on the left *y* axis. The amount of feed (right *y* axis) consumed by the animal 24 h before the analysis is indicated by the diamonds.

Table 1 Hexadecanal content in individual fecal pellets determined by external calibration

	Weight of fecal probe (mg)	Hexadecanal amount (ng)
Probe 1	22	56.3
Probe 2	43.2	116.4
Probe 3	36.1	138.1
Probe 4	31.7	143.1
Probe 5	45.1	122.1
Probe 6	18.2	21.4

The amount of hexadecanal of 6 freshly collected individual fecal pellets given in nanograms. The amount is not correlated with the weight of the pellet.

hexadecanal peaks (arrowhead in Figure 3A). The amount of hexadecanal in single fecal pellets varied up to 13-fold (Figure 3B), most pronounced for females; however, there was not a consistent gender difference. Analyses at 2.5-h intervals during 1 day (Figure 3C–F) revealed that for individual males (Figure 3C,E), the amount was highest around noon (see also Supplementary Figure 3A), whereas for individual females (Figure 3D,F), the amount increased until late afternoon (see also Supplementary Figure 3B). At the same time of the day, the samples from an individual male contained similar amounts of hexadecanal, whereas those of a female contained notably different amounts. The amount of hexadecanal thus fluctuates during a day, but with different duration from minimum to maximum for the genders. The amount of feed consumed by these mice was also determined and did not differ significantly during this period (diamonds in Figure 4). The data indicate that in males the fluctuation of hexadecanal content follows a near 24-h rhythm, whereas the rhythm seems to be extended in females.

In both genders, these variations are most likely not due to differences in feed consumption. We subsequently tried to determine how much hexadecanal is contained in single pellets. By using an external calibration method, the amount of hexadecanal in single fecal pellets was determined to range between 21.4 and 143.1 ng (Table 1). The amount is not correlated with the weight of the pellet.

Due to the fact that OR37 receptors are well conserved across mammalian species, it seems possible that hexadecanal is also present in feces of other species. To address this question, mice were exposed to feces from rats and the OR37B glomerulus examined. As seen in Figure 5B, many c-Fos positive JCs around the glomerulus were detectable ($16.2 \pm 7\%$, $n = 15$; statistically significant compared with clean air; $P = 0.0006$), indicating that feces from rat may in fact contain hexadecanal. GC/MS analyses of 3 rat fecal pellets showed that indeed a prominent peak for hexadecanal was detectable in the chromatogram of the headspace (arrowhead in Figure 5C).

The finding that the hexadecanal content was apparently not correlated with consumed feed suggests that its appearance is independent of the digestion process. Because previous studies have shown that several species cover their feces with secretions from their anal gland (Goodrich, Hesterman, Shaw, et al. 1981; Goodrich et al. 1990a), we set out to examine whether material that covers the fecal pellets can activate the OR37B glomerulus. To analyze this, several pellets were gently rolled on a piece of blotting paper to transfer the coating material, and mice were exposed to these impregnated papers. As shown in Figure 6B, a substantial number of JC at the OR37B glomerulus was c-Fos positive. Quantifications revealed $18.2 \pm 11.2\%$ ($n = 21$) of the JCs (Figure 6C). Control experiments in which mice were exposed to clean paper resulted in hardly any ($6.2 \pm 2.9\%$,

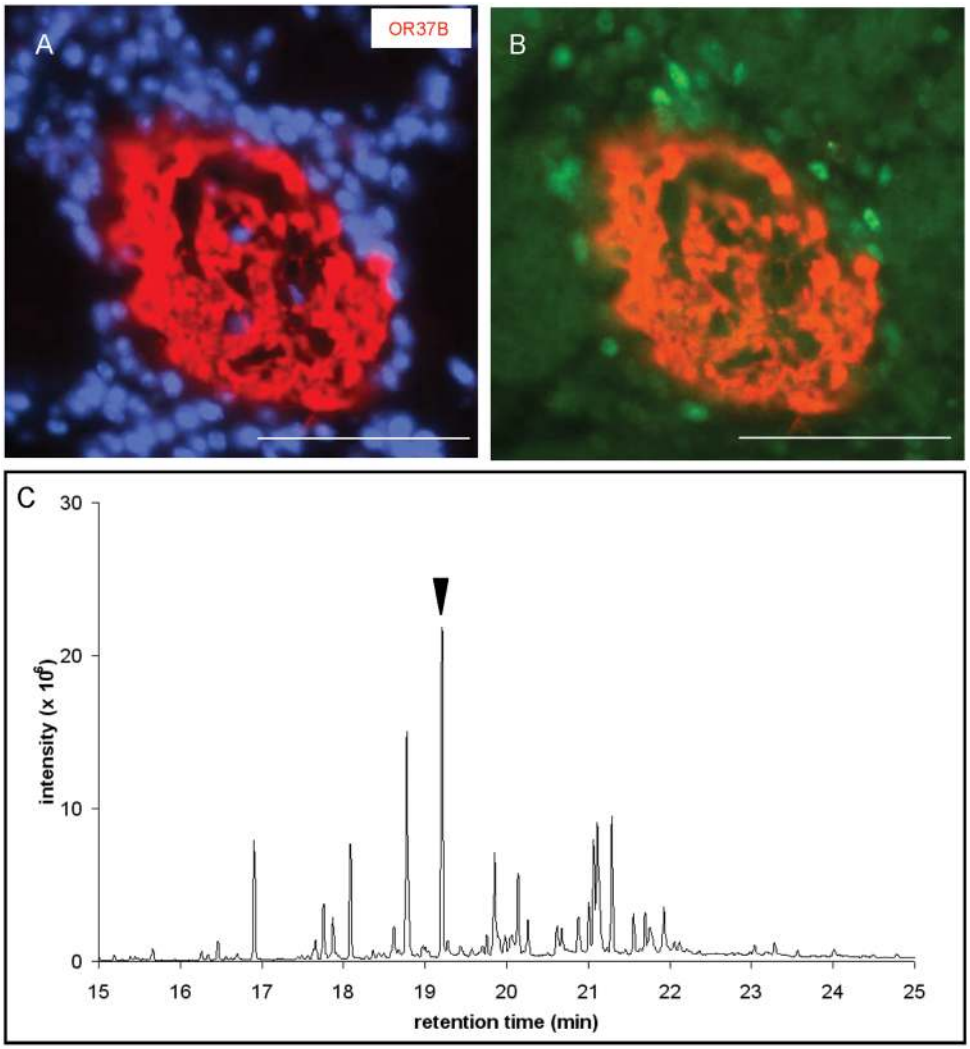

Figure 5 Rat feces contains hexadecanal, and exposure of mice activates the OR37B glomerulus. (**A**) Cross-section through an OR37B glomerulus of a mouse visualized by anti-β-galactosidase staining and counterstaining with DAPI. Scale bar: 50 μm. (**B**) After exposure of the mouse to rat feces, c-Fos positive cells are visible at the OR37B glomerulus. Scale bar: 50 μm. (**C**) Total ion current GC/MS chromatogram of volatiles from rat feces. The arrowhead indicates the peak of hexadecanal.

n = 13) c-Fos positive cells (Figure 6C). These results support the concept that AGS may be a source for hexadecanal. To further corroborate this idea, we next tested whether the odor of feces isolated from the intestine (before being voided through the anus) is able to activate OR37B glomeruli. As shown in Figure 6D, the number of c-Fos positive cells surrounding the OR37B was not significantly different from the air control (intestine: 7.9 ± 5.6%, n = 27; air: 3.7 ± 3.3%, n = 7; P = 0.1090), but significantly different from the numbers obtained after exposure of mice to deposited feces (deposited feces: 18 ± 8%, n = 45; P = 0.0001).

Attempts to isolate AGS from mice or rats failed because their glands do not store the secretion (Rowlatt et al. 1981). This is different for anal glands of dogs; therefore, we next analyzed the headspace of AGS from a dog. In fact, as shown for a representative sample in Figure 7A, a prominent peak for hexadecanal could be identified in the gas chromatogram (arrowhead in Figure 7A). Compared with the fecal probes from the rodent species, hexadecanal did not represent the highest peak; however, it was present in a considerable amount. These data strongly suggest that hexadecanal may be deposited from the anal gland. We next analyzed whether the exposure of mice to the AGS from dog can activate the OR37B glomerulus. As shown in Figure 7C, c-Fos positive JCs around this glomerulus were in fact detectable. The quantification revealed that 11.0 ± 6.4% (n = 11; see Figure 7D) of the cells were c-Fos positive, indicating that the amount of hexadecanal in the sample was sufficient to activate OR37B.

As a next step, first attempts were made to analyze whether exposure of mice to hexadecanal may affect their behavior. For this purpose, preference, locomotor activity, and open field tests were conducted (see Supplementary Methods).

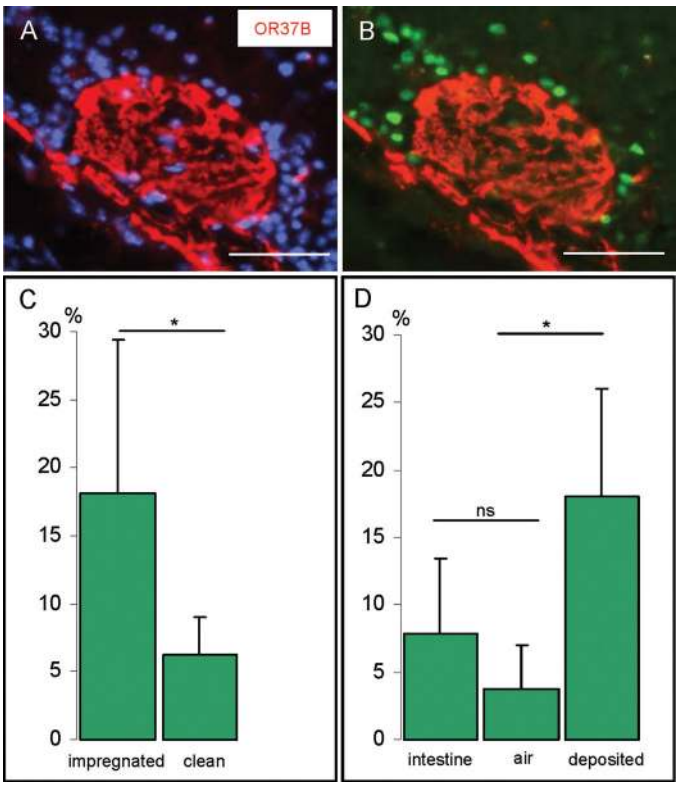

Figure 6 The OR37B glomerulus is activated by material coating fecal pellets, but not by feces from the intestine. (**A**) Cross-section through the OR37B glomerulus visualized by anti-β-galactosidase staining and counterstaining with DAPI. Scale bar: 50 μm. (**B**) After exposure of the mouse to material coating fecal pellets, c-Fos positive cells are visible at the OR37B glomerulus. Scale bar: 50 μm. (**C**) Percentage of c-Fos positive JCs at the OR37B glomerulus induced by material coating fecal pellets versus clean paper. *, statistically significant; data are given as means ± SD. (**D**) Percentage of c-Fos positive JCs at the OR37B glomerulus induced by feces isolated from the intestine versus air control and versus deposited feces. *, statistically significant; ns, statistically not significant. Data are given as mean ± SD.

However, no change in the behavior of animals exposed to hexadecanal compared with control mice could be observed under the tested conditions (see Supplementary Figure S5).

Discussion

In the present study, we have found that hexadecanal, a ligand for the receptor mOR37B, is deposited by mice together with their feces. With this approach, we succeeded to identify a biological source comprising a ligand for a distinct member of the unique OR37 receptor subfamily. Using a variety of analytical methods, we found that fecal pellets from mouse and rat contain the long-chain fatty aldehyde hexadecanal, and activity measurements have shown that exposure of mice to these samples elicited an activation of the OR37B glomerulus. From the various secretions and excretions that were analyzed in this study, significant amounts of hexadecanal were detectable only in feces. However, it cannot be ruled out that other body fluids also contain this compound. In previous studies, it has been shown that hexadecanal is a constituent of chicken feces (Cooperband et al. 2008) but to our knowledge, this is the first report that hexadecanal is a constituent of feces from mammals. In previous studies analyzing the components of feces from various mammalian species, including mouse, there was no evidence for this particular fatty aldehyde (Goodrich, Hesterman, Shaw, et al. 1981; Goodrich et al. 1990a, 1990b; Sankar and Archunan 2008; Apps et al. 2012; Karthikeyan et al. 2013). This discrepancy could be due to different procedures that have been used to analyze the samples. It is well known that the results of such analyses are critically dependent on the methodological approach (for a most recent review, see Drea et al. 2013). We have analyzed freshly collected samples covered by nitrogen to avoid the problem of oxidation of the aldehydes, and the samples were not frozen prior to analysis, as often done. Furthermore, we optimized an SPME procedure for the detection of such long-chain aldehydes and employed GC/MS runs that allow the detection of molecules with long retention times. Future studies can now clarify whether hexadecanal is a constituent, which is generally deposited with feces by mammals.

Our results using the activity marker c-Fos in JCs around receptor-specific glomeruli showed that an exposure of mice to feces activated the OR37B glomerulus to a significant degree; this is consistent with our previous results that the pure substance hexadecanal activated this particular glomerulus (Bautze et al. 2012), and accordingly also OSNs in the epithelium, which express this receptor (Supplementary Figure 4 and Supplementary Methods), whereas no activation was visible when mice were exposed to clean air. The percentage of JC around the OR37B glomerulus was not as high as when the pure compound was used as a stimulus. However—although it cannot be precisely measured—it is very likely that the amount of hexadecanal emitted from feces and thus present in the environment when the mouse investigates the sample, is lower compared with the situation when a mouse is exposed to the pure compound. Together, the results can be viewed in favor of the idea that the OR37 subtype is a detector with rather high sensitivity.

The mechanism(s) by which hexadecanal becomes a constituent of the fecal pellets are not entirely clear, yet. Our findings that an exposure of mice to feces collected from the intestine before it is voided through the anus did not result in an activation of the OR37 glomerulus, whereas it was activated by material transferred from feces onto a blotting paper—suggesting that hexadecanal is located on the surface of such a pellet—both support the view that hexadecanal is secreted from the so-called anal gland. For many species, it is known that they coat their feces with a secretion from this gland (Hesterman and Mykytowycz 1968; Goodrich, Hesterman, Shaw, et al. 1981; Goodrich et al. 1990a). Unfortunately, it was not possible to isolate AGS from mice or rats. However, our analysis of AGS from dog

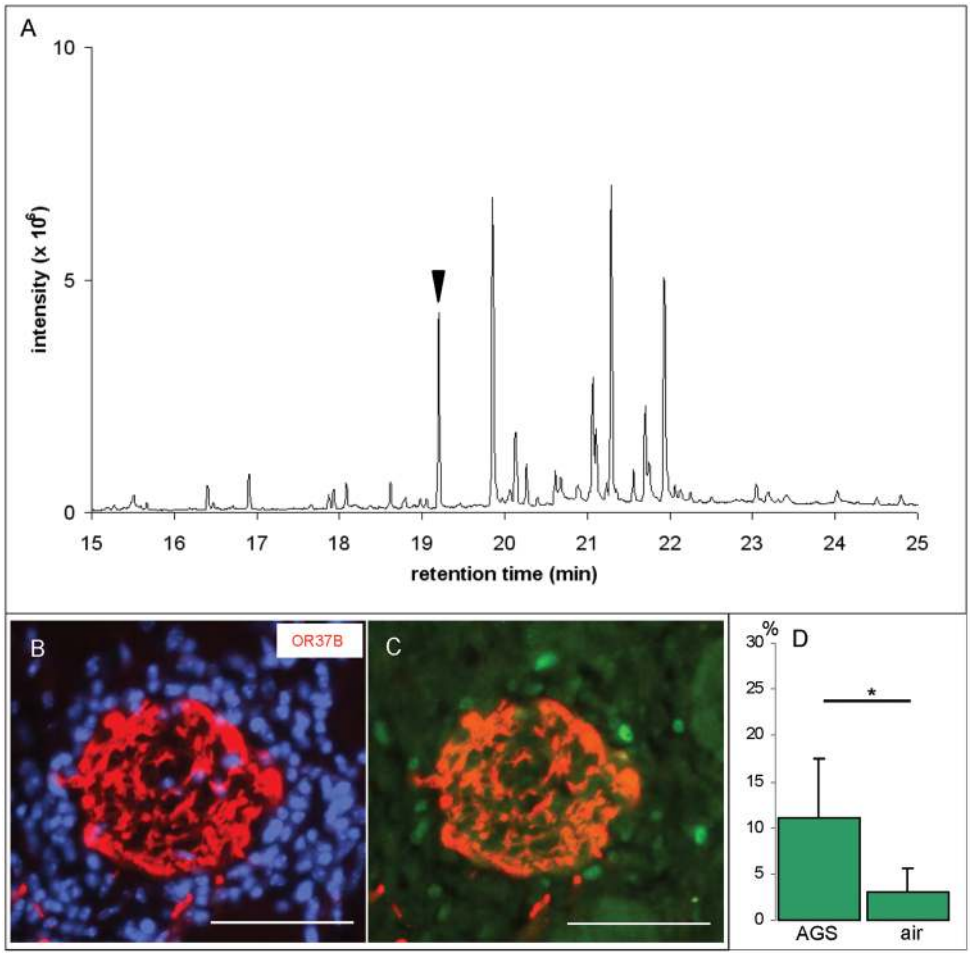

Figure 7 AGS from dog contains hexadecanal and activates the OR37B glomerulus. (**A**) Total ion current GC/MS chromatogram of dog AGS. The arrowhead marks the peak for hexadecanal. (**B**) Cross-section through the OR37B glomerulus of a mouse visualized by anti-β-galactosidase staining and counterstaining with DAPI. Scale bar: 50 µm. (**C**) After exposure of a mouse to AGS from dog, c-Fos positive cells are visible at the OR37B glomerulus. Scale bar: 50 µm. (**D**) Percentage of c-Fos positive JCs at the OR37B glomerulus induced by AGS from dog versus clean air; *, statistically significant; data are given as means ± SD.

has shown that it contained hexadecanal; moreover, exposure of mice to this material led to an activated OR37B glomerulus. Interestingly, previous studies have shown that also the AGS from the giant panda (Yuan et al. 2004) and 2 lemur species contains hexadecanal (Hayes et al. 2004). However, it has not been described for other species (Albone and Fox 1971; Preti et al. 1976; Goodrich et al. 1978, 1990a; Zhang et al. 2003, 2008; Apps et al. 2012), which could be due to the technical reasons outlined above. In this context, it is interesting to note that AGS from ferrets induced an activation of glomeruli in the ventral region of the ferret olfactory bulb (Woodley and Baum 2004; Batterton et al. 2006); in the ventral region of the mouse olfactory bulb, the OR37 glomeruli are positioned (Strotmann et al. 2000). Future studies are required to clarify whether AGS from ferrets does contain hexadecanal.

Urine and other body fluids contain numerous compounds, which are released into the environment and act as socially relevant cues (reviewed by Arakawa et al. 2008). Also feces with its associated secretions is employed by many species as a source for information in chemical communication with conspecifics (Sankar and Archunan 2008; Krueger and Flauger 2011). Accordingly, individuals intensely investigate feces from conspecifics (Ferkin and Johnston 1995; Zenuto et al. 2004; Ghosal et al. 2012). The effects and in particular the social significance of the volatiles emitted from feces have not been investigated as extensively as those comprised in the urine. Nevertheless, several studies have addressed these questions and have shown that mice deposit their feces preferentially in the proximity of their nesting and feeding site (Pennycuik 1973); thus, feces is placed where the animals "feel" comfortable. Feces from other mice causes individuals to also deposit their feces at that site (Goodrich et al. 1990b). Concerning this phenomenon, it has been discussed that the mice most likely show a preference for that location, rather than countermarking it. Studies with BALB/c mice have shown that in a surrounding with mouse feces, there are more nonaggressive encounters

with other individuals and moreover, an increased number of exploratory forays (Goodrich et al. 1990a). Most interestingly, it has been shown that volatiles from feces can have noticeable physiological effects, for example, they lead to a decreased heart rate; the most pronounced effect (almost 50% reduction) was visible when mice were exposed to feces from other individuals (Goodrich et al. 1990b). Altogether, these data provide some evidence that some volatiles emitted from conspecific feces may have positive, comforting, or encouraging effects. Interestingly, similar results have been obtained for rabbits, although the interindividual variability in their response was much higher; based on these differences, it was hypothesized that the response to the volatiles might be context and/or species specific (Goodrich, Hesterman, and Mykytowycz 1981). The question whether hexadecanal is in fact involved in mediating these effects, for example, in mice, cannot be answered at the present time. The finding that this compound is not only present in mouse but also in rat feces and in AGS from dog, which are predators for mice, implies that the reactions that hexadecanal elicits are different within and across species. This seems conceivable when it acts in combination with other molecules that are species specific and by this means triggers distinct physiological reactions or behavioral outputs. This may also be a reason for our finding that exposure of mice to hexadecanal alone did not induce any obvious behavioral changes.

Supplementary material

Supplementary material can be found at http://www.chemse.oxfordjournals.org/

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