NeuroImage xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

NeuroImage

journal homepage: www.elsevier.com/locate/ynimg

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

I. Wallrabenstein [a,*,1], J. Gerber [b,1], S. Rasche [a,1], I. Croy [c], S. Kurtenbach [a], T. Hummel [c], H. Hatt [a]

[a] Department of Cell Physiology, Ruhr University Bochum, Universitätsstr. 150, 44801 Bochum, Germany
[b] Department of Neuroradiology, Medizinische Fakultät Carl Gustav Carus, Technische Universität Dresden, Fetscherstr. 74, 01307 Dresden, Germany
[c] Department of Otorhinolaryngology, Smell and Taste Clinic, Technische Universität Dresden, Fetscherstr. 74, 01307 Dresden, Germany

ARTICLE INFO

Article history:
Received 13 December 2014
Accepted 11 March 2015
Available online xxxx

Keywords:
Social odors
Hedione
Pheromones
fMRI
Olfaction
Human VN1R1

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.

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 (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.,

* Corresponding author at: Ruhr-University Bochum, Department of Cell Physiology (ND4/169), Universitätsstr. 150, 44801 Bochum, Germany.
E-mail addresses: ivonne.wallrabenstein@rub.de (I. Wallrabenstein), johannes.gerber@uniklinikum-dresden.de (J. Gerber), sebastian.rasche@rub.de (S. Rasche), ilona.croy@tu-dresden.de (I. Croy), stefan.kurtenbach@me.com (S. Kurtenbach), thummel@mail.zih.tu-dresden.de (T. Hummel), hanns.hatt@rub.de (H. Hatt).
[1] Authors contributed equally.

http://dx.doi.org/10.1016/j.neuroimage.2015.03.029
1053-8119/

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.

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
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	

2.2. 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.

2.3. 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.

2.4. 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

Please cite this article as: Wallrabenstein, I., et al., The smelling of Hedione results in sex-differentiated human brain activity, NeuroImage (2015), http://dx.doi.org/10.1016/j.neuroimage.2015.03.029