Q3 The smelling of Hedione results in sex-differentiated human brain activity

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Q4 Human VN1R1

1. Introduction

“Social odors” or pheromones are defined as chemicals that are released from one animal and evoke a change in the behavior or hormone system of another animal of the same species (Karlsen and Luscher, 1959). Human chemosensory communication is highly complex and controversial (Wysocki and Preti, 2004). The axilla is an origin of human body odor, and axillary sweat is the most likely source of human pheromones. The effects of smelling the bouquets of axillary sweat were examined extensively in recent years. Functional imaging approaches to human brain activity, such as positron emission tomography (PET) or magnetic resonance imaging (MRI), were a focal point in recent studies. The sniffing of human body odor induces the activation of different neuronal networks than common olfactory stimuli (Lundstrom et al., 2008). Further, information about anxiety and emotional stress are transmitted via axillary sweat. Neuronal activation patterns show the involvement of areas that are known for the processing of emotions and the regulation of empathy and attention. Therefore, the olfactory system likely mediates emotional contagion, although participants cannot consciously differentiate perceived chemosensory stimuli (Mujica-Parodi et al., 2009; Prehn-Kristensen et al., 2009). A study of human tears provides another example of social chemical communication. Men showed reduced activity in brain substrates of sexual arousal when sniffing women’s tears induced by sadness (Gelstein et al., 2011). Derivatives of human sex hormones are discussed as single potent molecules that evoke physiological or behavioral responses. The steroids, 4,16-androstadien-3-one (AND) and estra-1,3,5(10),16-tetraen-3-ol (EST) are produced in a gender-specific pattern. AND and EST were linked to pheromone-like activities because they influence mood, physiological arousal, visual perception and brain activity (Grosser et al., 2000; Lundstrom et al., 2003; Bensaft et al., 2004; Villemure and Bushnell, 2007; Zhou et al., 2014).

There are two types of chemoreceptor families recognizing pheromones in rodents: ~240 vomeronasal-type 1 receptors (V1r) and ~61 vomeronasal-type 2 receptors (V2r). V1r and V2r are expressed in vomeronasal sensory neurons (VSNs) of the vomeronasal organ (VNO), which is a distinct structure besides the main olfactory epithelium (MOE) located in the nasal cavity (Dulac and Axel, 1995; Herrada et al., 2000; Villemure and Dulac, 1997; Yang et al., 2005; Young et al., 2010). Isolated VSNs are activated by nonvolatile peptides of the main histocompatibility complex (MHC) class I, thought to be responsible for kin recognition alongside other volatile pheromone substances (Leinders-Zufall et al., 2014).
Only one receptor — agonist pair in the vomeronasal system was identified so far, which showed that murine V1r2b expression is required to elicit a response to the pheromone 2-heptanone (Boschat et al., 2002). V1r family sizes show species-specific expansions throughout mammalian evolution, and the functional repertoire roughly correlates with anatomical observations of VNO size and quality. V1r family size declines in primates, and the V1r repertories of all Old World monkeys and apes consist primarily of pseudogenes (Young et al., 2010). There are over 100 functional V1rs in the rat and mouse genomes, but only five intact vomeronasal-type 1 receptor genes (VN1Rs) are found in human and chimpanzee genomes (Liman, 2006). At least one of the five intact VN1R genes is expressed in cells of the human olfactory mucosa (Rodriguez et al., 2000).

Humans also suffered inactivating mutations in the vomeronasal signal transduction gene Trpc2 (Liman and Liman, 2003). The loss of molecular components of VNO signaling is consistent with the absence of a functioning VNO in adult humans, but it does not necessarily indicate a loss of the sensing and functioning of “social odors” (Witt and Hummel, 2006). The detection of “social odors” was thought to be accomplished solely through the VNO, but it is now accepted that the main olfactory epithelium (OE) is also involved in the sensing of “social odors” (Brennan and Zufall, 2006; Frasnelli et al., 2011). In support of this view, surgical removal of the VNO in neonatal rabbits demonstrated that the stereotypic nipple search behavior occurred independently of the VNO via the main olfactory pathway (Distel and Hudson, 1985). Domestic pigs do not require the VNO for the detection of, and behavioral responses to, 5α-androst-16-en-3-one, which is a pheromone in boar’s saliva (Dorries et al., 1997). Additionally, the olfactory receptor (OR) OR7D4 responded to AND and 5α-androst-16-en-3-one, a putative pheromone in humans, in a heterologous cell system. A common variant of OR7D4, which expresses impaired function in vitro, correlated with variability in the perception of AND and 5α-androst-16-en-3-one in humans (Keller et al., 2007). Sensory neurons of the VNO that express the same vomeronasal receptors have neuronal projections to multiple glomeruli in the accessory olfactory bulb (AOB), which provides a direct pathway to hypothalamic areas where neuroendocrine levels can be regulated after pheromone detection (Li et al., 1990; Boehm et al., 2005). Receptors of the OE project to the main olfactory bulb (MOB). Individual mitral cells of the MOB in mice respond to volatile compounds in urine. Mutant mice possessing a non-functional dorsal MOB domain lack odor-driven innate aversive responses (Lin et al., 2005). Therefore, the MOB may also process social signals. Humans appear to lack a VNO and AOB (Brennan and Zufall, 2006; Frasnelli et al., 2011), but the smelling of AND causes hypothalamic activations that are gender-specific and similar to regions that control sexual orientation in other mammals (Savic et al., 2001, 2005; Savic, 2002; Berglund et al., 2006). If hypothalamic activations induced by AND are in fact gender-specific remains controversial (Burke et al., 2012).

The vomeronasal receptor VN1R1 is expressed in the human OE, and it is activated by volatile compounds in a heterologous system. However, the function of VN1R1 in vivo remains elusive (Shirokova et al., 2008). Our study indicates that not only VN1R1, but all intact VN1Rs are expressed in the human olfactory mucosa. We identified Hedione (HED) as a ligand for VN1R1 in two different heterologous expression systems and examined the pattern of cellular processing in response to the smelling of HED in humans.

2. Material and methods

2.1. Reverse-transcriptase polymerase chain reaction

RNA of nasal mucosa biopsies was isolated using the RNeasy Midi Kit (Qiagen). The cDNA was synthesized using a c-master RT Kit (Eppendorf). Polymerase chain reaction (PCR) was performed using 2 ng template cDNA. The primers and expected product sizes are given in Table 1.

Table 1

<table>
<thead>
<tr>
<th>Primer sequences and expected PCR product sizes.</th>
<th>Exp. size</th>
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<tr>
<td>VN1R1fw AGGCTGGCCACACAGACTTC</td>
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<tr>
<td>VN1R1rw CGTCCCTGTGCTGAACACT</td>
<td>515</td>
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<tr>
<td>VN1R2Nfw TCTTGACAGACAGGAGAAC</td>
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<tr>
<td>VN1R2Nrw CGATGCCACCAACACAAAC</td>
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<td>VN1R3rw GCATTCAGTTACCTGGGCCTTTCCTAAGGATGTC</td>
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<tr>
<td>VN1R4fw GCATCGTGCATGGCCGCTGGGTTTGCAACTC</td>
<td>949</td>
</tr>
<tr>
<td>VN1R4rw GCATCGTGCATGGCCGCTGGGTTTGCAACTC</td>
<td>1112</td>
</tr>
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</table>

2.2. Cloning of full-length VN1R1

The human VN1R1 coding sequence was amplified from genomic DNA using the primer pair VN1R1fw (GCAAGCTTACATGCTGGAAG) and VN1R1rw (AGCTCGAGCTCGTCCTTCAACAT), which amplified the entire open reading frame (NM_020633), which was cloned into pcDNA3 (Invitrogen) (Fig. 2). The generated plasmids were verified using sequencing, and two genetic variants were identified to yield the plasmids pVN1R1-CC and pVN1R1-AT.

2.2.1. Cell culture and transfection

Cell culture and transfection were performed as described previously (Wetzl et al., 1999). Reagents for cell culture use were purchased from Invitrogen, unless stated otherwise. HEK293 cells were maintained under standard conditions in minimum Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin and streptomycin, and 2 mM L-glutamine at 37 °C and 5% CO2. Transfections were performed using standard calcium phosphate precipitation. Cells were transfected with 2.5 μg DNA per 3.5-mm dish and incubated for 12 h for DNA uptake.

2.3. Single-cell Ca2+ imaging

Ca2+ imaging experiments were performed as described previously (Wetzl et al., 1999). Culture medium was removed and replaced by the standard experimental solution (in mM: 140 NaCl, 5.4 KCl, 1.8 CaCl2, 1.0 MgCl2, 10 HEPES, and 5 glucose, pH 7.4) containing 7.5 μM fura-2/AM (Invitrogen) prior to experiments. Cells were incubated for 45 min at 37 °C and washed with a fura-2/AM-free solution. Ratiometric Ca2+ imaging was performed using an inverted microscope (Axiovert 100; Zeiss) and a monochromatic illuminator (T.I.L.L. Photonics GmbH, Planegg, Germany) to generate alternating monochromatic wave-lengths. A CCD camera captured fluorescence signals (PXL 37; Photometrics). Camera controlling and data recording from randomly selected fields of view were performed using WinNT based-software.
(T.I.LL-Photonics; Vision 3.3). All fluorescence ratios \( \frac{f_{340}}{f_{380}} \) were background-corrected.

Exposure to odors was accomplished using a specialized switch-operated superfusion device. ATP (10 μM; Thermo Scientific) was used as a positive control. Applied odorant mixture H-100 (200 μM of each substance) induced transient Ca\(^{2+}\) signals in VN1R1-transfected cells. Subdivision of the mixture into mixtures of 10 different odorants (H-10; 1 mM of each substance) and further subdivision into single substances led to the identification of HED as a ligand for VN1R1. An HED-reduced H-10 mix (H-10\(^{-}\)) failed to induce transient Ca\(^{2+}\) signals. AND (1 mM) did not activate VN1R1. In total, no significant differences between the two isoforms, VN1R1-CC and VN1R1-AT, were observed. The positive control was 10 μM ATP. Gray trace: Ca\(^{2+}\) signals in non-transfected cells.

**2.4. Cre-luciferase assay**

We used the well established Cre-luciferase reporter gene assay for high throughput functional chemosensory receptor screening. We adapted the optimized protocol of Zhuang and Matsunami for measuring receptor activity with the Dual-Glo Luciferase Assay System (Promega) (Zhuang and Matsunami, 2008). The method is commonly used for screening chemoreceptor activity with a broad panel of different chemicals and different concentrations in parallel. Thus, after the agonist...
identification with the help of Ca$^{2+}$-imaging experiments, we used the
Cre-luciferase assay for an extended screening and the construction of
concentration–response relationship. At the same time, two independent
recombinant systems revealing the same agonists, improve the reliability
of results as they were performed in previous studies (Wallrabenstein et al., 2013; Busse et al., 2014). Whereas Ca$^{2+}$-imaging measures an intra-
cellular Calcium increase after receptor activation, the Cre-luciferase
assay uses the intracellular CAMP accumulation, which is typical for signal
transduction of canonical odorant receptors and was also shown for
VN1R1 (Shirokova et al., 2008).

HANA3A cells were maintained under standard conditions in DMEM
supplemented with 10% FBS and 100 units/ml penicillin and streptomycin
in 37 °C. Cells (approximately 15,000 cells/well) were plated on
poly-l-lysine-coated 96-well plates (NUNC) and transfected after 24 h
with FuGENE® HD (Promega) according to the manufacturer’s protocol. We used for one complete 96-well plate 18 μl
transfection reagent, 5 μg pVN1R1, 2 μg pGL4-luciferase reporter, 1 μg
pRL-TK-Renilla reporter, 0.5 μg G-protein αolf and 1 μg receptor trans-
port protein (RTPI5) to ensure cell surface expression (Zhuang and Matsunami, 2007). Cells were stimulated 24 h after transfection for
four hours at 37 °C with agonists (purchased from Sigma Aldrich or
received from Synrise AG, Holzminden, Germany) diluted in CD 293
medium (1 ×) (Life Technologies) with 2 mM L-glutamine added.

After 4-h stimulation with test substances, recombinant VN1R1 activ-
ation elevated CAMP and the subsequent expression of the CAMP-
dependent reporter gene constructs Cre-luciferase, as described previ-
ously for chemosensory receptors of the OE (Zhuang and Matsunami, 2008; Wallrabenstein et al., 2013). Expression rates of Cre-luciferase
were monitored by luminescent enzymatic reactivity. Thus, lumines-
cence signals correlate with receptor activation. Renilla luciferase re-
porter driven by a constitutively active TK-promoter (pRL-TK-Renilla)
was served as an internal control to determine cell viability and transfection
efficiency. We normalized firefly luciferase activity to Renilla luciferase
signal for a certain wells. These ratios were normalized to the negative
control signal of DMSO to calculate an activation factor above basal
level. In addition, mock-transfected cells were stimulated to exclude un-
specific responses to the tested substances. We applied a one-tailed un-
paired Student’s t-test to calculate whether there was a significantly
stronger activation in pVN1R1-CC cells compared to mock-transfected
cells. We stimulated cells in control wells with forskolin (10 μM; Sigma Aldrich), an activator of adenylyl cyclase, to test the functionality
of the assay system.

2.5. Subjects

Seventeen right-handed subjects (9 women and 8 men, mean age
23.6 ± 3.6 years) participated in the functional imaging (fMRI) experi-
mment. Handedness was assessed using the modified Edinburgh Invento-
ry (Oldfield, 1971). All participants provided informed consent, and the
local ethics committee approved all procedures (application # 10603
2011).

2.6. Odorants and olfactometry

Odorants were delivered via a cannula (4-mm inner diameter) to the
left nostril using a computer-controlled olfactometer (OM6b; Burghart,
Wedel, Germany) with a flow rate of 7.1 l/min. The odorants used
included phenylethyl alcohol (Aldrich, Steinheim, Germany; order #
77861) and the V1R-agonist Hedione (Firmenich, Meyrin, Switzerland;
order # 947325, used as a 5% solution in propylene glycol). Both odors
produce a floral, pleasant smell. Therefore, both stimuli were presented
in concentrations clearly perceivable and without causing any trigemi-
nal sensation to ensure a similar intensity, as determined by a small
panel of healthy subjects prior to the present experiments; for the ex-
periments proper the same concentrations of both odors were used
across all participants (HED 27.3% v/v; PEA 33% v/v). The odorants were
embedded in a warm, humidified airflow and presented in blocks of
24 s each. Multiple odorous stimuli were presented with a duration of
1 s within the blocks, and the interstimulus interval was randomized be-
 tween 1 to 3 s. The no-odorant condition was identical except for the
embedded odorant. Subjects performed velopharyngeal closure and
breathed only through their mouth during the experiments (Kobal and
Hummel, 1988). Subjects were trained in this technique using biofeed-
back prior to testing.

2.7. Experimental design

This study used a passive smelling paradigm in a block design. There
were six blocks of odor and six blocks of the no odor conditions. The par-
adigm was replicated once. Odorants were presented pseudorandomly
and in a different order for the two sessions; Subjects rated the intensity
and valence of the odors following each block using visual analogue
scales that ranged from 0 to 10 (0 = no odor perceived; 10 = very
strong) or −5 to +5 (−5 = very unpleasant; +5 = very pleasant), re-
spectively. None of the subjects reported a stinging or burning sensation
in response to odorous stimulation.

2.8. Imaging parameters

A 1.5 T MRI scanner (Siemens Sonata, Erlangen, Germany) and a full-
head eight-channel receiver coil were used for image acquisition. A gra-
dient echo T2*-sensitive echo planar imaging (GE-EPI) sequence was
employed (TR 3000 ms, TE 35 ms, image matrix 64 × 64, in-plane reso-
lution 3 mm, through-plane resolution 3.75 mm). The time of echo was
close to the optimal 50 ms that was established using a 1.5 T by Stocker
and colleagues (2006) for the imaging of limbic structures (Stocker
et al., 2006). Images were acquired in the axial plane oriented parallel
to the planum sphenoidale to minimize bone artifacts. A total of 96 func-
tional volumes per run in twenty-six slice locations (covering nearly the
entire head) were acquired per session. A full brain T1-weighted turbo
FLASH 3D-sequence was acquired to overlay functional data (TR 2150,
TE 3.93, slice thickness: 1 mm).

2.9. Imaging analysis

Data were analyzed using SPM8 (http://www.fil.ion.ucl.ac.uk/spm/)
in Matlab (Matlab 6.5 R3, The MathWorks Inc., Natick, MA). Images
were realigned and corrected for motion based on the realignment pa-
rameters. No subject was excluded from further analysis due to head
movement. Spatial normalization into the Montreal Neurological Insti-
tute standard space was followed (MNI template supplied with SPM).
Images were smoothed using an 8-mm smoothing kernel to render the
images suitable for statistical analyses. SPM uses the General Linear
Model to describe the data in respect to experimental and confounding
effects and residual variability. Hypotheses testing were ensued. First
level analysis was performed individually, and the results were taken to
the second level in a group random effect analysis. Analyses were
performed on eight predefined regions of interest (ROI): olfactory
eloquent areas (piriform cortex, amygdala, thalamus, hippocampus,
insula, orbito-frontal cortex), hypothalamus, and midbrain. These
areas underwent an analysis of variance with the between subject factor
gender and the within subject factor odorant. Statistical maps were
thresholded at p < 0.05corrected with a cluster criterion of three voxels
for whole brain analysis and Bonferroni-corrected for multiple compar-
isons of the eight ROIs (p < 0.05/8 = 0.006) with a cluster criterion of
three voxels for ROI analysis. Statistical maps for explorative whole
brain analyses were thresholded at p < 0.001 uncorrected with a cluster
of three voxels. Predefined areas of interest (ROI) were ana-
alyzed. All masks were created using the "automated anatomical labeling
(‘aal’) atlas (Tzourio-Mazoyer et al., 2002), embedded in WFU PickAtlas
2.4 software (Maldjian et al., 2003), except for the piriform cortex
(defined according to the criteria described in (Zelano et al., 2005)).
and the hypothalamus (6-mm sphere around (−6 | 0 | −14)). The values of the ROI analyses were entered into a statistics package (SPSS 21, SPSS Inc., Chicago, IL, USA) to perform a second-level group random effect analysis for inference. Activation estimation data was extracted for the peak value of the hypothalamus region using the marshall toolbox (Brett et al., 2002) for all 8 scans following the HED stimulation and following the OFF conditions and contrast estimates were calculated by time wise subtraction of OFF from HED conditions. The impact of pleasantness rating on neuronal response was calculated using an ANOVA for repeated measurements with the within subject variable “time” (8), the between subject variable “gender”, and “pleasantness ratings” as covariate. Time and gender were included because those variables were found to significantly impact neuronal response in the hypothalamus.

3. Results

3.1. mRNA of all intact VN1Rs is present in human olfactory mucosa

We analyzed the expression of all potentially functional members of the human VN1 gene family and detected mRNA transcripts by PCR in the human olfactory mucosa. All VN1R transcripts clearly showed distinct bands and accordingly robust mRNA transcript levels. The VN1R10P gene, which is annotated as a pseudogene in the NCBI database (Gene ID: 387316), was also detected in human olfactory mucosa (Fig. 1A). Beside the non-coding RNA (NR_045612.1) a complete coding sequence can be found as well (AF352327.1). Like VN1R8 and VN1R5, VN1R10P might occur as a segregated pseudogene. Control experiments without reverse transcription (RT) ensured the specific detection of the amplified transcripts (Fig. 1A). PCR products of amplified VN1Rs were verified using Sanger sequencing and the corresponding PCR primers (Table 1). We verified the expression of different canonical signaling proteins that are typically present in olfactory sensory neurons; such as G-protein αolf, adenylyl cyclase type 3 (ACII), and the cyclic nucleotide-gated channel subunit A2 (CNGA2), as well as the expression of randomly canonical ORs, supporting that the analyzed biopsies truly contained OE (Fig. 1A).

3.2. Hedione activates human VN1R1

As VN1R1 revealed the most striking signal in our RT–PCR experiments, we cloned the full open reading frame and recombinantly expressed the common variant pVN1R1-CC in HEK293 cells for Ca2+-imaging experiments. A complex mixture of 100 different compounds (Henkel 100: H-100) was initially used to identify VN1R1 ligands (Wetzel et al., 1999; Spehr et al., 2003). Progressive subdivision of the H-100 mixture (20 mM) into smaller fractions (H-10; 10 mM) leads to only one active substance: Hedione (HED). The other single substances of the activating H-10 mixture: cymol, eugenol, geraniol, helional, lyril, (R)-(-)-carvon, S- (+)-carvon, citral, and benzyl acetate (1 mM of each), did not induce any intracellular Ca2+ increase. In addition, application of an H-10 mixture lacking Hed did also not induce any responses, which confirmed HED as the active ligand (Fig. 1B). The application of 1 mM HED induced transient Ca2+ signals in 82 cells in 52 independent experiments of approximately 250 cells each. Even lower concentrations down to 1 μM induced VN1R1 activation (Fig. 1B). Ca2+ increases were never observed for tested mixtures or single substances in non-transfected HEK293 control cells (Fig. 1B). In addition, we tested the putative human pheromone AND (1 μM) on VN1R1 action, but found no Ca2+ rises after stimulation (Fig. 1B).

Genetic variations of chemoreceptors caused by single nucleotide polymorphisms (SNPs) that result in an amino acid change (S201F; A229D), were described for VN1R1 (Rodriguez et al., 2000). The two VN1R1 variants contain either a C at mRNA pos. 720 and 804 or T at mRNA pos. 720 and A at pos. 804 in the coding sequence (VN1R1-CC; VN1R1-AT). In following experiments, we tested the potential impact of the reported SNPs on receptor function and found that HED activated both VN1R1 variants. HED activation was normalized to the corresponding ATP control. The mean values exhibited high standard deviations (nearly half of normalized responses) and showed no statistically significant differences (p > 0.05 unpaired Student’s t-test) between the two VN1R1 isoforms: VN1R1-CC and VN1R1-AT (Fig. 1B).

After Ca2+ imaging experiments lead to the identification of an agonist, we employed the Cre-luciferase reporter gene assay to test more possible ligands (see Material and methods). The tested substances were comprised of HED, H-10, to retest the odorant mixture that led to the identification of HED in our Ca2+-imaging experiments (Fig. 1B), and the most related chemical structure of Hed, namely methyl jasmonate, the VN1R1 agonists previously reported by Shirokova et al. (α-ionone, β-ionone, C7–C10 aldehydes, carveol and myrtrenal) and 5α-androst-16-en-3-one, which is beside AND also present in human sweat (Bird and Gower, 1981; Shirokova et al., 2008). HED (4.10 ± 0.45-fold basal level) and H-10 (4.17 ± 0.42-fold basal level) significantly activated VN1R1 compared to mock-transfected controls, which confirmed our Ca2+-imaging data. In addition, methyl jasmonate significantly activated VN1R1, but with a lower efficacy (1.92 ± 0.10-fold basal level) as well as the previously reported ligands α-ionone and β-ionone (1.66 ± 0.19 and 1.70 ± 0.2-fold basal level, respectively). 5α-androst-16-en-3-one, C7–C10 aldehydes, carveol and myrtrenal did not significantly activate VN1R1. Amongst all agonists, HED showed the highest efficacy and activated the recombinant VN1R1 in a concentration-dependent fashion with a detection threshold concentration of 30 μM (*p < 0.05) (Fig. 3).

3.3. fMRI characterization of HED action

Following the heterologous identification of HED activation of a putative human pheromone receptor, we investigated whether HED activates brain regions beyond the canonical olfactory network. For inducing activation of the canonical olfactory network as a control, the common odor phenylethyl alcohol (PEA) was used (see Material and methods). We report the results of the group random effect analysis of the 17 subjects enrolled in the study. The effects of individual odors on brain activity were studied in respect to the baseline condition air.

Ratings provided during sessions indicated that HED and PEA were rated at similar intensities (PEA: M = 5.93, SD = 1.69; HED: M = 5.47, SD = 1.60; F[1,15] = 0.78, p = 0.39). There was also no major difference between the valence of odors because both odors were perceived as mildly pleasant (PEA: M = 0.65, SD = 2.19; HED: M = 1.19, SD = 1.77; F[1,15] = 1.27, p = 0.26).

Fig. 3. Concentration–response relationship for VN1R1 and HED. Curve fitting was performed using the Hill equation in SigmaPlot V8.0 (Systat Software, San Jose, CA). The calculated EC50 is 56 μM ± 0.094 μM (n = 4). Detection threshold concentration was 30 μM (p < 0.05).
Table 2

<table>
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<th>Cluster level</th>
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The VN1R1 agonist HED led to activation in both insulae (−39 6 12; −30 24 6; 27 18 12; 42 15 0), the left amygdala (−21 3 6), the right amygdala approaching the right hippocampus (36 9 6; 24 15 6), and the left and right orbital regions (−30 45 15; −51 39 9; −45 30 6; 39 42 6) (Table 2; see also Supplementary Table 1). Activations of the insula approached the piriform cortex, and extended further medially when statistical thresholds were lowered. HED significantly enhanced activation compared to the PEA stimulus in a cluster reaching from the right amygdala to the right hippocampus (33 9 18). There were no differential activations induced by PEA compared to HED in olfactory eloquent brain areas (Fig. 4).

Furthermore, the VN1R1 agonist led to activation in two clusters in the left and right midbrain (−6 3 6; 3 18 9), and the left midbrain reached into the hypothalamic region. None of these regions showed suprathreshold activation in response to PEA. Accordingly, the VN1R1 agonist activated the hypothalamus significantly more than PEA (0 0 6). Notably, this result remained significant after correction for family-wise error (p = 0.018).

The effect of selective hypothalamic activation of the VN1R1 agonist was assessed for gender differences. A significant differential activation was observed in women compared to men in the hypothalamus (3 3 6; p = 0.003). No gender effects in the hypothalamus were found for the PEA odor, which suggests that this effect was not a general effect of olfactory activation. Notably, women also perceived HED as significantly more intense than men (p = 0.034). There were no significant gender effects on the hedonic perception.

Fig. 4. Results from fMRI analyses. In the top row (A), activations in olfactory regions of interest are shown separately for PEA and HED. Enhanced activation in HED vs. PEA was found in the hippocampus (B) and hypothalamus (C). Contrast estimates for the HED vs. PEA comparison and the time course of activation in the hypothalamic region are provided. D) Contrast estimations plotted against pleasantness ratings showed a significant correlation. The less pleasant that HED was perceived, the more activation was found in the hypothalamic region. The activation was more pronounced in women compared to men (bar plot). For the visualization purpose, activations are shown with a threshold of p < 0.005, uncorrected and plotted on a T1-weighted image provided by the MRicron program (Rorden et al., 2007).
Moreover, the hedonic perception of the VN1R1 agonist was related to the strength of activation in the hypothalamus region (F(1,14) = 5.0, p = 0.042). The decreasing pleasantness of the VN1R1 agonist corresponded to increasing activity in the hypothalamus region. No such effect was observed for the intensity perception of the VN1R1 agonist.

4. Discussion

4.1. Human VN1R expression

Our RT–PCR experiments showed that not only VN1R1, but all intact human VNR transcripts (VN1R1–VN1R5) are present in the olfactory mucosa. Consistent with a previous study, VN1R1 exhibited the most robust signal (Fig. 1A) (Rodriguez et al., 2000). With very low expression levels, VN1R1 mRNA was detected beyond the human olfactory mucosa as well, in the brain, lung and kidney (Rodriguez et al., 2000). A recent study of chemosensory receptors analyzed transcriptome data and established a comprehensive overview of gene expression. These results also indicated a widespread VN1R1 expression, but in total confirm very low expression rates in non-olfactory tissues (Fiegel et al., 2013). A ligand screening and subsequent binding study demonstrated heterologous VN1Rs activation by some volatile compounds, which in turn functions for a functional role in olfaction, especially for VN1R1, as it has shown the most robust expression in the olfactory mucosa (Shirokova et al., 2008; Corin et al., 2011). This possibility is supported by the fact that all human VNR1s use the canonical cAMP signaling via G-protein Gαolf when recombinitely expressed in Hela/Olf cells (Shirokova et al., 2008), or HANA3A in this work, which is a characteristic of ORs. Another class of a chemosensory receptor family expressed in vertebrate OE is called trace amine-associated receptors (TAARs). TAARs mediate odor-driven innate responses within the OE and use cAMP signaling as well (Liberles and Buck, 2006; Wallrabenstein et al., 2013).

4.2. Activation of recombinitely expressed human VN1R1

To characterize the activation of VN1R1, we further examined VN1R1 in ligand screenings. The initial screening was performed using Ca²⁺-imaging experiments, which led to the identification of HED as an agonist of VN1R1. As previously reported, we had only a small quantity of responding cells, which was likely due to weak transfection rates (Wetzel et al., 1999). Further, successful transfection is not imperatively equivalent to the functional integration of the receptor into the cell membrane. Responses showed a clear Ca²⁺ increase, in some cases almost with the same magnitude as the activation level of ATP control for maximum stimulation, whereas non-transfected cells were not affected (Fig. 1B). Two SNPs within the coding sequence of the VN1R1 gene were described (Rodriguez et al., 2000). These SNPs occur in pairs and result in an exchange of amino acids (S201F; A229D). One exchange is located within a transmembrane domain, and the other exchange affects an intracellular loop. These SNPs are annotated in the dbSNP (rs61744949; rs28649880), and both occur with an allele frequency of 30%. However, only 9% of the analyzed people are homozygous for these SNP alleles. Allele frequency data were based on the genotypes of 4550 European American individuals from the NHLBI Grand Opportunity Exome Sequencing Project (ESP) Exome Variant Server, release ESP6500 (https://esp.gs.washington.edu/drupal/).

Ca²⁺-imaging experiments revealed that the amino acid exchange did not alter the response to HED (p > 0.05), and the SNPs did not appear to affect receptor functionality. For an extended ligand screening, we switched over to a commonly used high throughput reporter gene assay system. We confirmed the HED action on VN1R1 and moreover, determined a concentration–response curve with an EC50 value of 56 ± 0.09 μM. Methyl jasmonate is the most structurally related compound to HED, and it differs in a double bond that occurs within the pentencyl side chain. This compound also activated VN1R1. Methyl jasmonate is a volatile organic compound and stress hormone that induces defense-related mechanisms in plants. Notably, different studies demonstrated anticancer activity of this compound (Rotem et al., 2005; Ezekwudo et al., 2007; Cohen and Flescher, 2009; Zheng et al., 2013). Using the Cre-luciferase system we screened previously reported agonists (α-ionone, β-ionone, C7–C10 aldehydes, carveol and myrtenal) (Shirokova et al., 2008). Ionones activated VN1R1 with an approximately 2.5-fold lower efficacy than HED. The relative efficacies of ionones and methyl jasmonate did not differ from each other (p > 0.05). The C7–C10 aldehydes, carveol and myrtenal did not activate VN1R1 significantly compared to the mock controls. This disagreement may be explained by the different assay systems and possible lower efficacies, which were not sufficient to reach significant activation levels for the tested screening concentrations in our system. Different substances may also activate the receptor with different kinetics and desensitization rates. Shirokova et al. used a Ca²⁺–FIPLR assay, collected Ca²⁺-dependent fluorescence data 5–10 min after application and monitored direct receptor activation in much shorter time scales. This result contrasts our luciferase system, in which cAMP accumulation was measured after a 4-h stimulation with the test substances, and the possible quick desensitization of the cAMP rise after receptor activation would not be captured.

However, because HED was the most efficient agonist in our VN1R1 ligand screening, we used HED for subsequent fMRI experiments to clarify, if smelling a substance activating a putative human pheromone receptor elicits neuronal networks described for mediating pheromone effects in other mammals.
To exclude concentration-dependent differences between the two test odors when comparing neuronal activations, we ensured that subjects reached the same odor intensity of each by rating. We applied test odors at perceivable concentrations and thus, cannot exclude OR activation for HED. In fact, dual properties of some odorant molecules might be mediated by different receptors. Accordingly, HED might be detected by a so far unknown OR (or different ORs) to mediate the general odor perception, while pheromonal receptors like VN1R1 are connected to neuronal circuits, capable to alter the hormonal state. This seems to be a perfect match for our results, since we did not observe any differential activation induced by PEA compared to HED in olfactory eloquent brain areas, but significant differences in prespecified hypothalamic regions, associated with regulation of hormone release in mammals. The hypothalamic regions were also shown to be activated by the endogenously occurring steroid AND out of human sweat (Savic et al., 2001), whereas HED is obtained by chemical synthesis, only. Noteworthy, the smelling of the non-natural compound HED shows similar hypothalamic activation as AND does. Challenged by capturing the absolute smell of jasmonate, methyl dihydrojasmonate was discovered in 1958 by a chemist (Keller, A., Zhuang, H., Chi, Q., Vosshall, L.B., Matsunami, H., 2007). Genetic variation in a GPC human vomeronasal type 1 receptor (Savic et al., 2001), whereas human odorant receptor alters odor perception. Nature 449, 468–472.

6. Conclusion

The current data show that HED is an odorous substance that activates the VN1R1 receptor, expressed in the OE. We postulate an involvement of VN1R1 activity in observed sex-differentiated hypothalamic activation. Future studies might clarify whether VN1R1 activation indeed leads to modulation of hormonal secretion in humans. But hypothalamic Gonadotropin-releasing hormone (GnRH) neurons within the OE (Liberles and Buck, 2006; Liberles and Buck, 2005; Yoon et al., 2005). Thus, one might speculate that the hypothalamic regions were also shown to be activated by the endogenously occurring steroid AND out of human sweat (Savic et al., 2001), whereas the chemical structure of HED might be similar to a currently unknown naturally occurring ligand, which has the potential of gender-specific modulation of hormonal secretion in humans.

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None of the authors declares a conflict of interest.


