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

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ABSTRACT

A large family of vomeronasal receptors recognizes pheromone cues in many animals including most amphibia, reptiles, rhodents, and other mammals. Humans possess five vomeronasal-type 1 receptor genes (VN1R1–VN1R5), which code for proteins that are functional in recombinant expression systems. We used two different recombinant expression systems and identified Hedione as a ligand for the putative human pheromone receptor VN1R1 expressed in the human olfactory mucosa. Following the ligand identification, we employed functional magnetic resonance imaging (fMRI) in healthy volunteers to characterize the *in vivo* action of the VN1R1 ligand Hedione. In comparison to a common floral odor (phenylethyl alcohol), Hedione exhibited significantly enhanced activation in limbic areas (amygdala, hippocampus) and elicited a sex-differentiated response in a hypothalamic region that is associated with hormonal release.

Utilizing a novel combination of methods, our results indicate that the putative human pheromone receptor VN1R1 is involved in extra-olfactory neuronal activations induced by the odorous substance Hedione. The activation of VN1R1 might play a role in gender-specific modulation of hormonal secretion in humans.

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 (Karlson 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 these steroids 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; Bensafi 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). V1rs and V2rs 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 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.,

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2000, 2004). Only one receptor — agonist pair in the vomeronasal system was identified so far, which showed that murine V1rb2 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 repertoires 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 Innan, 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 necessarily 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 exhibits 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 (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 cerebral processing in response to the smelling of HED in humans.

Material and methods

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
Primer sequences and expected PCR product sizes.
Primer Sequence (5′–3′).

		Exp. size
VN1R1fw	AGGGTGGGCACAAGAGTTTCC	528
VN1R1rw	CTGGCCTGGGTTTGCAACTAC	
VN1R2NWfw	TCTCTGCACACGGAGAGAAAC	602
VN1R2NWrw	CATGAGCCCCAGACACAAAAC	
VN1R3fw	GCATAAGCTTACCATGGCGGCCGCCTCCAAGGATTTTGC	979
VN1R3rw	GCATCTCGAGTCATGCGGCCGCTCTGACCAGCTTAGGAAAC	
VN1R4fw	GCATAAGCTTACCATGGCGGCCGCAGCCTCCCGGTATGTG	949
VN1R4rw	GCAGTG GCATCTCGAGTCATGCGGCCGCTCTTTTCCAGGCAAAAC	
VN1R5NWfw	CTGAGTGTGCTCCAGGCCATC	515
VN1R5NWrw	TTGGCCACAATAACCTGGAGC	
VN1R10Pfw	GCATAAGCTCCACCATGTGGAATATATTTATGCTGC	1050
VN1R10Prw	GCATCTCGAGTTAAAAACACATTCTCTTGTGG	
ACIIIfw	CTCAGCTGTCTCCAGTACTAC	323
ACIIIrw	CTGTCAGTGCCATTGAGCCCA	
Golffw	GAGGCCAACAAAAAGATCGAGA	327
Golfrw	CTGGGAATATTGAAAGTCAGTG	
CNGfw	AGCCTGCTTCAGTGATCTACACAC	572
CNGrw	AAATAGGTACTCCTCATCCTTTAC	
OR11A1fw	GCGGATCCACCATGGAAATTGTCTCCACAGGAAACG	948
OR11A1rw	GCCTCGAGTAAGGCGGCCGCATCAAGTGTTTCAGTTTGTTTG	
OR2C1fw	GCGGATCCACCATGGACGGGGTGAATGATA	939
OR2C1rw	GCCTCGAGTAAGCCAACTTCTCTTCCTTTC	
OR10R2fw	GCGGATCCACCATGTTTTACTTCTTTCCCCTTTGC	1008
OR10R2rw	GCCTCGAGTAAGGCGGCCGCATTATATAGTTTTAGAGAACC	
OR6C1fw	GCGGATCCACCATGAGAAACCATACAGAAATAAC	939
OR6C1rw	GCCTCGAGTAAGGCGGCCGCTGTGCTTGTGAAAAATACAG TC	

Cloning of full-length VN1R1

The human VN1R1 coding sequence was amplified from genomic DNA using the primer pair VN1R1fwd (GCAAGCTTACCATGGTTGGAGA CACATTAAAACTTC) and VN1R1plrw (AGCTCGAGACCTCATGGCATGA CAACCAGATTAGG), which amplified the entire open reading frame (NM_020633), which was cloned into pcDNA3 (Invitrogen). The generated plasmids were verified using sequencing, and two genetic variants were identified to yield the plasmids pVN1R1-CC and pVN1R1-AT.

Cell culture and transfection

Cell culture and transfection were performed as described previously (Wetzel 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% CO_2. Transfections were performed using standard calcium phosphate precipitation. Cells were transfected with 2.5 μg DNA per 3.5-cm dish and incubated for 12 h for DNA uptake.

Single-cell Ca^{2+} imaging

Ca^{2+} imaging experiments were performed as described previously (Wetzel et al., 1999). Culture medium was removed and replaced by the standard experimental solution (in mM: 140 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 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 Ca^{2+} 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 wavelengths. 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.L.L-Photonics; Vision 3.3). All fluorescence ratios (f_{340}/f_{380}) were background-corrected.

Exposure to odorants was accomplished using a specialized switch-operated superfusion device. ATP (10 μM; Thermo Scientific) was used as a positive control. Applied odorant mixture of 100 different compounds (Henkel 100, Henkel AG & Co. KGaA, Düsseldorf, Germany) was subdivided into smaller groups of 10 different compounds (Henkel 10). Further subdivision of the activating Henkel 10 mixture (H-10) into single substances led to identification of the activating substance.

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 expression systems revealing the same agonists, improve the reliability of results (Wallrabenstein et al., 2013; Busse et al., 2014). Whereas Ca^{2+} imaging measures an intracellular Ca^{2+} 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 at 37 °C. Cells (approximately 15,000 cells/well) were plated on poly-D-lysine-coated 96-well plates (NUNC) and transfected after 24 h with FuGENE® HD (Promega) transfection reagent 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 α_{olf} and 1 μg receptor transport protein (RTP1S) 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 Symrise 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 activation elevated cAMP and the subsequent expression of the cAMP-dependent reporter gene Cre-luciferase, as described previously 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, luminescence signals correlate with receptor activation. *Renilla* luciferase reporter driven by a constitutively active TK-promoter (pRL-TK-*Renilla*) 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 unspecific responses to the tested substances. We applied a one-tailed unpaired Student's t-test to calculate whether there was a significantly stronger activation in pVN1R1-CC cells compared to mock-transfected controls. 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.

Subjects

Seventeen right-handed subjects (9 women and 8 men, mean age 25.6 ± 3.6 years) participated in the functional imaging (fMRI) experiment. Handedness was assessed using the modified Edinburgh Inventory (Oldfield, 1971). All participants provided informed consent, and the local ethics committee approved all procedures (application # 106032011).

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 trigeminal sensation to ensure a similar intensity, as determined by a small panel of healthy subjects prior to the present experiments; for the experiments proper the same concentrations of both odors were used across all participants (HED 27% 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 between 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 biofeedback prior to testing.

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 paradigm 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), respectively. None of the subjects reported a stinging or burning sensation in response to odorous stimulation.

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 gradient echo T2*-sensitive echo planar imaging (GE-EPI) sequence was employed (TR 3000 ms, TE 35 ms, image matrix 64 × 64, in-plane resolution 3 mm, through-plane resolution 3.75 mm). The time of echo was close to the optimal 40 ms that was established using a 1.5 T by Stocker and colleagues (2006) for the imaging of limbic structures. Images were acquired in the axial plane oriented parallel to the planum sphenoidale to minimize bone artifacts. A total of 96 functional 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).

Imaging analysis

Data were analyzed using SPM8 (http://www.fil.ion.ucl.ac.uk/spm/) in Matlab (Matlab 6.5 R3, The MathsWorks Inc., Natick, MA). Images were realigned and corrected for motion based on the realignment parameters. No subject was excluded from further analysis due to head movement. Spatial normalization into the Montreal Neurological Institute 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 ensued. First level analysis was performed individually, and the results were taken to the second level in a group random effect analysis. Analyses were

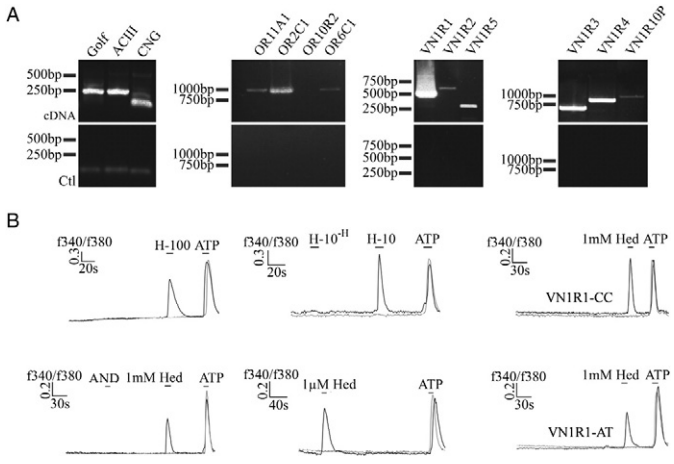

Fig. 1. A) RT–PCR experiments on human olfactory epithelium. Upper row: PCR amplification showing the presence of mRNA coding for known signaling components of the olfactory signal transduction cascade, *e.g.*, the olfactory G-Protein α_{olf}, adenylyl cyclase type 3 (ACIII), CNG2A channel subunit and various ORs. In addition, mRNA encoding for all five known VN1Rs and VN1R10P was identified using RT–PCR. Lower row: RT control (Ctl). B) Cytosolic Ca^{2+} levels of VN1R1-transfected HEK293 cells. All substances were applied for 10 s. The complex 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 ($H\text{-}10^{-H}$) 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.

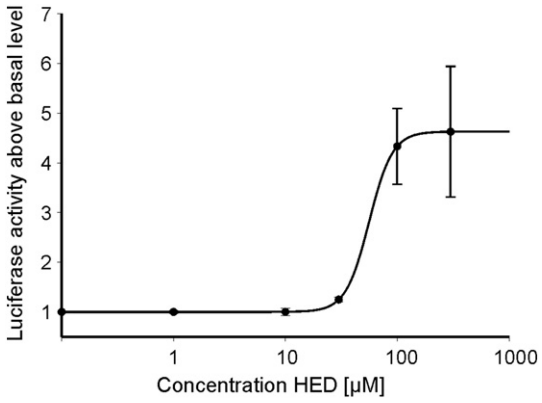

Fig. 3. Concentration–response relationship for VN1R1 and HED. Curve fitting was performed using the Hill equation in SigmaPlot V8.0 (Systat Software, San Jose, CA). The calculated EC_{50} is 56 µM ± 0.094 µM (n = 4). Detection threshold concentration was 30 µM ($p < 0.05$).

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 $p < 0.05_{corrected}$ with a cluster criterion of three voxels for whole brain analysis and Bonferroni-corrected for multiple comparisons 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 criterion of three voxels. Predefined areas of interest (ROI) were analyzed. 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)) (Berglund et al., 2006). Data are reported in MNI space. The contrast 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 marsbar 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.

Results

mRNA of all intact VN1Rs is present in human olfactory mucosa

We analyzed the expression of all potentially functional members of the human VN1r gene family and detected mRNA transcripts by PCR in the human olfactory mucosa. All VN1R transcripts clearly showed

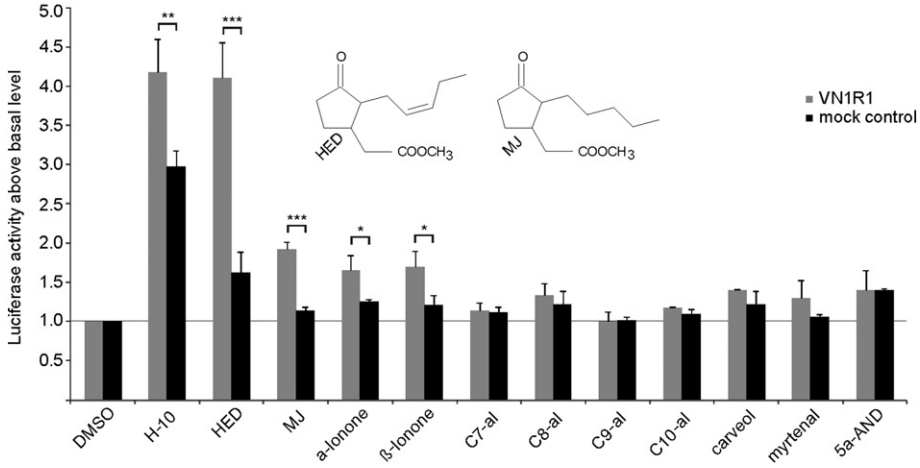

Fig. 2. Cre-Luciferase activity of human VN1R1. Responses of human VN1R1 to different tested substances. The screening concentration was 100 µM. Data are given as means ± SEM of 3–8 independent experiments, each performed in duplicate. The responses were normalized to the response to DMSO, and signals are given as activation above basal level. H-10, HED, methyl jasmonate (MJ), α-ionone and β-ionone significantly activated VN1R1 compared to mock-transfected controls. Error bars represent SEM. *$p < 0.05$; **$p = 0.01$; ***$p < 0.001$.

Table 2
Results of fMRI analyses of regions of interest ($p < 0.005$, $k = 3$).

		Cluster level	x, y, z
HED vs. baseline			
Olfactory eloquent areas	Amygdala left	10	−21 −3 −18
	Amygdala to hippocampus	6	24 −15 −6
	Hippocampus	9	36 −9 −18
	Insula left	4	−39 6 −12
		27	−30 24 −6
	Insula right	14	27 18 −12
		4	42 15 0
	Orbitofrontal	48	39 42 −6
		20	−30 45 −15
		12	−51 39 −9
		27	−45 30 −6
Other regions of interest	Midbrain	3	3 −18 −9
	Midbrain to hypothalamus	7	−6 −3 −3
HED (to baseline) vs. PEA (to baseline) inclusively masked by HED (to baseline) $p < 0.05$			
	Hippocampus	4	33 −9 −18
	Hypothalamus	5	0 0 −6

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 VN1R3 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 α_{olf}, adenylyl cyclase type 3 (ACIII), 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).

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 Ca^{2+} 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, lyral, (R)-(−)-carvon, S-(+)-carvon, citral, and benzyl acetate (1 mM of each), did not induce any intracellular Ca^{2+} 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 Ca^{2+} 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). Ca^{2+} 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 mM) on VN1R1 action, but found no Ca^{2+} 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

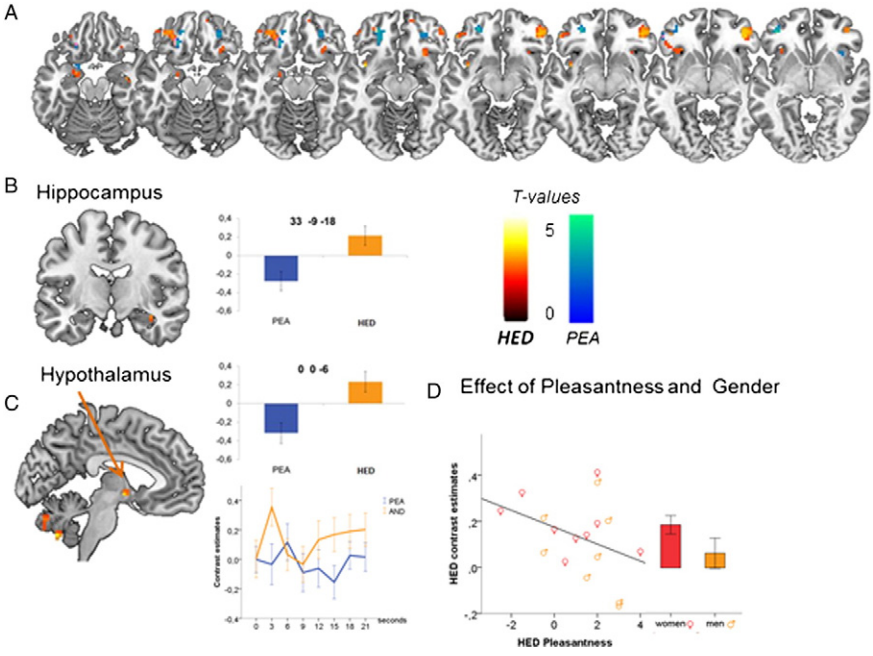

Fig. 4. Results from fMRI analyses. In the top row (A), activations in olfactory regions of interest are shown separately for PEA and HED. Enhanced activation in HED *vs.* PEA was found in the hippocampus (B) and hypothalamus (C). Contrast estimates for the HED *vs.* PEA comparison and the time course 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 $p \leq 0.005$, uncorrected and plotted on a T1-weighted image provided by the MRIcron program (Rorden et al., 2007).

significant differences ($p > 0.05$ unpaired Student's t-test) between the two VN1R1 isoforms: VN1R1-CC and VN1R1-AT (Fig. 1B).

After Ca^{2+} 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; Fig. 2). The tested substances were comprised of HED, H-10, to retest the odorant mixture that led to the identification of HED in our Ca^{2+} imaging experiments, and the most related chemical structure of HED, namely methyl jasmonate, the VN1R1 agonists previously reported by Shirokova et al. (α-ionone, ß-ionone, C7–C10 aldehydes, carveol and myrtenal) 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 Ca^{2+} imaging data (Fig. 2). 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 ß-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 myrtenal 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).

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 (Fig. 4).

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: $M = 1.19$, $SD = 1.77$; $F[1,15] = 1.27$, $p = 0.26$).

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 −18), the right amygdala approaching the right hippocampus (36 −9 −18; 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 Supplemental 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.

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.

Discussion

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 (Flegel et al., 2013). A ligand screening and subsequent binding study demonstrated heterologous VN1Rs activation by some volatile compounds, which in turn argues 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 VN1Rs use the canonical cAMP signaling *via* G-protein α_{olf} when recombinantly 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).

Activation of recombinantly expressed human VN1R1

To characterize the activation of VN1R1, we further examined VN1R1 in ligand screenings. The initial screening was performed using Ca^{2+} 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 Ca^{2+} 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/). Ca^{2+} 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 reporter gene assay system. We confirmed the HED action on VN1R1 and moreover, determined a concentration–response curve

with an EC_{50} 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 pentenylic 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, ß-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 Ca^{2+} FLIPR assay, collected Ca^{2+}-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.

fMRI characterization of HED action

Before starting fMRI scanning, we made sure that both odors HED and the control PEA were perceived at similar intensities and pleasantness. Therefore, the differences revealed with fMRI are likely to be due to the binding characteristics of the two odors. Both stimuli were also floral odors. The two striking results of our fMRI experiments were (1) that HED elicited a significant activation in the hypothalamus compared to PEA, and (2), the brain response to HED was sex-differentiated, with larger responses found in women compared to men. Both findings indicate a specific role of the VN1R1 agonist, which may relate to a sex-differentiated modulation of hormone release. However, these effects are currently unknown. Notably, the degree of hypothalamic activation was related to the pleasantness ratings of HED; more activation was found with increasing unpleasantness. Similar effects were claimed by Savic and Berglund, who found that androstenol, which is an odor frequently claimed to exhibit pheromone-like effects in humans, produced a sex-specific activation in the hypothalamus (Savic and Berglund, 2010). This odor elicited a larger hypothalamic response in women compared to men, which is similar to the present results. These authors also claimed that androstenol had specific hormonal effects, which had been suggested previously (Cowley and Brooksbank, 1991; Gower and Ruparelia, 1993; Shinohara et al., 2000). Most interestingly, Wyart et al. found that odorous stimulation with AND led to a specific increase in systemic cortisol release (Wyart et al., 2007). These results warrant future studies to investigate the presence of these hypothetical effects.

Notably, HED in the present study produced stronger activation compared to PEA in the amygdala and hippocampus, areas that are closely connected to the processing of valence and memories (Gottfried, 2006). These differences were noted despite the similar perceptions of the qualitative and quantitative values of HED and PEA.

Pheromonal substances in mice, eliciting extra-olfactory brain activations, were primarily not only detected in vomeronasal sensory neurons (VSNs) of the VNO, but also in olfactory sensory neurons (OSNs) of the OE (Spehr et al., 2006; Lopez et al., 2014). The receptor repertoire expressed in murine and human OSNs comprises canonical ORs as well as the putative pheromone receptor families TAARs and VNRs (Buck and Axel, 1991; Liberles and Buck, 2006; Carnicelli et al., 2010; Ibarra-Soria et al., 2014; Kanageswaran et al., 2015). TAARs constitute an olfactory subsystem mediating odor-driven innate responses. Because they are shown to be exclusively activated by volatile amines within the OE (Liberles and Buck, 2006; Ferrero et al., 2012), in our experiments HED in principle is able to activate ORs and/or VN1R1. ORs contribute to general odor perception and are widely believed to activate olfactory neuronal networks. So far, there are no studies examining the axonal projections of VNR-expressing OSN to cortical areas. But hypothalamic Gonadotropin-releasing hormone (GnRH) neurons in mice, receive sensory information from VSNs of the VNO and from a discrete population of OSNs of the OE respectively (Boehm et al., 2005; Yoon et al., 2005). Thus, one might speculate that the hypothalamic input from OE derived from VNR-expressing OSNs and that this is also true for humans, who most probably lack a functional VNO (Witt and Hummel, 2006; Frasnelli et al., 2011; Hummel et al., 2011).

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 jasmine, methyl dihydrojasmonate was discovered in 1958 by a chemist from the fragrance company Firmenich and later named as HED, from greek “hedone” for “pleasure” promoted in many high-class fragrances. The chemical structure of HED might be similar to a currently unknown naturally occurring ligand, which has the potential of gender-specific hormonal regulation in humans.

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.

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None of the authors declares a conflict of interest.

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