Large scale analysis of olfactory receptors

with highly genetically variations in relation with specific anosmia

Dissertation to obtain the degree
Doctor Philosophiae (Doctor of Philosophy, PhD)
At the Faculty of Biology and Biotechnology
Ruhr-University Bochum

International Graduate School of Biosciences
Ruhr-University Bochum
(Department of Cell Physiology)

Submitted by
Kaveh Ashti Baghaei

From

(Tehran, Iran)

Bochum, Germany

(June, 2013)

First supervisor: Prof. Dr. Dr. Dr. Hanns Hatt

Second supervisor: Prof. Dr. Stefan Wiese

Umfassende Analyse Olfaktorischer Rezeptoren

in Bezug auf genetische Varianten im Zusammenhang mit spezifischer Anosmie

Dissertation zur Erlangung des Grades
eines Doktors der Naturwissenschaften
der Fakultät für Biologie und Biotechnologie
an der Internationalen Graduiertenschule Biowissenschaften
der Ruhr-Universität Bochum

angefertigt im
Lehrstuhl für Zellphysiologie

vorgelegt von
Kaveh Ashti Baghaei

Aus
Tehran, Iran

Bochum
Juni, 2013

Referent: Prof. Dr. Dr. Dr. Hanns Hatt

Korreferent: Prof. Dr. Stefan Wiese

To my parents, brother and wife
whose love and support sustained
me throughout.

Erklärung

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1. Introduction:

1.1 Olfactory system

The human olfactory system has the remarkable capacity to recognise and discriminate a large number of different chemical components and odour molecules. As a chemical sensor, the olfactory system is effective on social and sexual behaviour and quality of life since odourants can evoke memories and emotions or influence our mood.

Odourant perception starts with the chemical interaction between an odourant molecule and an olfactory receptor expressed in an olfactory sensory neuron (OSN). These neurons are located in the olfactory epithelium (OE), which is covered with a mucous layer which supplies lipid/protein-rich secretions for the OE surface. Most parts of the human nasal cavity consist of ciliated columnar respiratory epithelial tissue. The upper section of the nasal cavity, lining the cribriform plate, consists of OSNs (Morrison & Costanzo 1990). OSNs are bipolar cells with a single unbranched dendrite with axons penetrating through the cribriform plate to the olfactory bulb of the brain (Fig. 1.1). Axons of mitral cell then leave the olfactory bulb (OB) to higher brain structures including the piriform cortex, hippocampus and amygdale. About 10–25 cilia, each ~5 µm long, extend from the knob of each OSN (Menco 1980; Morrison & Costanzo 1992). Each OSN expresses just one type of olfactory receptor (OR) protein which is the first level for the primary events of chemo-sensory transduction.

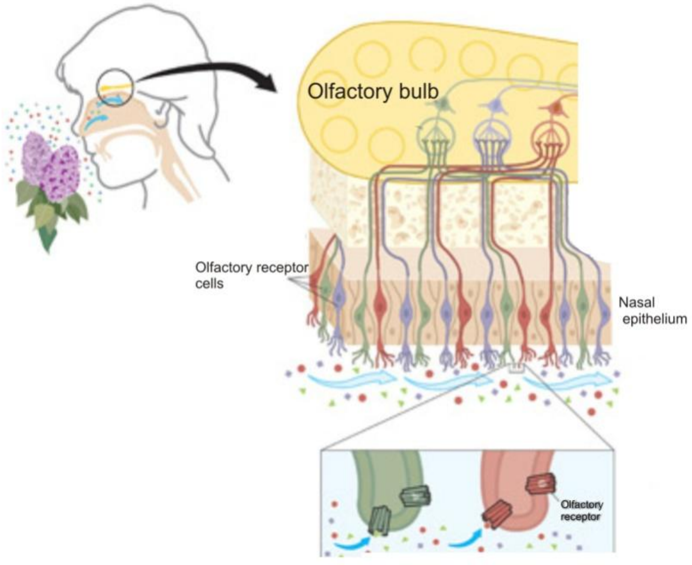


Figure 1.1: Different part of olfactory system. It is including: olfactory bulb, olfactory mucosa, olfactory nerve cells, and olfactory receptors in humans. (Andrea Rinaldi. 2007).

1.2 Olfactory receptors (ORs)

The olfactory system can differentiate among thousands of chemical components by using olfactory receptors as one of the key elements of the molecular decoding device of the nose. ORs, as well as visual opsins, bitter and sweet taste receptors (TAS2R1 and TAS1R1) and vomeronasal receptors (V1Rs, V2Rs and V3Rs), are encoded by a large gene super family, belonging to G-protein-coupled receptors (GPCRs) (Mombaerts 2004). They are identified by several characteristic sequence motifs and constitute the largest gene family in the mammalian genome (Young et al. 2002; Zhang & Firestein 2002; Godfrey et al. 2004). GPCRs have a common structural feature with seven α-helical transmembrane (TM) regions. They can be classified into six groups by sequence similarities and OR genes belong to a rhodopsin-like the GPCR superfamily, which is the largest of them (Fredriksson et al. 2003).

This gene super family is organised into 18 gene families and 300 subfamilies, these are localised in multiple clusters of varying sizes, distributed on all autosomes except chromosomes 20 and Y (Safran et al. 2003; Olender et al. 2004). The two largest OR gene clusters are located on chromosome 11 with 38 functional genes on 11q and 44 functional genes on 11p. Based on the percentage of similar amino acid sequences, OR genes can be grouped into a particular family and furthermore into a particular subfamily. ORs with >40% protein sequence identity are considered to be within the same family, and if they share >60%, they belong to the same subfamily (Glusman et al. 2000). In accordance with this concept, OR genes are classified by the HUGO Gene Nomenclature Committee.

ORs are ~310 amino acids long on average. The sequencing of OR genes revealed remarkable diversity in transmembrane (TM) helices 3 to 6 between paralogs (Fig. 1.2), likely accounting for the high diversity in ligand specificity (Buck & Axel 1991). There are several motifs that are characteristics of ORs. One such motif is 'MAYDRYVAIC', located at the junction of TM3 and the intracellular loop between TM3 and TM4. Within this motif, the stretch of three amino acids, 'DRY' (aspartic acid–arginine–tyrosine), is highly conserved among rhodopsin-like GPCRs (Niimura 2012). The DRY motif is possibly important for G-protein coupling (Rovati et al. 2007). It is supposed that in the OR repertoire, contact positions show pronounced variability between paralogs (Wu & Kabat 2008). Later studies have tried to consider odourant binding residues in olfactory receptors based on sequence analysis. Some approaches predicted 22 putative contact residues, located on TMs 3 to 7 in their models (Singer et al. 1995; Singer et al. 1996; Anselmi et al. 2011). In a recent study, a molecular model of the ligand-binding site is provided by combining dynamic homology modelling with site-directed mutagenesis and

functional analysis, allowing the prediction of receptor function based on computational information (Gelis et al. 2011).

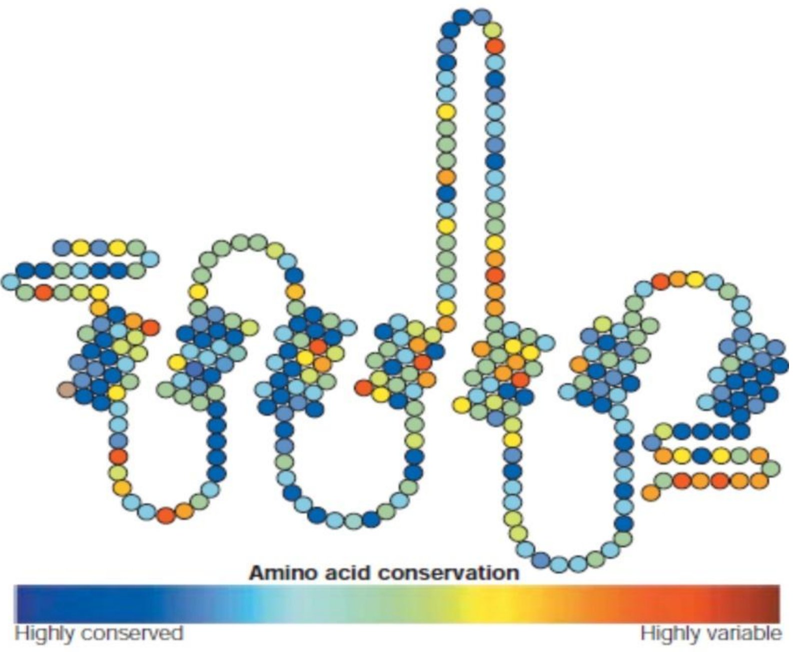


Figure 1.2: The degree of amino acid conservation in olfactory receptor. It is shown as a color in the rainbow spectrum. The blue color is an indicator for highly conserved and red color shows highly variable amino acids. Modified picture after *Peter Mombaerts* (2004).

1.2.1 Segregating pseudogenes (SPGs)

Human ORs present a high number of pseudogenes, whereby more than 50% of the loci are nonfunctional due to frame-disrupting mutations (Glusman et al. 2001; Niimura & Nei 2003; Olender et al. 2008; Niimura & Nei 2006).

As proposed by Menashe et al., a fraction of the human olfactory receptors could be segregated between an intact and pseudogene form of the olfactory receptor (Menashe et al. 2003). In other words, some olfactory receptor genes display both functional and non-functional allels, which are called segregating pseudogenes. These are explained as genes that segregate in populations between intact genes and pseudogenes due to a disruptive single nucleotide polymorphism

(SNP). This divider mutation can introduce a stop codon, or alter a highly conserved amino acid that is important for the proper function of the protein.

The first studies in 2003 revealed 12 SPGs in humans (Gilad & Lancet 2003). These results show frequencies of intact alleles in Pygmies (an African population) more than in Caucasians. In the same year Menashe et al. extrapolated the number of segregating olfactory receptor pseudogenes in the entire human genome to at least 60 (Menashe et al. 2003). They found that African Americans have more functional ORs than non-African participants. In 2012 the number of SPGs jumped to 244 when Lancet's group reported an unusually high genetic diversity in the OR gene repertoire among individuals (Fig. 1.3) and suggested that individual humans have highly personalised "barcodes" of functional olfactory receptors (Olender et al. 2012). In Olender's study every human individual is personalised by a different combination of such SPGs (Olender et al. 2012). One of the most accepted hypotheses is that allelic variants of OR genes may produce different functional characteristics that can lead to the generation of different odourant sensitivity phenotypes in the human population (Young & Trask 2002; Hasin-Brumshtein et al. 2009). An association study between SPGs and odourant sensitivity showed a high association between forms of the OR11H7P gene and sensitivity to isovaleric acid (Menashe et al. 2007). Individuals who are heterozygous or homozygous for the intact allele of OR11H7P were more likely to be hyperosmic to isovaleric acid than individuals who are homozygous for the disrupted allele. It could be mentioned that potential ligands for deorphanized ORs can generally be used in association studies to determine detailed relationships between individual olfactory receptor disruption and odourant perception variability.

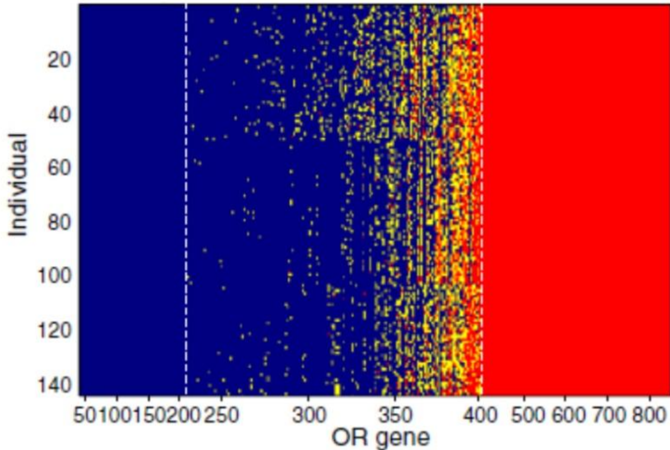


Figure 1.3: Personalized OR repertoires in 145 individuals. The inter-individual variability is quite vast; it doesn't seem even two individuals having same pattern in their olfactory repertoires. Colors explain in following order: Blue- homozygotes for an intact allele, red- homozygotes for a disrupted allele, yellow- heterozygotes. (Olender et al. 2012).

1.2.2 Copy number variations (CNVs)

CNVs caused by genomic rearrangements can generate phenotypes by different molecular mechanisms such as: gene dosage, gene interruption, gene fusion and position effects or unmasking of recessive alleles (Lupski & Stankiewicz 2005). Some of the associations between CNVs and phenotype variation are involved in human diseases. The best–known example is Down syndrome caused by trisomy of human chromosome 21; another would be thalasemia due to alpha globin gene rearrangements (Higgs et al. 1979).

CNVs have been defined as "a segment of DNA that is 1 kb or larger and is present at a variable copy number in comparison with a reference genome" (Feuk et al. 2006). This definition was modified, based on a functional definition, and it was suggested that it should choose an average exon size (~100 bp) as a parameter for defining CNV (Zhang et al. 2009). Copy number variations are a source of genetic diversity in humans. When the breakpoint of a deletion, insertion or duplication is located within a functional gene, it may interrupt the gene and cause a

loss of function by inactivating a gene as described by red-green opsin genes and colour blindness (Nathans et al. 1986).

In genome-wide studies, CNVs for a larger number of OR genes or gene clusters were detected (Redon et al. 2006; Wong et al. 2007). In a previous study, individual CNVs of OR genes were systematically investigated (Young et al. 2008). In a panel of approximately 50 human individuals, copy-number variations of 18 ORs were confirmed. No individual had the full number of functional OR genes expected from the reference genome data and virtually every individual had a unique combination of functional losses and gains among the ORs, thus demonstrating that CNVs generate individual patterns of OR genes. In a study by Waszak, it is reported that deleterious variants including CNVs and SNPs affect 15% and 20% of the human OR gene repertoire, respectively (Waszak et al. 2010). They revealed that OR loci display an extensive range of locus copy-numbers across individuals, with zero to nine copies in OR loci. A recent study by Olender in 2012 showed an increased number of CNVs to 66% of intact OR loci. They reported that of the 851 human genomic OR loci, 438 have a frame-disrupting pseudogene apparently fixed in the entire population. Of the 413 remaining loci, 271 (66%) have at least one allele lacking an intact open reading frame, including frame disruptions and deletion CNV alleles (Olender et al. 2012).

The presence of these copy number variations can induce genomic structural variations in specific gene families as well as at a genome-wide scale by gene duplication or exon shuffling (Zhang et al. 2009). So, CNVs could be one of the reasons for the significant variations in olfactory capabilities among human individuals. However, the relation of CNV to odour perception remains to be evaluated.

1.3 Olfactory signal transduction pathways

The canonical pathway of signal transduction in OSNs of mammals is composed of the OR as one variable component and four constant elements: the $G_{\alpha olf}$-containing heterotrimeric G-protein; adenylyl cyclase, which produces the second messenger cAMP; a cyclic nucleotide-gated cation channel and a Ca^{2+}-activated chloride channel (Fig. 1.4) (Mombaerts 2004).

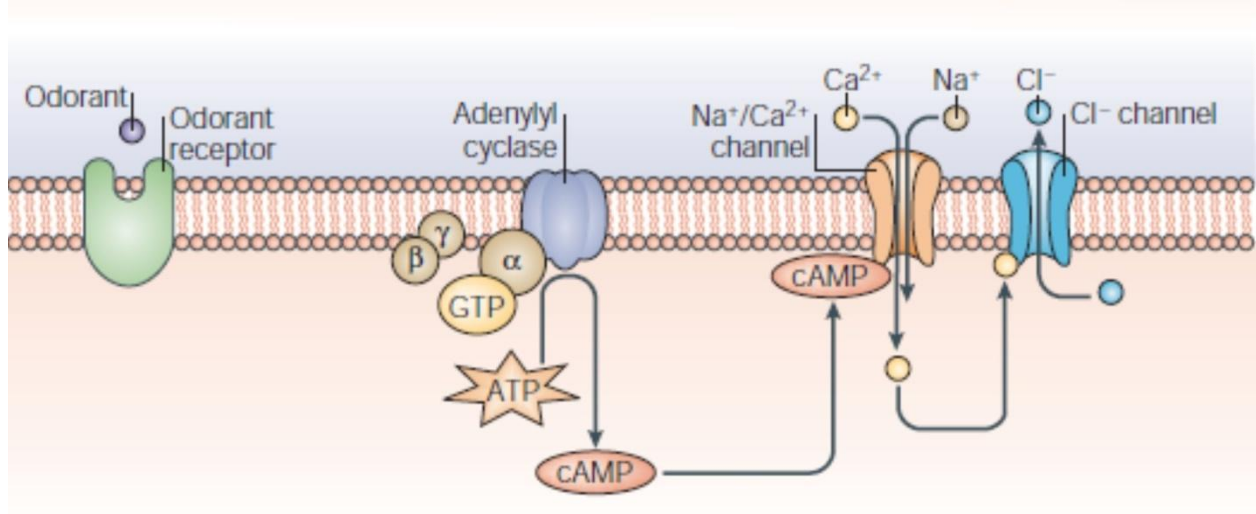


Figure 1.4: Pathway of transduction in OSNs (Mombaerts 2004)**. After** odourant binding to olfactory receptor, a G-protein cascade would be activated leading to increased production of cAMP, a second messenger that opens cyclic nucleotide-gated ion channel (CNG). Final result being the triggering of an action potential.

Olfactory sensory neurons express a G-protein with a specific subunit named $G_{\alpha olf}$ (Belluscio et al. 1998). Upon binding of an agonist to the OR, $G_{\alpha olf}$ activates an olfaction-specific adenylate cyclase that uses ATP to produce cAMP. Following this cascade, cyclic nucleotide-gated channels (CNG) will be opened by cAMP. Inactive OSNs normally maintain a resting potential across their plasma membrane of about −65 mV (inside with respect to outside). The opening of the channel depolarises the neuron membrane as a consequence of the influx of sodium (Na^{+}) and calcium (Ca^{2+}) ions. The Ca^{2+} -influx also causes the opening of a Ca^{2+}-dependent-Cl^{-}-channel, which enhances the depolarisation of the cell membrane and generates action potentials

along the sensory axons, leading to signal transmissions to the olfactory bulbs (Benbernou et al. 2011).

The adenylyl cyclase (AC)/cAMP pathway is essential for olfactory responses in vertebrates (Sklar et al. 1986; Brunet et al. 1996; Wong et al. 2000). However, odourants activate more than one transduction cascade; IP_3 has also been shown to be an efficient second messenger (Restrepo et al. 1990; Stengl et al. 1992; Bruch 1996; Spehr et al. 2003). Fundamental to an emerging concept of multiple olfactory signalling pathways was the observation that some odours did not elicit a rise in cAMP in biochemical assays (Sklar et al. 1986). Instead, such 'non-cAMP odours' appeared to induce phospho-inositide signalling in olfactory cells (Boekhoff et al. 1990; Ronnett et al. 1993). Also, a possible involvement of inositol 1, 4, 5-trisphosphate ($InsP_3$) in olfaction was reported for a variety of species (Restrepo et al. 1990; Fadool & Ache 1992; Schild et al. 1995; Kashiwayanagi et al. 1996; Kaur et al. 2001; Spehr et al. 2002; Klasen et al. 2010). In 2005 it was reported that phosphatidyl-inositide-related signaling proteins, including phospholipase C beta-2 (PLC b2), InsP3-receptor type III (InsP3R-III) and classical transient receptor potential channel 6 (TRPC6), are co-localised in a distinct population of microvillous cells in the olfactory epithelium of mammals (Elsaesser et al. 2005). These results showed the presence of a novel class of secondary chemosensory cells in the olfactory epithelium of mammals that utilise phosphatidyl-inositides as a second messenger in signal transduction.

However, it was reported that *in vivo,* different chemical components could activate the cAMP or IP_3 pathways or both of them (Restrepo et al. 1990; Bruch 1996; Nakamura 2000; Wong et al. 2000; Spehr et al. 2002). The correlation with the *in vitro* tests performed in heterologous cell system is not totally clear (Katada et al. 2004; Shirokova et al. 2005; Ko & Park 2006; Kato et al. 2008).

1.4 Functional expression of olfactory receptors in recombinant systems

The understanding of OR function has improved slowly due to a lack of suitable heterologous systems for expression and assays on odourant responses (Malnic 2007; Touhara 2007). Expression of GPCRs is a complex process that includes protein folding, posttranslational modifications, and transport through cellular compartments including the endoplasmic reticulum and Golgi apparatus. One main reason for the inadequate functional expression of ORs is that the receptors do not reach the cell membrane of heterologous cells. They remain in the endoplasmic reticulum as a result of inefficient folding and poor coupling to the export machinery, combined with aggregation and degradation through both proteosomal and autophagic pathways (Gimelbrant et al. 1999; Matsunami 2005; Touhara 2007).

Studies in the last decade have improved the expression of ORs in heterologous systems. Several attempts have been made to achieve functional expression of ORs on the cell surface in heterologous systems. In some cases, fusion of the 20 N-terminal amino acids of the rhodopsin or serotonin receptor to the N-terminal region of ORs resulted in a expression of functional ORs in the plasma membrane and induced a successful odourant-response in a heterologous system such as HEK293 cells (Krautwurst et al. 1998; Wetzel et al. 1999; Kajiya et al. 2001). It has been shown that glycosylation of the N-terminus of ORs is required for proper translocation to the plasma membrane (Katada et al. 2004). Tagged ORs could be cotransfected in heterologous cells with the G_{α} subunits. The dissociated G_{α} and $G_{\beta\gamma}$ subunits activate a widespread variety of effectors, including adenylyl cyclases, phospholipases and plasma membrane channels (Hepler & Gilman 1992; Chen et al. 1995). The primary effectors of G_s and G_q coupled signalling pathways are adenylyl cyclase and phospholipase C, respectively (Chen et al. 1995). The G_s subunit usually involves the activation of adenylyl cyclase and measurement of cAMP concentration by

radioimmunoassay, while activation of the G_q subunit is commonly assayed by measurement of the production of inositol triphosphate or diacylglycerol from phosphatidiylinositol 4,5-bisphosphate or by changes in intracellular calcium (Shirokova et al. 2005; Gaillard et al. 2002; Katada et al. 2004; Levasseur et al. 2003; Matarazzo et al. 2005).

Other important cofactors that help to transport ORs to the cell membrane are receptor transporting proteins (RTPs) 1 and 2 and receptor expression enhancing protein (REEP) 1 (Saito et al. 2004). One transmembrane protein, RTP1, which has been referred to as an OR chaperone, appears to enhance cell surface expression of ORs, and many ORs have been deorphanized by co-expressing them with RTP1 (Saito et al. 2004; Saito et al. 2009).

Additionally, some G-protein activating enhancers have been co-expressed in association with ORs, in order to improve the olfactory signal transduction. Guanine nucleotide exchange factors (GEF) could increase activation of G-proteins (Tall et al. 2003). Ric-8B, as a known GEF expressed in olfactory sensory neurons, is able to interact with $G_\alpha$olf (Dannecker et al. 2005). GEFs catalyse the exchange of GDP for GTP to generate an activated form of G_α, which is then able to activate a variety of effectors (Tall et al. 2003). Ric-8A and Ric-8B are GEFs in the mammalian olfactory system. While Ric8A can interact with the Gαq, Gαi and Gαo classes of Gα subunits, Ric-8B interacts with the Gαs class (Nishimura et al. 2006). It has been shown that Ric-8 (A and B) promotes efficient functional expression of ORs in heterologous systems (Dannecker et al. 2006). It has also been shown that a myristoylation sequence–conjugated mutant of Ric-8A (Myr-Ric-8A) could be used as a signal amplifier and Myr-Ric-8A greatly enhances Ga15-mediated Ca^{2+}responses of ORs in HEK293 cells. Myr-Ric-8A as a cofactor that enhances OR-mediated Ca^{2+} signaling in HEK293 cells should be helpful in functional expression of ORs in heterologous cells by using Ca-imaging (Yoshikawa, Touhara 2009).

Finally, the expression of ORs could be supported by the presence of the Hsc70 proteins. Hsc70t, which belongs to the Hsp70 family of genes, is expressed exclusively after meiosis in mouse spermatogenesis (Matsumoto & Fujimoto 1990; Matsumoto et al. 1993). However, it might be involved in the folding or trafficking of olfactory receptors (Neuhaus et al. 2006). HEK293 cells cotransfected with Hsc70t and odourant receptors (ORs) from mice and humans show a significantly enhanced OR expression. Hsc70t expression also changes the quantity of cells functionally expressing olfactory receptors at the cell surface so the number of cells responding to odourants in Ca-imaging experiments will be increased significantly (Neuhaus et al. 2006).

Thus, introduction of the factors required for OR expression indicate that cell surface expression of ORs as a critical level for OR assays can be significantly improved by co-expression with auxiliary proteins that associate in OR trafficking to the cell surface, and it is expected that these proteins help to solve the problems of cell surface expression of ORs.

1.5 Odourant perception

An *odourant* is a substance capable of eliciting an olfactory response whereas *odour* is the sensation resulting from stimulation of the olfactory organs. Odours play an important part in our everyday life, from appetite stimulation to serving as warning signals (Hatt 2004). The general criterions for an odourant are: it should be volatile, hydrophobic and have a molecular weight less than approximately 300 Daltons. In this explanation of odour perception, odour threshold should be noted. This term used to determine the lowest concentration of particular odourant to which animals (humans) responded 50 per cent of the time to repeated presentations of an odourant. Thresholds for odourants vary greatly between individual persons and it is base of classification of odourants in terms of the intensity from weak to strong according to efficacy and affinity.

The perception of odourants always involves the perception of a complex mixture of different odour molecules. For example, the smell of a rose is composed of 260 chemical components. In some sensory modalities, the magnitude of mixed stimuli is perceived as the sum of the magnitudes of the individual stimulus; this property is known as additivity (Keller & Vosshall 2004). The estimated intensity of the smell of the mixture of two odourants is frequently perceived as being non-additive. This phenomenon is called "counteracting". There are three types of counteracting: "partial addition, in which the mixture smells more intense than the stronger component; compromise, in which the smell intensity of the mixture is in between the intensities of the components; and compensation, in which the mixture smells less intense than the weaker component" (Keller & Vosshall 2004).

Many theories have been proposed in the past to describe the mechanism of smelling odourants (Rossiter 1996) but advances in biological understanding, not least of which being the discovery of odourant receptors, have gradually ruled them out. One of these is the physical theory that proposes the shape of the odourant molecule determines which olfactory receptor cells will be reacted (Keller & Vosshall 2004). This theory is based on fragments of molecular shape and molecular vibrations (Mori & Shepherd 1994).

1.5.1 Chemical structure basis of odourant

To understand the association between odourants and receptors, it is necessary to know which properties of odourant or odour descriptors are critical for interaction with olfactory receptors. It has been proposed that the majority of olfactory receptors respond to multiple odours. Although different in many respects, they should share certain molecular properties such as functional groups (Duchamp-Viret & Duchamp 1997; Firestein et al. 1993). These properties mostly gravitated toward the chemical structure of odourants, carbon atom number (CAN), functional

group type and position, which are parts of odour descriptors that have been determined in recent olfaction research (Zhao et al. 1998; Meister & Bonhoeffer 2001; Malnic et al. 1999; Spehr et al. 2003; Guerrieri et al. 2005). It has been shown by different studies that olfactory sensory neurons respond to molecules with similar CAN, pending these molecules share the same functional group. ORs that respond to odourants with 5 carbons have similar responsiveness to odourants with 4 or 6 carbons but are less likely to respond to odourants with 7 or more carbons (Sato et al. 1994; Rubin & Katz 1999; Uchida et al. 2000; Wachowiak & Cohen 2001; Sachse et al. 1999; Johnson et al. 2002; Spehr et al. 2003; Takahashi et al. 2004). This means odourants that have similar CAN and functional groups elicit similar response patterns. With regard to functional groups, the testing of one rat olfactory receptor (I7) with 90 odourants of different chemical structures suggested the carbonyl aldehyde group is critical for the activity of odourant at the I7 receptor (Araneda et al. 2000). Also, a similar experiment was done with *Drosophila melanogaster* olfactory sensory neurons and 110 odourants of diverse functional groups (Hallem & Carlson 2006).

It should be noted that not all descriptions of odourants are in accordance with chemical structure and, in some examples, conception of odours is not explicable by the structure. Two well-known instances are musks and ambers.

Musk is one of the most famous odourants. Because of its universal inclusion in fragrance and due to expense and legislation it has been synthetic for a long time. The molecules that indicate musk odour properties are diverse in structure. The other group of odourants is amber which was originally produced by whales. Ambers make an interesting combination of closely related smells with widely different structures: timberol, karanal and cedramber are close enough that a perfumer will occasionally confuse them with each other (Fig. 1.5).

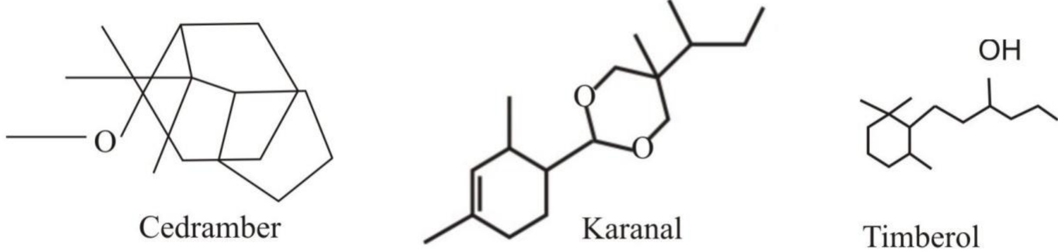


Figure 1.5: Three examples of odourant molecules in amber group, with widely different structure but similar odourant perception.

In other part there are enantiomers which present completely different smell, one of the examples is R and S carvone since the smell of R carvone is minty and S carvone is caraway (Fig. 1.6) (van Boelens 1993; Rossiter 1996).

Figure 1.6: Some enantiomers smell completely different. (R)-carvone on the left hand with minty smell and (S)-carvone with caraway on the right hand.

Also Ohloff (Ohloff 1995) presented isomers of different chemical substances have effect on odourant intensity (Fig. 1.7).

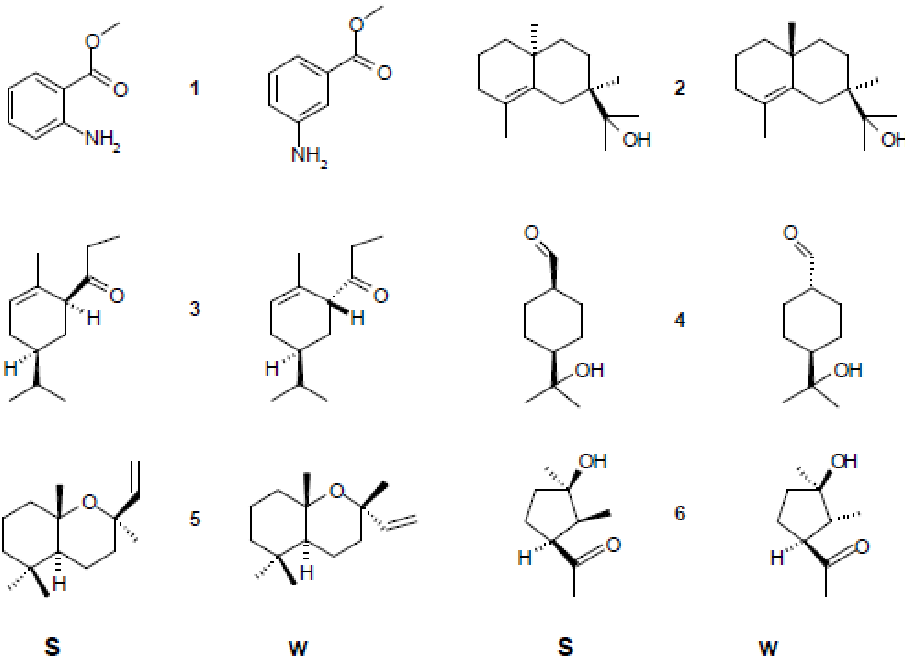


Figure 1.7: A sample of strong-weak isomer pairs. Presented by Ohloff (1994). 1- methylanthranilate, 2-eudesmol, 3-neron, 4-p-menthane derivatives, 5-caparrapi oxide, 6- iridanes. In every case, the strong isomer is in the left and weak isomer is in the right.

In agreement with these responses of the olfactory system, Wetzel et al (Wetzel et al. 1999) reported that a receptor only responded to helional at very low concentrations but not to piperonal with possess a closely related chemical structure.

1.5.2 Olfactory Dysfunction

Odourant perception is the most variable sense among different individuals (Zhang, Firestein 2007). This variation is reflected in olfactory dysfunctions and normal physiological variation such as specific anosmia.

Distribution of olfactory dysfunction in human population is a matter of debate. However, most studies reported frequencies of 1% to 3% of chemosensory disorders (Wysocki, Gilbert 1989; Murphy et al. 2002). Different reasons are reported as factors in occurrence of smell dysfunction

(Doty et al. 1984; Frye et al. 1990; Deems et al. 1991). One of the main reasons of olfactory dysfunction is a process that directly affects and impairs either the olfactory epithelium or olfactory pathways (Fig. 1.8). Olfactory disorders can be categorized in different groups (Jones, Rog 1998; Simmen, Briner 2006) : 1) anosmia: missing olfactory sensations and complete loss of smell function, 2) partial anosmia: ability to perceive some, but not all of odourants, 3) hyposmia or microsmia: a general decreased sensitivity to odourants, 4) hyperosmia: unusual acute smell function (increasing sense of smell), as an increased sensitivity to all odors, 5) olfactory agnosia: inability to recognize an odor sensation, 6) dysosmia (cacosmia or parosmia): When a normal pleasant odor is perceived as a unpleasant odourant, 7) phantosmia: dysosmic sensation perceived in the absence of an odor stimulus and 8) specific anosmia: The inability to smell one of a few odourants in the presence of an otherwise normal sense of smell.

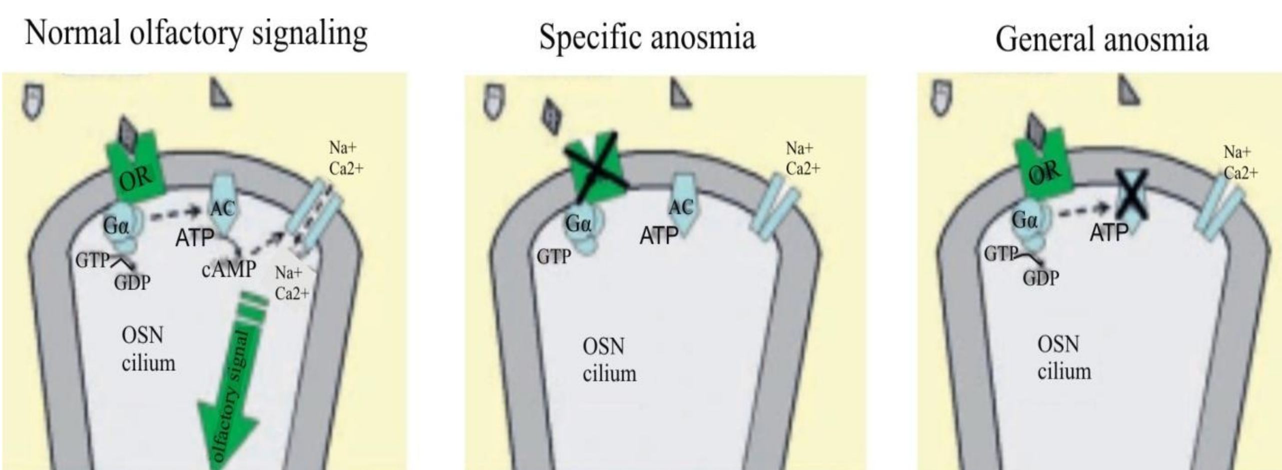


Figure 1.8: A schematic drawing of the olfactory signaling pathway and olfactory dysfunctions. The picture shows specific anosmia and general anosmia in compare with normal olfactory signaling. Modified from Menashe (Menashe et al. 2006).

1.5.3 Specific anosmia

A major theoretical of specific anosmia was made by Marcel Guillot (Guillot 1948) in his paper entitled 'Anosmies partielles et odeurs fondamentales'. Amoore used new term instead of *partial* as '*specific* anosmia', and explained it as “the condition which a person of otherwise normal olfactory acuity cannot perceive a particular compound, at a concentration such that its odor is obvious to most other people” (Amoore 1977). A complimentary description of specific anosmia describes it as a condition in which, most often a person has a 10–100 fold diminished sensitivity to a given odourant, out of this range it could be refer to exact term as specific hyposmia (Menashe, Lancet 2006). The first anosmic defects were pointed out for isovaleric acid, 1-pyrroline, trimethylamine, isobutyraldehyde, 5α-androst-16-en-3one and pentadecalactone by Amoore (Amoore 1977).

A known example for anosmia is androstenone (5α-androst-16-en-3-one), with 30% rates of specific anosmia (Hasin-Brumshtein et al. 2009), some individuals with normal sense of smell are unable to detect the odor of androstenone (5α-androst-16-en-3-one) at the concentrations tested, and those who are able to perceive it describe the odor in different ways as: sweaty, urinous, musky, sweet, or even perfume-like(Griffiths, Patterson 1970; Knaapila et al. 2012; Hummel et al. 2005; Wysocki et al. 1989).

Diversity of quality descriptors for a one given odourant is named specific allosmia (O'Connell et al. 1994), and the term specific anosmia describes the inability of some people to smell an odourant (Amoore 1967). Therefore, the perception of androstenone is an example of both a specific allosmia and anosmia.

Geneticists have been interested in the ability to smell androstenone because the expectation is that individual differences can be explained by a deleterious allele in a particular narrowly tuned

olfactory receptor (Amoore 1967) and it is shown by various studies that anosmia to androstenone is highly concordant in monozygotic twins and the ability to detect androstenone is a heritable trait (Wysocki, Beauchamp 1984; Gross-Isseroff et al. 1992; Keller et al. 2007; Wysocki et al. 1989). This hypothesis has proved to be partially true, A novel approach showed that a combination of two non-synonymous SNPs (R88W and T133M) in the human OR gene OR7D4 accounts for 19–39% of the variation in sensitivity and quality perception of androstenone. The study found that subjects with at least one copy of the WM haplotype are less sensitive to androstenone than those that do not carry a WM allele. These results provided for the first time the link between genetic variation in OR and odor perception (Keller et al. 2007).

Associations between olfactory receptor alleles and perception are observed not only for androstenone but also for isovaleric acid (Menashe et al. 2007), asparagus metabolites (Eriksson et al. 2010; Pelchat et al. 2011), and cis-3-hexen-1-ol (Knaapila et al. 2012). Isovaleric acid is one of the first evidence for specific anosmia in humans (Russell et al. 1993). A genetic study (Hasin-Brumshtein et al. 2009) showed an association between isovaleric acid sensitivity and the genotype of a segregating OR pseudogene OR11H7P on human chromosome 14. Also in early studies heritability has been shown for the sensitivity to pentadecalactone as a musky odourant (Whissell-Buechy, Amoore 1973).

Regarding to subject of relation between specific anosmia and genetic variation, it is supposed that if an odourant is recognized by one particular OR, mutations in that OR could be lead to specific anosmia for the odourant, but if an odourant is recognized by more than one OR, specific anosmia would not occur unless all of the relevant ORs were mutated. However, it should be noticed that these genetic associations could not explain specific anosmia entirely.

Olfactory sensitivity may be influenced by gender or some environmental and behavioral factors have also been suggested to affect olfactory proceeds (Menashe, Lancet 2006).

Aim of study

The interaction of odourants with olfactory receptors is the initial step in odourant detection. Olfactory receptors are the largest group of G protein-coupled receptors. These receptors present genetic variations worldwide which are hypothesized to influence their functions. However, still just a small number of agonists were identified out of thousands of odourants. Due to this compelling complexity, the identification of receptor-ligand pairs was and is still in its infancy and just a handful of human cognate receptor-ligand pairs are known so far. Elucidation of general properties of the olfactory system, such as determination of the general similarity between some odourants and ORs, requires investigation of a large number of diverse ORs with chemically diverse odourants in a consistent assay. Availability of the complete sequence of human genome and quantitative data for millions of SNP gained from the 1000 genomes project and other sources provides enormous opportunities to relate olfactory phenotype to the underlying genotype of odourant receptor genes. It would be of interest, to relate specific anosmias (encountered in various populations) with the underlying gene mutations. Also previous studies identified ligands for several ORs. However, numerous olfactory receptors of the CNV and SPG groups remain as orphan receptors. Further, their connection to specific anosmia is still elusive. To explore these questions, we conducted a large-scale analysis of human olfactory receptors responses with a multitude of odourants related to specific anosmia.

The proposed project will elucidate the genetic basis of variations in human odor perception and moreover enhance our understanding about the mechanisms creating an individual 'unique smeller' in terms of odor sensitivity and rating. In addition it is planed to use findings of deorphanized receptors to address several distinct questions such as quantification of odourant similarity, quantification of receptor similarity and receptor code of an odourant is composed of

ORs that are "narrowly tuned" to a few odourants or "broadly tuned" to recognize many odorants.

2. Materials and Methods

2.1 DNA constructs and plasmids

Human ORs (SPGs /CNVs) were amplified from human genomic DNA by PCR using specific primers, which amplify the complete open reading frame and contain restriction sites(EcoRI, NotI, ApaI and SalI) for further subcloning into pCI expression vector (Invitrogen). Amplified ORs were cloned in multiple cloning site of pCI vector contain with Rho-tag (Fig. 2.1). PCR reactions were done with 100 ng genomic DNA and specific primers for human olfactory receptors. The generated plasmid was verified by sequencing. Primers were designed with Primer3plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi), with parameters including of melting temperature (Tm) between 55 and 65^0 C, GC-content between 30% and 70% and length from 18 to 28 bp (Tab. 2.1). The restriction site was chosen identical with the restriction site in pCI-vector. It was controlled with the Webcutter 2.0 software (http://users.unimi.it/~camelot/tools/cut2.html) to ensure that the restricted nucleotide site is not identical with any nucleotide region inside the target gene.

pCI plasmid containing Rho-tag was a kindly gift from H. Matsunami. The expression cofactor RTP1 was cotransfected to support the expression of recombinant ORs on the cell surface (Saito et al. 2004). The other cofactors Gα15, Myr-Ric8A, and HSC70 were also cotransfected for augmentation of olfactory signals which are produced by OR activations. HSC70 was a kindly gift from E. M. Neuhaus (Neuhaus et al. 2006; Krautwurst et al. 1998; Yoshikawa, Touhara 2009).

Myr-Ric8A was generated by using Ric8A primer including Myr site (Yoshikawa, Touhara 2009) and cloning the PCR products into a pcDNA3 vector. Forward and reverse primers used in construction of Myr-Ric8A were as follow:

gcatatGAATTCACCATGGGTAGCAACAAGAGCAAGCCCAAGGATGCCAGCCAGCGGA TGGAGCCCCGGGCGGTTGC as forward primer included restriction site of EcoRI and gcatatGCGGCCGCTCAGTCAGGGTCCGAGTCAGGGT as reverse primer with NotI restriction site.

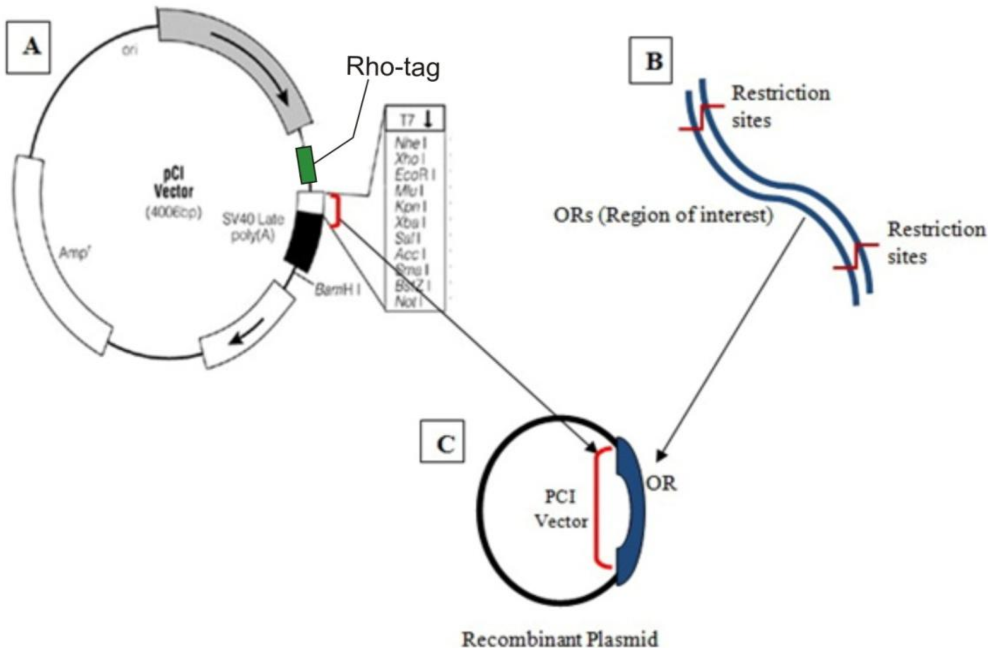


Figure 2.1: cloning of ORs. A) pCI expression vector (Invitrogen) including Rho-tag as a leader peptide sequence. B) ORs were amplified with 100 ng genomic DNA and specific primers for human olfactory receptors by PCR. The restriction site was chosen identical with the restriction sites in pCI-vector. C) The generated recombinant plasmids were verified by sequencing.

Table 2.1: Oligonucleotides

Gene	Fwd Primer	Rev Primer	Cloning sites
OR1B1	gcatatGAATTCATGATGAGCTTTGCCCCTAATG	gcatatGCGGCCGCGGGGTCTACCTTCACCCATTC	Ecorl/Notl
OR1N1	gcatatGAATTCATGGAAAACCAATCCAGCATTTC	gcatatGCGGCCGCAGAGGAAACAATACTCCTGTGACTG	Ecorl/Notl
OR1S1	gcatatGTCGACATGCATCAAGGAAACCAAACC	gcatatGCGGCCGCAAGGGAAGAAATTTTTCTATTGATG	Sall/Notl
OR1S2	gcatatGTCGACATGCATCAAGGAAACCAAACC	gcatatGCGGCCGCAAGGGAAGAAATTTTTCTATTGATG	Sall/Notl
OR2A5	gcatatGTCGACATGACAAAAAATCAGACATGGG	gcatatGCGGCCGCTCACTTTGATCTCTGTTTCCAC	Sall/Notl
OR2AG1	gcatatGAATTCATGGAGCTCTGGAACTTCACC	gcatatGCGGCCGCTCAGAGCGTGGAGTGTGCTGG	EcoRI/Notl
OR2G3	gcatatGAATTCATGGGATTGGGCAATGAGAG	gcatatGCGGCCGCTCACAATTTTCCCGAGAGAAGTTTC	EcoRI/Notl
OR2G6	gcatatGAATTCTTTCTTCTCCTGGGATTTTCA	gcatatGCGGCCGCTCAGTCCTTGTGGCTTTGTCCAG	EcoRI/Notl
OR2J1	gcatatGAATTCATGTTGATGAAAAAAAATGCAAG	gcatatGCGGCCGCTCACATCCCCCATTCCCACC	EcoRI/Notl
OR2L13	gcatatGAATTCATGGAGAAATGGAATCACACTTC	gcatatGCGGCCGCTCATTCTTTCAGGAAAGAGAATATCCC	EcoRI/Notl
OR2L8	gcatatGAATTCATGGAAAATTACAATCAAACATCAA	gcatatGCGGCCGCTCACATTTTCACAGAGCAGATTCTCTG	EcoRI/Notl
OR2S2	gcatatGAATTCATGGAAAAAGCCAATGAGACC	gcatatGCGGCCGCTCACTGAGTGAAGCCTTTTGGTCTC	EcoRI/Notl
OR2T10	gcatatGAATTCATGCGGCTGGCCAAC	gcatatGCGGCCGCTCAATATGGAGGTTTCTGCACGC	EcoRI/Notl
OR2T6	gcatatGAATTCATGAATGAAAACAATGAAACCTTG	gcatatGCGGCCGCTCAACATCTTGCCACAACTCTCTTC	EcoRI/Notl
OR2V1	gcatatGAATTCATGGGAAGATGGGTGAACC	gcatatGCGGCCGCTCAGTGCTGGCTGCCAATCCT	EcoRI/Notl
OR4A47	gcatatGAATTCATGGAGCCAAGGAAAAATGTG	gcatatGCGGCCGCTGTACTACTTGATATGAGGTCTCTTC	EcoRI/Notl
OR4C11	gcatatGTCGACATGCAGCAAAATAACAGTGTGC	gcatatGCGGCCGCTCATCCTTTGTTTTCTGAAATAATTTTGC	Sall/Notl
OR4C16	gcatatGAATTCATGCAACTGAATAATAATGTGACTG	gcatatGCGGCCGCTCTTTTGTCATCTGTGATCAATTTC	EcoRI/Notl
OR4C3	gcatatGAATTCATGGACATACCACAAAATATCAC	gcatatGCGGCCGCCCATGTAAAGAGCTTTCTCATGG	EcoRI/Notl
OR4C5	gcatatGTCGACATGTATGTGTCAAATTGCAATCC	gcatatGCGGCCGCATCACACAAATTGTTACCCCAG	Sall\Notl
OR4C6	gcatatGTCGACATGGAAAATCAAAACAATG	gcatatGCGGCCGCTTTCCCAGCCAAAGCC	Sall/Notl
OR4E2	gcatatGAATTCATGGACAGTCTAAACCAAACAAGA	gcatatGCGGCCGCTCATGTATATGATTTCGTGAAAAAAACTTG	EcoRI/Notl
OR4F15	gcatatGAATTCATGAATGGAATGAATCACTCTGTG	gcatatGCGGCCGCCAAAATCTTTGTAAAATGCGC	EcoRI/Notl
OR4F21	gcatatGAATTCATGGATGGAGAGAATCACTCAG	gcatatGCGGCCGCTGAGATCTTCTTGTAAATCACTAGCTG	EcoRI/Notl
OR4K1	gcatatGAATTCATGGCTCACACAAATGAATCG	gcatatGCGGCCGCGTTTTTCCAGGAGTTCACATGA	EcoRI/Notl
OR4K14	gcatatGAATTCATGGACCCACAGAACTATTCCTT	gcatatGCGGCCGCTCATTGAAAAGTCACCCGTCGGT	EcoRI/Notl
OR4K2	gcatatGAATTCATGGATGTGGGCAATAAGTCT	gcatatGCGGCCGCTGAAGGCATTGCCTTATTAAAA	EcoRI/Notl
OR4K5	gcatatGAATTCATGGATAAGTCCAATTCTTCAGTG	gcatatGCGGCCGCATGAAAGGAAGTTCTCACTACTAGTG	EcoRI/Notl
OR4M2	gcatatGAATTCATGGAAACTGCAAATTACACC	gcatatGCGGCCGCCTTCTCTTTACACAAAATATATTTGG	EcoRI/Notl
OR4N2	gcatatGTCGACATGGAAAGCGAGAACAGAAC	gcatatGCGGCCGCGGCTATGTGCTTATTAAACACC	Sall/Notl
OR4P4	gcatatGAATTCATGTTCTATCTACACTGGACCATGG	gcatatGCGGCCGCTCAGAAAAGTTGATTTCTTTTCAGGAG	EcoRI/Notl
OR4S2	gcatatGTCGACATGGAAAAAATAAACAACGTAACTGA	gcatatGCGGCCGCTCATTTCCCTTTAGCCTCCAAGA	Sall/Notl
OR4X1	gcatatGCGGCCGCTATGGTTGCTACAAACAATGTG	gcatatGGGCCCTCAAATTACTTTTCCCCCAATAAATCTCC	Notl/Apal
OR4X2	gcatatGTCGACAATGACTGAATTCATTTTTCTGG	gcatatGCGGCCGCTTATTTCTCATTTAGTCTCAATGTC	Sall/Notl
OR5AS1	gcatatGAATTCATGTTGGAGAGTAATTACACC	gcatatGCGGCCGCGATATTGACTATTCTTAAACG	EcoRI/Notl
OR5D13	gcatatGAATTCATGATGGCATCTGAAAGAAATC	gcatatGCGGCCGCGTGGTAAATCAATTTGGTGAC	EcoRI/Notl
OR5H6	gcatatGAATTCATGGAAGAGGAAAATGCAAC	gcatatGCGGCCGCAACATTGCTTTTGAACATTTTTG	EcoRI/Notl
OR5I1	gcatatGAATTCATGGAATTTACAGATAGAAACTAC	gcatatGCGGCCGCTGAAGAATCTACCTTTGATCTTAG	EcoRI/Notl
OR5L1	gcatatGAATTCATGGGCAAGGAAAACTGCAC	gcatatGCGGCCGCGGAGTGAATTTTGGAGCCCATC	EcoRI/Notl
OR5M9	gcatatGAATTCATGCCTAATTTCACGGATGTG	gcatatGCGGCCGCCTGCCTCACATATGTCTTGG	EcoRI/Notl
OR5R1	gcatatGAATTCATGGCTGAAGTTAATATCATTTATG	gcatatGCGGCCGCATAAAGTTTTCTTATTTTTAAAAATG	EcoRI/Notl
OR6C1	gcatatGTCGACATGAGAAACCATACAGAAATAACAGA	gcatatGCGGCCGCTCATGTGCTTGTGAAAAATACAGTCTTC	Sall/Notl
OR6J1	gcatatGAATTCATGGGTAACTGGACTGCAGC	gcatatGCGGCCGCTCAACACTGGAGCTTTACAGAATAGACA	EcoRI/Notl
OR6Q1	gcatatGAATTCATGCAACCATATACCAAAAACTG	gcatatGCGGCCGCTCACTGTCCCTTCCAAAAGTTGAG	EcoRI/Notl
OR7C2	gcatatGAATTCATGGAAAGAGGAAACCAAACAGAAG	gcatatGCGGCCGCTTAGCAATGGTCCCCTCTTTG	EcoRI/Notl
OR8B4	gcatatGAATTCATGACTCTGAGAAACAGCTCCTC	gcatatGCGGCCGCTCAGAAGAGCACTCTCTTCAGGGTT	EcoRI/Notl
OR8D2	gcatatGAATTCATGGCTACTTCAAACCATTCTTC	gcatatGCGGCCGCTCAGGATGACTGCCTTCCCCTAG	EcoRI/Notl
OR8G1	gcatatGAATTCATGTCAGGAGAAAATAATTCCTCA	gcatatGCGGCCGCTCAACTTGAAATATAAATTAAATTAGATTAG	EcoRI/Notl
OR8J2P	gcatatGAATTCATGGCTTCAGGAAATCTCACATGGG	gcatatGCGGCCGCATTTACATTAGTTTG	EcoRI/Notl
OR8K3	gcatatGTCGACGAACAACACAATCTAACAACG	gcatatGCGGCCGCTCAAACAAAAATATTACATAAGTTATTCC	Sall/Notl
OR10A6	gcatatGAATTCATGGAAAGACAAAATCAAAGCTG	gcatatGCGGCCGCTCAGATTGTGTGTAAAACCACTCGC	EcoRI/Notl
OR10AG1	gcatatGAATTCATGGAATTTGTTCTTTTGGGG	gcatatGCGGCCGCTCATGTTAATAACTTAGCTAGTAATTTTC	EcoRI/Notl
OR10C1	gcatatGAATTCATGAGTGCAAACACCTCCATGGTG	gcatatGCGGCCGCTCAAATCTCCATAGGCACCG	EcoRI/Notl
OR10Q1	gcatatGTCGACATGCCTGTGGGGAAACTTGT	gcatatGCGGCCGCTCAGTTGGCGTCAGAGGCTG	Sall/Notl
OR10X1	gcatatGTCGACATGAAGATCAACCAGACAATCCT	gcatatGCGGCCGCTCATTTTTTCAAGGCAACTGTGTTT	Sall/Notl
OR12D1P	gcatatGAATTCATGCTGAATACAACCTCAG	gcatatGCGGCCGCTCACTACTTCAAGATTTCTTTAGGC	EcoRI/Notl
OR12D2	gcatatGAATTCATGCTGAATACAACCTCAGTCACCG	gcatatGCGGCCGCTCAAAGCCTTCTGATCACTCTAC	EcoRI/Notl
OR13C7P	gcatatGAATTCATGGTAAGTGCCAATCAGACAG	gcatatGCGGCCGCGGCAAAGCATTTCTGAAATATCAAGTCCC	EcoRI/Notl
OR51A4	gcatatGTCGACATGTCCATTATCAACACATCATATG	gcatatGCGGCCGCTCATTAAATCTTCCGTTGACACAATTT	Sall/Notl
OR51B2	gcatatGAATTCATGTGGCCCAATATTACTGC	gcatatGCGGCCGCACTACTAAACCTATGTTTAGATAAAAGGCG	EcoRI/Notl
OR51F1	gcatatGAATTCATGGAAATCCTAAGCAACTCAA	gcatatGCGGCCGCTCATTTTGTAAGCAGCAGACTGA	EcoRI/Notl
OR51G1	gcatatGAATTCATGACAATTCTTCTTAATAGCAGCC	gcatatGCGGCCGCTTAATCCTTCCAAAAACACCTAA	EcoRI/Notl
OR51J1	gcatatGAATTCATGAAAATTTCTAATAACTCTTTGGG	gcatatGCGGCCGCCACTCTGCGTCTTTTCTCTAATAGC	EcoRI/Notl
OR51Q1	gcatatGAATTCATGTCCCAGGTGACTAACACCAC	gcatatGCGGCCGCTCATCTTGAATGCATATTTTTGAGG	EcoRI/Notl
OR52B4	gcatatGAATTCATGCCTACTGTAAACCACAGTG	gcatatGCGGCCGCTTATTTCTGTTTTATAAACAAAAACTGAACC	EcoRI/Notl
OR52E8	gcatatGAATTCATGTCTACGTCTAATCACACCCA	gcatatGCGGCCGCTCAGTGATTGGTCTTGAGAAAAATCC	EcoRI/Notl
OR52H1	gcatatGTCGACATGCCATCTGCCTCTGC	gcatatGCGGCCGCTCATCCTGTACCCTTAGAAAACA	EcoRI/Notl
OR52N4	gcatatGTCGACATGCTAACACTGAATAAAACAGACC	gcatatGCGGCCGCTTGGTATCCCATGCTGTAGGAT	EcoRI/Notl
OR52N5	gcatatGAATTCATGCCTCTATTTAATTCATTATGCTG	gcatatGCGGCCGCTCAACCTGCACCCTTATCACCCT	EcoRI/Notl

2.2 Mutagenesis by Overlap Extension PCR

Mutations were introduced for OR1B1 and OR10Q1 (Tab. 2.2) by Overlap Extension PCR (OEP).

Table 2.2: Primers for site-directed mutagenesis of OR1B1 and OR10Q1 genes. Flanking primers in combination with internal primers provided mutated gene segments, Full length primers included EcoRI and NotI restriction sites for further cloning into pCI expression vector. Site-directed mutations were selected for base of minor allele frequency of nucleotide.

Flanking Primer	Internal primers	Point of Mutation and Frequency
OR1B1 Forward(Fwd): GCATATGAATTCATGATGAGCTTTGCCCCTAATG Reverse(Rev): GCATATGCGGCCGCGGGGTCTACCTTCACCC ATTC	OR1B1-688 (Fwd): GAATTGGGGCCACTATTCTACG (Rev): CGTAGAATAGTGGCCCCAATTC	G(standard) ⟶ A(mutation) Nucleotide: 688, MAF: 0.39
	OR1B1-574 (Fwd): CCGGCCACTTCTGTGAGCCTCTTG (Rev): CAAGAGGCTCACAGAAGTGGCCGG	C (standard) ⟶ T (mutation) Nucleotide:574, MFA: 0.31
	OR1B1-789 (Fwd): CACCATCATTTGGGTCTACTTCC (Rev): GGAAGTAGACCCAAATGATGGTG	T (standard) ⟶ G (mutation) Nucleotide: 789, MAF: 0.25
OR10Q1 Fwd: gcatatGTCGACATGCCTGTGGGGAAACTTGT Rev: gcatatGCGGCCGCTCAGTTGGCGTCAGAGGCTG	OR10Q1-540 (FWD): GGAAATCAATCACTTCCTCTGC (Rev): GCAGAGGAAGTGATTGATTTCC	C (standard) ⟶ T (mutation) Nucleotide: 540, MAF: 14%

In mutagenesis by overlap extension PCR method, initial PCRs provided mutated gene segments, with overlapping complementary 3' ends carrying desired point mutation that were then mixed and used as template for a subsequent PCR to generate the full-length product. Overlapping strands of these intermediate products hybridize at this 3' region in a subsequent PCR and are extended to generate the full-length product amplified by flanking primers (Fig 2.2). Full length primers included restriction sites for further cloning into pCI vector. The nucleotide sequence of the mutants was verified by sequencing.

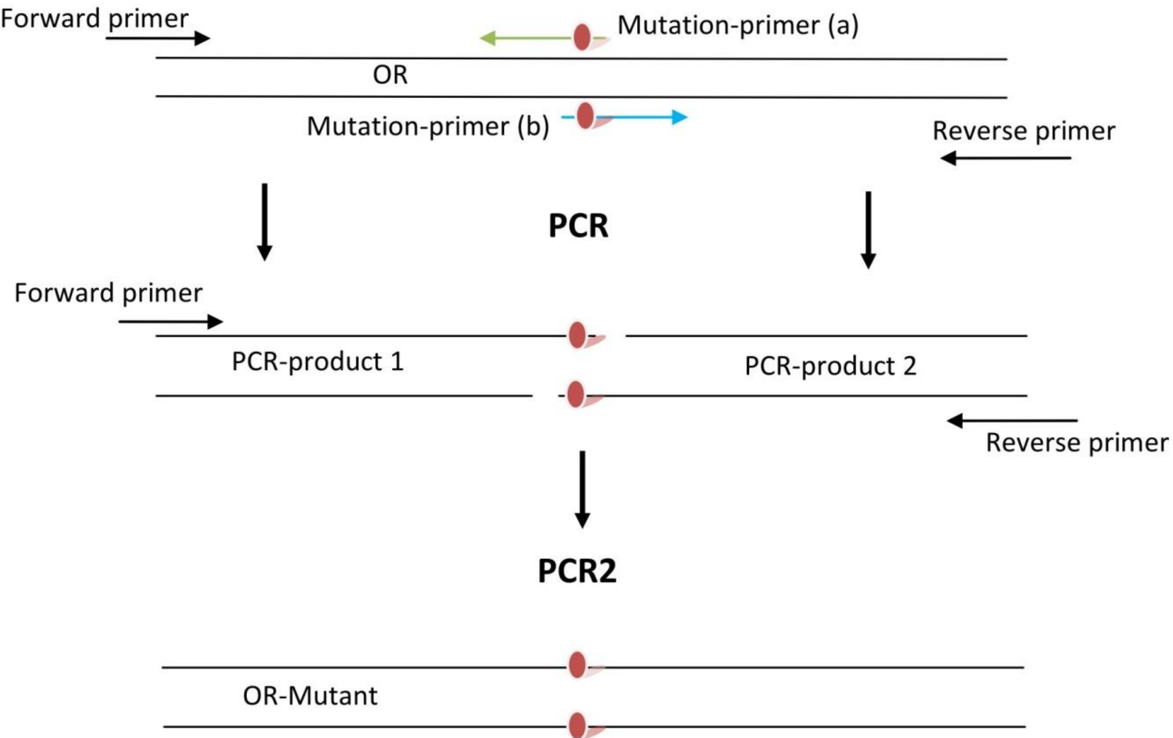


Figure 2.2: Generation of OR1B1 and OR10Q1 mutants by Overlap Extension PCR. First PCRs generate overlapping gene segments that are then used as template DNA for second PCR to create a full-length product. Internal primers generate overlapping, complementary 3′ ends on the intermediate segments and introduce nucleotide substitutions for site-directed mutagenesis. Overlapping strands of these intermediate products hybridize at this 3′ region in a subsequent PCR and are extended to generate the full-length product amplified by flanking primers.

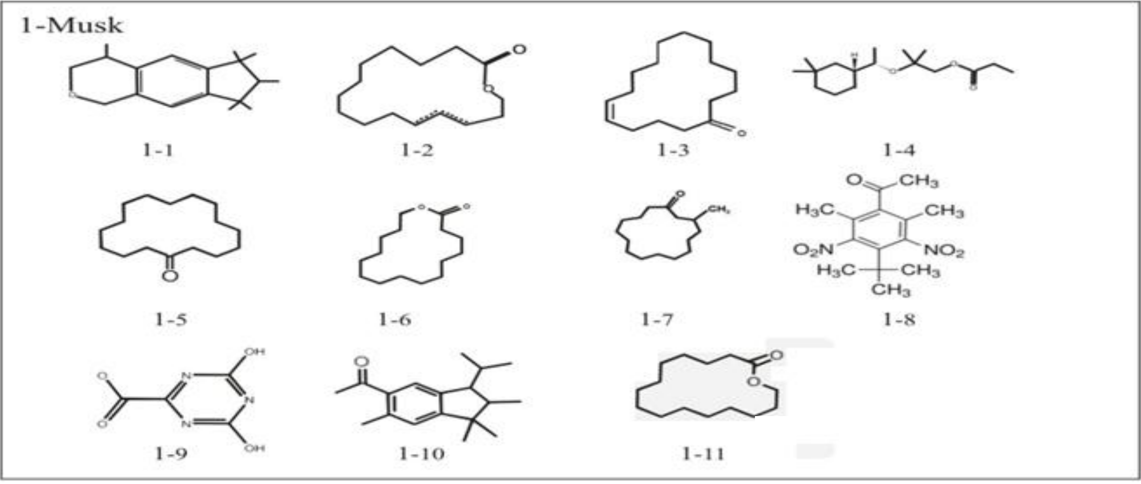
2.3 Odourant library

Odourants which are related to specific anosmia (Amoore 1974; Amoore 1977; Lawless et al. 1995; Gilbert, Kemp 1996) were used for odourant explorations for OR expressions. These 66 odourants were divided to deferent groups according to chemical structures (Tab. 2.3) and smelling. Odourants contained in each mixture are shown below.

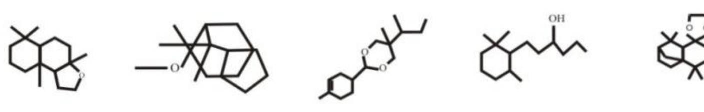
Musk group: (1.1) Galaxolide, (1.2) Globalide, (1.3) Globanone, (1.4) Helvetolide, (1.5) Isomuscone, (1.6) ω-cyclopentadecanolide, (1.7) Muscone, (1.8) Musk Ketone, (1.9) Oxonate, (1.10) Traseolide, (1.11) Macrolide Supra. **Amber Mixture** (2.1) Ambroxan, (2.2) Cedramber,

(2.3) Karanal, (2.4) Timberol, (2.5) Ysamber K. **Ketone Mixture** (3.1) 2-Aminoacetophenone, (3.2) 2-Butanone, (3.3) 3-Hydroxy-2-Methyl-4-Pyran-4-one, (3.4) 5α-Androst-16-en-3-one (Androstadien-3-one), (3.5) Hedion, (3.6) α-Ionone, (3.7) 1-Octen-3-one, (3.8) Civetone, (3.9)Methyl naphthyl keton B, (3.10) Methylhexylketon, (3.11) β-Damascone , (3.12) Calone **Carboxylic acids Mixture** (4.1) Acetic acid, (4.2) Isobutyric acid, (4.3) Isocaproic acid, (4.4) Isovaleric acid, (4.5) N-Hexanoic acid, (4.6) Propionic acid **Alcohol Mixture** (5.1) Alcohol C6, (5.2) Alcohol C9, (5.3) Anisylalcohol, (5.4) Benzylalcohol, (5.5) Cinamylalcohol, (5.6) Muguetalcohol, (5.7) Phenylethylalcohol, (5.8) Phenylpropylalcohol, (5.9) Geraniol **Aldehydes-Lactones Mixture** (6.1) 3-Phenylpropionaldehyde, (6.2) Aldehyd C6, (6.3) Aldehyde C12, (6.4) Aldehyde C14, (6.5) Aldehyde C18, (6.6) Anisic Aldehyde, (6.7) Benzaldehyde, (6.8) Heptanaldehyde, (6.9) Hydratropicaldehyd, (6.10) Isobutyraldehyde, (6.11) Octanal, (6.12) Trans-2-Nonenal **Mixture of Other** (7.1) 2,3-Butanedione , (7.2) 2-4-6-Trichloroanisole, (7.3) Geosmin, (7.4) L-Carvone, (7.5) Lyral , (7.6) Sandranol, (7.7) 2-Isobutyl-3-Methoxypyrazin, (7.8) 1-Butanthiol, (7.9) Methyldisulfide , (7.10) Skatole, (7.11) Ozonil

Table 2.3: 66 chemical odourant component in related with specific anosmia were divided to 7 different groups according to smelling and chemical structure.

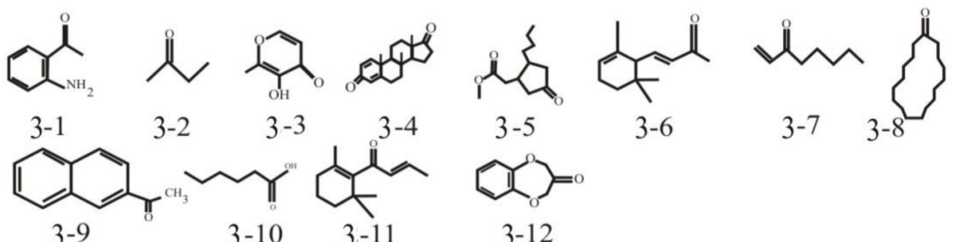


2 - Amber



2-1 2-2 2-3 2-4 2-5

3- Ketone



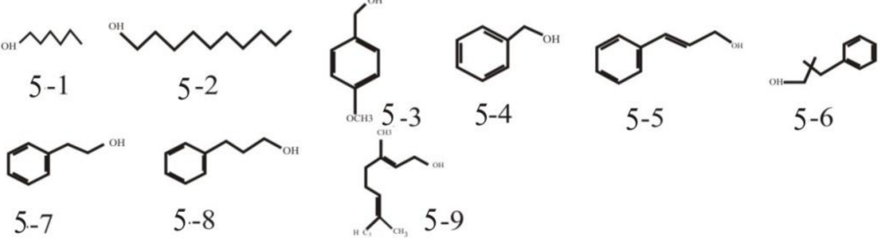
3-1 3-2 3-3 3-4 3-5 3-6 3-7 3-8

3-9 3-10 3-11 3-12

4 - Acids

4-1 4-2 4-3 4-4 4-5 4-6

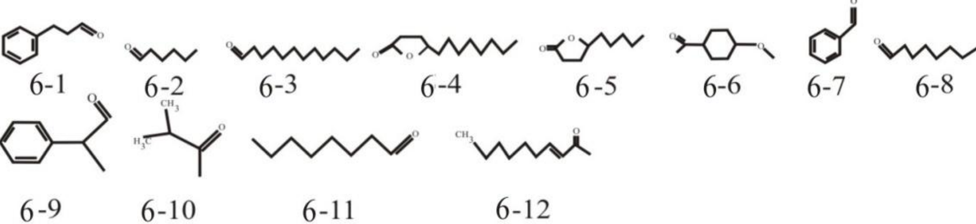
5 - Alcohols



5-1 5-2 5-3 5-4 5-5 5-6

5-7 5-8 5-9

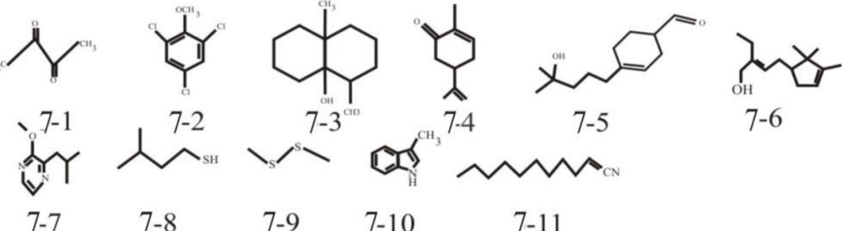
6- Aldehyde Lactone



6-1 6-2 6-3 6-4 6-5 6-6 6-7 6-8

6-9 6-10 6-11 6-12

7- Others



7-1 7-2 7-3 7-4 7-5 7-6

7-7 7-8 7-9 7-10 7-11

All the chemicals used in functional assay were kindly gift from Symrise AG Company or purchased from Sigma/Aldrich. Odourant stock solutions were prepared in dimethyl sulfoxide (DMSO). Chemical component were diluted with Ringer's solution to concentration for Ca-imaging (final DMSO concentration was maximum 0.2%).

2.4 Cell culture and transient DNA transfection

HEK293 cells were cultivated in Petri dishes (Ø 35 mm) as previously described (Benbernou et al. 2007). We grow up HEK293 cells in culture medium containing standard DMEM with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin in a humidified atmosphere (37 °C, 5% CO_2). After 2-3 days, the transfection process was carried out, whereby the cell culture was 70-80% confluent. The calcium-phosphate method was applied to transfect DNA plasmid transiently in HEK293 cells in case of Ca-imaging method.

Calcium cations and phosphate anions created crystal precipitations coupled with DNA which could invade into the cells through endocytosis. Cells were transiently transfected with plasmid DNA which consists of pCI expression vector and PCR-amplified full-length cDNA of olfactory receptors candidates, and also Gα15, RTP1, Myr-Ric8A, HSC70, and m-Cherry as cofactors. The precipitation could be created after 15-20 min, with dropping the transfection solution on HEK293 cells. Before measuring by Ca-imaging cells were incubated 48-60 hours at 37^0 C, 5% CO_2. Cationic liposome-mediated transfection method was applied to transfect DNA plasmid transiently in HANA3A cells in case of CRE-Luciferase and CRE-SEAP assays.

HANA3A cells were plated in 96 well plate covered with poly-D-lysine at roughly density about 4000 cells/well (in 50µl volume). After 24h incubation (37^0 C/ CO_2), the following was added in two different tubes (Scale according to one well):

Tube a)	52 ng receptor plasmid
	20.8 ng pCRE-Luci plasmid
	10.4 ng pSV40-Renilla
	DMEM (no serum) 5 µl per well.
Tube b)	0.15 µl Fugene transfection reagent (according to protocol)
	DMEM (no serum) 5 µl per well.

To prepare the transfection complex, we mixed solution (a) and (b) together and incubate them for 15-20 min at room temperature and in last step 45µl DMEM (with 5% FBS) was added to the "ab" mixture. After aspiration media from cells in the 96 well plates 50 µl of DMEM (with 5% FBS and AB mixture) was added to each well.

2.5 Ca-imaging

Calcium imaging of the transfected HEK293 cells was done by using chambers determinate an inverted microscope (Olympus; IX70) using a 10x objective (CPlanFL N Olympus). Fluorescence emission was determined every 4 s by using a CCD camera (Charge Coupled Device, C9100 Hamamatsu). The capillary tube which was responsible to supply odourants was generated closely above the cells. The columns filled with odourants, DMSO and ATP. Odourants were manually monitored to drop each 4-5 s to transfected cells. Solutions with drop wise form spread on HEK293 cells in 35mm dishes.

In experiments, Fura-2 Acetoxymethylester (Fura-2/AM) was applied. The non-polar Fura-2/AM is fluorescent but not yet calcium-sensitive. Once diffuses into cell plasma, Fura-2/AM is

hydrolyzed to Fura-2 which is calcium-sensitive and functions as Ca^{2+} chelator. Regardless of the presence of calcium, Fura-2 emits at the wavelength of 510 nm. Once Fura-2 binds to free intracellular Ca^{2+} in cell plasma, its ratio of excitation at wavelengths of 340 nm and 380 nm is changed and correlated to the amount of intracellular Ca^{2+}. According to this feature, the analysis of 340/380 nm excitation ratio for Fura-2 allows to quantify intracellular calcium levels.

In preparation for Ca-imaging measurements, transfected HEK293 cells were added 3 µl Fura-2/AM (3 µM) and incubated 30-45 min at 37^0C, 5% CO2. After this step, experiments were carried out in a dark condition (Fura-2 is light-sensitive). After the incubation, medium of cell culture was replaced with 2 ml Ringer's solution.

The transfection rates of cells controlled with transfected m-Cherry plasmid. Transfected cells by m-Cherry could be observed as red cells under fluorescence of microscope (emission maximum at 610 nm) (Fig. 2. 3).

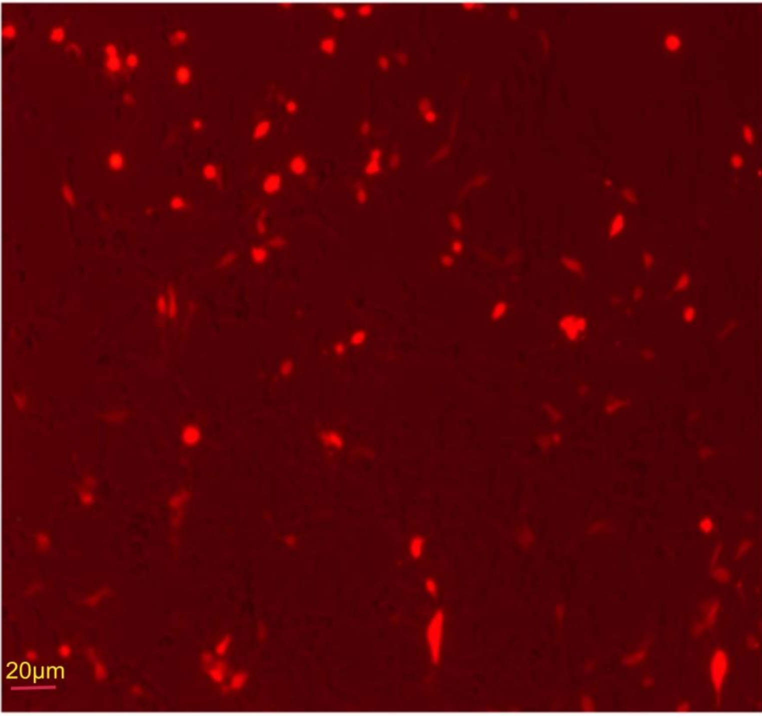


Figure 2.3: m-Cherry as indicator for transfected HEK293 cells in a transient transfection. Identification of transfected cells by m-Cherry genes that it expressed in HEK293 cells and detected by excited red fluorescence of the microscope. Transfection rate is approximately under 50%.

2.6 Stimulation and screening with odourants

Odourants were freshly prepared with DMSO as 1 M stocks solutions. Six mixtures (200 µM each chemical) were applied to cells sequentially (20s each mixture/30s Ringer). Those ORs tested with single odourants that had generated a response to odourant mixture group previously. Odourants were prepared in 100-200 µM concentration.

Ringer’s solution was used for washing cells between measuring by two different odourants. Positive response was determined by the timing of the response, the strength of the response (more than twofold higher than the noise amplitude of the baseline), and the shape of the response curve (sharp rise in curve with gradual recovery). The typical shape of response curves was established by observing responses to repeated stimulation with 200 µM of odourants. After

testing with single odourants at 100 µM, stimulating odourants were often retested at 200µM concentrations. To confirm the result of every receptor that produced a significant response curves, the experiment was repeated for twelve times. Finally, cells were exposed to ATP (0.25 mM) which could activate P_2Y receptor channel and induce Ca^{2+} influx into cells.

2.7 Strategy of screening

For screening ORs with all 66 odourants, they were classified into 7 different chemical groups. Odourants were applied in two steps, during the first one; ORs were screened by odourant groups and in the second step particular odourants were applied for deorphanization. In first step of ORs screening, odourant groups were applied for ORs during three different times of measuring. ORs that responded to same odourant groups at least for two times were selected for next step of screening with particular odourants in same responded odourant group. In the second step, if the OR after three times of measuring responded significantly to particular odourants, it would be selected for more measuring otherwise it would be excluded from the experiments. To get final conformation of deorphanized olfactory receptors they were repeated six times during two sets of experiments that each set was including of three measurements (Fig. 2.4). In this study those of ORs were presented as deorphanized receptors that during all of sets showed significant responses ($P<0.05$).

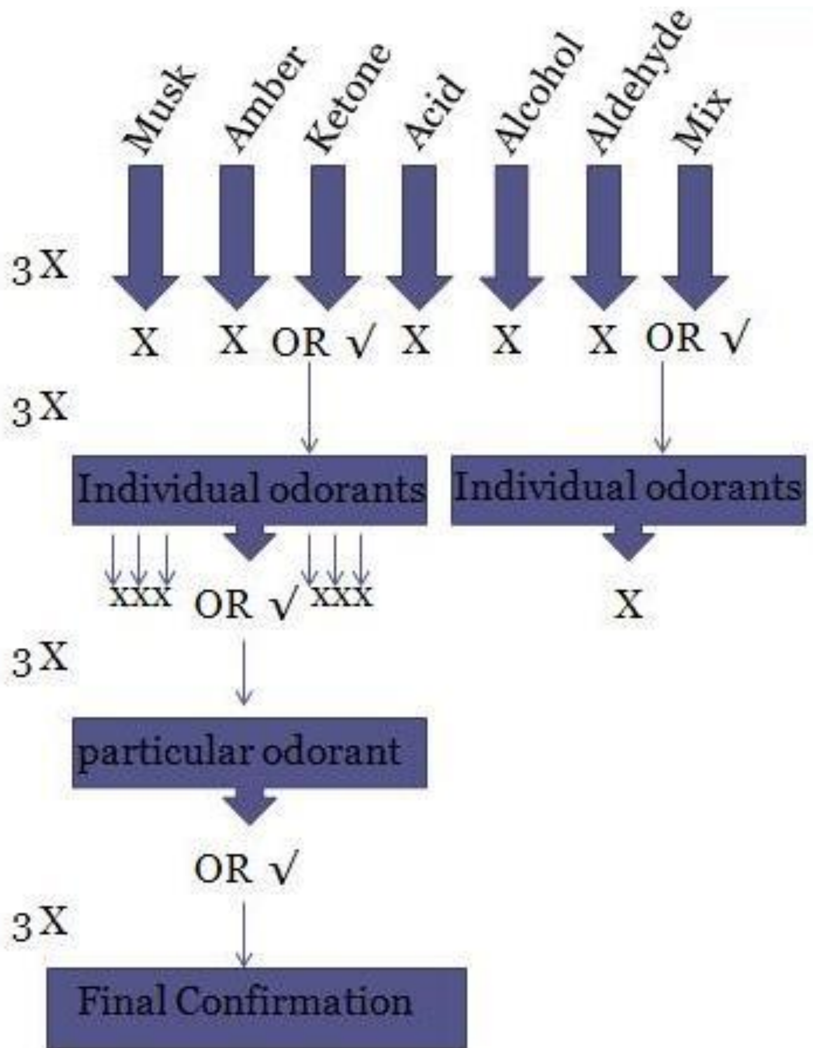


Figure 2.4: ORs were screened in by odourant groups and individual odourants. At first odourant groups were applied three times (3X) by odourant groups. In second approach all individual odourants from same responded odourant group were applied to responded OR. If OR responded to particular odourants, measuring would be repeated up to 6X time for final confirmation.

2.8 CRE-Luciferase

The Dual-Luciferase® Reporter (DLR™) Assay and Dual-Glo™ Assay enable the sequential measurement of both firefly and renilla luciferases from one sample. Odourant receptor activation leads to an increase in intracellular cAMP; we used CRE-luciferase to measure this

change. renilla luciferase driven by a constitutively active SV40 promoter (pRL-SV40; Promega) served as an internal control for cell viability and transfection efficiency. At the end data were normalized to renilla activity levels by dividing the value obtained for firefly luciferase by the renilla luciferase value.

We stimulated the ORs responded by Ca-imaging with seven separate odourant mixtures formed from 66 odourants. Odourants were applied at 5 different concentrations (50 µM, 100 µM, 150 µM, 200 µM, 250 µM, 300 µM) and all ORs that did not show activity with Ca-imaging were eliminated. We used Dual-Glo™ Luciferase Assay System (Promega) for the luciferase assay in order that it is described below.

1. Transfected HANA3A cells on poly-D-lysine-coated 96 well plates are ready for stimulation after 24 h. The cells in each well should be 50-80% confluent at the time of stimulation.

2. Replace DMEM medium with CD293 (chemically defined medium) and incubation the plate for 30min at 37^0C and 5% CO_2.

3. Add 25 µl of odourant solution diluted in CD293 and incubation 4h at 37^0C and 5% CO_2.

4. Incubate the plate with firefly luciferase buffer (20 µl per well) in 10min at room temperature.

5. Measuring by plate reader (Luminometer).

6. Add second buffer (renilla/20 µl per well) and incubation in 10min at room temperature.

7. Measuring by plate reader (Luminometer).

8. According to manufacturer’s protocols for measuring luciferase and renilla activities, calculate normalized luciferase activity with using the formula (Ln-Lmin)/(Lmax-Lmin), where Ln is the luminescence of firefly in response to the odourant, L min is the minimum luciferase value on a plate, and L max is the maximum luciferase value on plate.

2.9 CRE-SEAP assay

Secreted embryonic alkaline phosphatase (SEAP) is a reporter widely used to study promoter activity or gene expression. It is a truncated form of human placental alkaline phosphatase (PLAP) by deletion of the GPI anchor. Unlike endogenous alkaline phosphatases, PLAP is extremely heat stable and resistant to the inhibitor L-homoarginine. SEAP is secreted into cell culture supernatant and therefore allows determining reporter activity without disturbing the cells (Fig. 2.5).

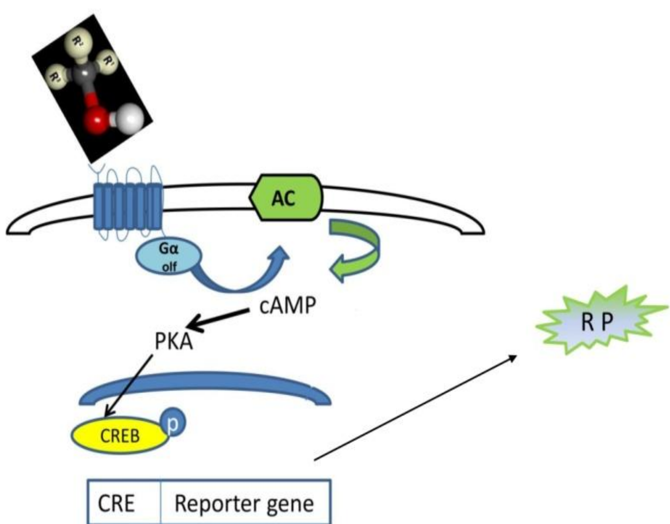


Figure 2.5: Schematic of CRE-SEAP as a reporter gene assay mediated with cAMP. Suitable chemical component activates Gαolf, leading to activation of adenylate cyclase (AC) and increase in cAMP. This second messenger activates phosphokinase A (PKA) that followed by CREB phosphorylation and translation of the SEAP gene as a reporter gene and produces a secreted form of alkaline phosphatase as a reporter protein (RP).

For the SEAP assay, cells from each well were cotransfected with Gα olf, Ric8b, RTP1, OR and a pCRE-SEAP plasmid in order that coming bellow. The protocol of CRE-SEAP assay provided kindly by L. Buck.

Day1- Cell seeding

Seeding the HEK293 cells in DMEM (10% fetal calf serum), 6000-7000 cells per well.

Day 2 – Transfect cells and add test ligands

1. For 1 well, the followings are added in an eppendorf tube (scale according to number of wells needed and use excess to cover loss during pipetting):

OR	CRE-SEAP plasmid	$G_{\alpha olf}$	RTP1
25-50ng	25ng	25ng	25ng

Transfection with Lipofectamine:

Per well:

1- Master mix(OR+ CRE-SEAP+ Gα olf+RTP1) + 25 µl opti-Mem medium
2- Lipofectamine (0.2 µl per well) +25 µl OptiMem medium (wait for 5 min please)
   1+2= Incubate 15-20min at room temperature

Whole the mixture (1+2) added to each well (without medium replacing)

3- Incubated at 37 for 24h.

Day3-

24 h after transfection, Medium will be replaced by 200µl of serum free DMEM medium containing different agonists (odourants).

1- Incubated for 6h at 37^0 C.
2- 200µl of media from each well will be transferred to a new plate (96 well plates).
3- Heating 65^0 C for 30 min.

4- 100 µl supernatant from each well will be transfer to new 96 well plates (Black well plates) & adding 100 µl SEAP Buffer (0.5mM $MgCl_2$, 1M diethanolamine, 1.2mM 4-MUP, 10mM Homoarginine, pH=10) to each well.

5- Incubated for 10min at 37^0 C.

It should be noted that, assay becomes time-sensitive after addition of Reaction Buffer.

6-Read plate for fluorescence at wavelength: Excitation 335nm & Emission 449nm (the name of program on Packard tetra plate reader system is CRE-SEAP).

2.10 Immunohistochemistry

We used immunoassay system to consider the expression of olfactory receptors in HEK293 cells and expression on the cell surface. For immunohistochemistry studies HEK293 were grown on polylysine-coated coverslips (80–100 µm thickness; Menzel Gläser, Germany). After the transfection of the HEK293 cells, coverslips were fixed by incubation in 3% paraformaldehyde in Ringer’s solution containing 10 mM glucose at room temperature for 30min. Cells were permeabilized with 0.1% Triton X-100 in PBS containing 1% cold-water fish skin gelatin (Sigma) and incubated with Rho-tag antibody 4D2 (primary antibody) in PBS/gelatin/Triton X-100 (1:200). After washing, coverslips were incubated with fluorescently labeled secondary antibodies (488-Goat-Anti mouse 1:1000) and mounted in ProLong Antifade (Molecular Probes). All fluorescence images were obtained with a confocal microscope (LSM510 Meta; Zeiss). Also to investigate of OR expression in cell surface of the cells we tried to live cell-surface staining plasma membrane.

As it is described before (Zhuang, Matsunami 2008), for live cell-surface staining Plasma membrane expression of N-terminally tagged Rho-tag was assessed using the primary anti-rhodopsin antibody, 4D2 in staining solution (1:100, 1 h incubation on ice) and HEK293 cells

were incubated with 4D2 anti body for 30min. After aspiration of staining solution including 4D2 anti body, cells would be incubated with second antibody on ice. Labeled OR protein was visualized by using a 488-Goat Anti-Mouse secondary antibody (1:200, goat-anti-mouse) and confocal microscopy (LSM510 Meta; Zeiss, 100_ HCXPL APO oil immersion).

2.11 Data analysis

The graphs of individual cells from Calcium imaging data (fluorescence intensity vs time in seconds) done by using Excel software (Microsoft). Responses were analyzed by the fractional change in fluorescence intensity: $\Delta F/F$o or (F _ Fo)/Fo, where F is intensity at each time point after stimulation and Fo is the value of emitted fluorescent light before the stimulus application (baseline). To determine significant cell responses in regard of OR activations to a particular odourant, the statistical chi-square test (four fields) was applied. P-value < 0.05 was considered as significant result.

3. Results:

3.1 Identification of SPG and CNV OR candidates

By the HORDE data base, in total around 60 olfactory receptors were presented as SPGs. In the first approach, we attempted to clone all of the 60 SPGs and in most cases we selected ORs as SPGs with high SNP variation. After cloning, recombinant plasmids were sequenced and results were compared with the gene data bank of the NCBI data base. Those ORs determined as pseudogenes were excluded from experiments. In the end, 40 SPGs were selected for deorphanization (Tab. 3.1). These 40 SPGs are distributed in most OR families with SNP frequency between 0.02% in OR2S2 and roughly 50% in OR1E3p. Olfactory receptor families 2, 5 and 8 with 15 ORs were the largest families considered in this study. SPGs were mostly those presented by Gilad and Menashe in different studies (Gilad & Lancet 2003; Menashe et al. 2003). Data about olfactory receptors with SPGs was obtained from the Human Olfactory Data Explorer (HORDE).

Table 3.1: 40 ORs known as SPGs from different OR families distributed in different chromosomes. Single nucleotide polymorphisms (SNPs) occurred as a point mutation in a highly conserved amino acid. Minor allele frequencies (MAFs) and the number of samples are shown in right column.

OR family	Olfactory receptors	Chromosome	SNP	MAF
1	OR1B1	9q	a nonsynonymous SNP rs1476860 (AA change=R/*)	A=0.333/727
	OR1S1	11q	a nonsynonymous SNP in a highly conserved amino-acid (rs1966834)	G=0.496/1083
	OR1E3P	17p	a polymorphism of C->del at position 54 (rs11377766)	
2	OR2F1	7q	a polymorphism in a highly conserved amino-acid (rs2072164)	T=0.120/263
	OR2L8	1q	a nonsynonymous SNP in a highly conserved amino-acid (rs4925583)	A=0.224/489
	OR2J1	6q	a nonsynonymous SNP rs2394517 (AA change=*/Q)	T=0.386/843
	OR2S2	9p	a nonsynonymous SNP in a highly conserved amino-acid (rs2233563)	T=0.021/45
	OR2AG1	11	a nonsynonymous SNP in a highly conserved amino-acid (rs11826041)	G=0.038/83
4	OR4X1	11p	a nonsynonymous SNP rs10838851 (AA change=Y/*)	T=0.340/742
	OR4X2	11p	a nonsynonymous SNP rs7120775 (AA change=Y/*)	G=0.167/364
	OR4C16	11q	a nonsynonymous SNP rs1459101 (AA change=Q/*)	T=0.277/605
	OR4E2	14q	a nonsynonymous SNP in a highly conserved amino-acid (rs2874103)	G=0.259/566
5	OR5D13	11q	a nonsynonymous SNP in a highly conserved amino-acid (rs297118)	G=0.026/57
	OR5H6	3q	a nonsynonymous SNP in a highly conserved amino-acid (rs9289564 and rs9853887)	G=0.286/625, T=0.359/784
	OR5AL1P	11q	a polymorphism of 2 bp del at positions 468-469 (rs10633383)	
	OR5L1	11q	a nonsynonymous SNP in a highly conserved amino-acid (rs12790505)	C=0.098/213
	OR5R1	11q	a nonsynonymous SNP in a highly conserved amino-acid (rs7111634 and rs6591324)	C=0.039/85 , A=0.244/534
6	OR6J1	14q	a nonsynonymous SNP in a highly conserved amino-acid (rs3751484)	T=0.167/364
	OR6Q1	11q	a polymorphism of C->del at position 685	-=0.132/289
7	OR7C2	19p	a nonsynonymous SNP in a highly conserved amino-acid (rs11883178)	A=0.035/76
8	ORB4	11q	a nonsynonymous SNP in a highly conserved amino-acid (rs4057749)	G=0.266/580
	OR8K3	11q	a nonsynonymous SNP in a highly conserved amino-acid (rs960193)	T=0.238/520
	OR8G1	11q	a nonsynonymous SNP rs4268525 (AA change=Y/*)	C=0.500/1092
	OR8J2P	11q	a nonsynonymous SNP T->C at position 190 (AA change=*/R)	
	OR8D2	11q	a nonsynonymous SNP in a highly conserved amino-acid (rs2512219)	T=0.238/520
10	OR10A6	11p	a nonsynonymous SNP in a highly conserved amino-acid (rs4758258)	A=0.221/482
	OR10X1	1q	a nonsynonymous SNP rs863362 (AA change=W/*)	C=0.465/1016
	OR10C1	6p	a nonsynonymous SNP rs17184009 (AA change=Q/*)	T=0.025/55
12	OR12D1P	6P	a polymorphism of 16 bp del at positions 556-572	
	OR12D2	6P	a nonsynonymous SNP in a highly conserved amino-acid (rs2073153)	C=0.370/809
13	OR13C7P	9P	a polymorphism of 2 bp insertion (AA) at positions 435-436	
51	OR51G1	11p	a nonsynonymous SNP in a highly conserved amino-acid (G->A at position 371, R130H)	
	OR51B2	11p	a nonsynonymous SNP in a highly conserved amino-acid (rs7952293)	A=0.245/536
	OR51Q1	11p	a nonsynonymous SNP rs2647574 (AA change=R/*)	T=0.442/966
	OR51F1	11p	a polymorphism of C->del at position 274	-=0.227/495
	OR51J1	11P	a nonsynonymous SNP in a highly conserved amino-acid (rs1909261)	A=0.159/347
52	OR52H1	11p	a nonsynonymous SNP in a highly conserved amino-acid (rs1566275)	T=0.133/291
	OR52R1	11p	a nonsynonymous SNP in a highly conserved amino-acid (rs7941731)	G=0.312/682
	OR52N4	11p	a nonsynonymous SNP rs4910844 (AA change=R/*)	T=0.215/469
	OR52B4	11p	a polymorphism of C->del at position 119 (rs11310407	-=0.328/716

hCNV-OR candidates were chosen based on large-scale CNV-identifications (Iafrate et al. 2004; Waszak et al. 2010). CNV which involves gains or losses of between several and hundreds of kilobases of genomic DNA were identified by different techniques.

Table 3.2: 34 ORs known as CNVs from different OR families distributed in different chromosomes. Number of copy number variations (CNVs) and number of samples and also distribution of them by OR family and chromosome is shown according to gain or loss of alleles.

OR family	Olfactory receptors	Chromosome	Number of CNVs	Gain/Deletion	Reference
1	OR1N1	9q	2/150	Gain	Waszak et al. 2010
	OR1S2	11q	1/150	Gain	Waszak
2	OR2T10	1q	48/150	Deletion	Waszak
	OR2G6	1q	1/150	Gain	Waszak
	OR2G3	1q	2 of 55	Deletion	Iafrate et al. 2005
	OR2L13	1q	1 of 55	Deletion	Iafrate
	OR2T6	1q	1/150	Gain	Waszak
	OR2A5	7q	1/150	Deletion	Waszak
	OR2V1	5q	1/150	Gain	Waszak
4	OR4C11	11q	56/150	Deletion	Waszak
	OR4K14	14q	1 / 55	Deletion	Iafrate
	OR4P4	11q	60/150	Deletion	Waszak
	OR4S2	11q	61/150	Deletion	Waszak
	OR4C6	11q	19/150	Deletion	Waszak
	OR4A47	11p	2/150	Deletion	Waszak
	OR4C5	11p	149/150	Gain	Waszak
	OR4F15	15q	1/150	Gain	Waszak
	OR4F21	8P	1/55	Deletion	Iafrate
	OR4K1	14q	71/150	Gain	Waszak
	OR4K2	14q	82/150	Gain	Waszak
	OR4K5	14q	75/150	Gain	Waszak
	OR4C3	11p	145/150	Gain	Waszak
	OR4M2	15q	79/150	Gain	Waszak
	OR4N2	14q	50/150	Gain	Waszak
5	OR5AS1	11q	1/150	Deletion	Waszak
	OR5L1	11q	1/150	Deletion	Waszak
	OR5M9	11q	2/55	Deletion	Iafrate
	OR5I1	11q	1/150	Deletion	Waszak
6	OR6C1	12q	1/150	Gain	Waszak
10	OR10AG1	11q	2 /55	Deletion	Iafrate
	OR10Q1	11q	1/150	Gain	Waszak
51	OR51A4	11q	2/55	Deletion	Iafrate
52	OR52E8	11p15	16/150	Deletion	Waszak
	OR52N5	11p15	42/150	Deletion	Waszak

The method of array-based comparative genomic hybridisation (array CGH) was applied for the analysis of the genomes of 55 unrelated individuals. By use of large insert DNA fragments, the arrays distinguished every 1 Mb throughout the human genome. The genomic DNA from 39 unrelated healthy control individuals was compared with genomic DNA from 16 individuals from whom chromosomal imbalances had previously been characterised. The comparative analyses allowed detection of all expected CNVs. This project has contributed as the main source

for genomic variation databases, e.g. Database Genomic Variants (DGVs) and Human Olfactory Data Explorer (HORDE). In the project of Iafrate (Iafrate et al. 2004), a novel computational approach with an underlying statistical framework, namely CopySeq, was applied for the analysis of the genomes of 150 unrelated individuals with different ancestries. 34 hCNV-OR candidates were performed in pairs with CNV-features which were identified in the above mentioned projects (Tab. 3.2). These ORs were selected from two groups of variations in CNV as those that gain or loss of the genes. We also tried to select olfactory receptors that were reported in both the studies of Iafrate and Waszak as CNVs. In addition, ORs as CNV were selected by their different distribution in the human population from 0.01% in OR1S2 to 99% in OR4C5. Most of these olfactory receptors were located in family 4.

3.2 Large scale investigation of olfactory receptors

To better understand the interaction between olfactory receptors with chemical odourants and the role of genetic variations in the phenotype of smelling, we analysed the responses of 40 SPGs and 34 CNVs to 66 odourants with different chemical structures and different perceptions in humans (Tab. 2.3). We generated libraries of human ORs by the cloning of ORs in pCI plasmid as an expression vector that represents a large fraction of the SPGs and CNVs in human OR families (Tab. 3.1, 3.2).

pCI plasmids containing Rho-tag were used for cloning. The inclusion of the first 20 amino acids of rhodopsin (Rho-tag) at the N-terminal end has been shown to promote the cell-surface expression of some ORs (Fig. 3.1) (Kajiya et al. 2001). PCRs were all done as described earlier (2.1) and cloned in pCI vector supplemented with Rho-tag. After the cloning procedure all extracted plasmids were sequenced and those that were identified as pseudogenes were excluded from the experiments.

Our human OR library comprises 74 human ORs which represents almost 20% of the total 387 human OR genes.

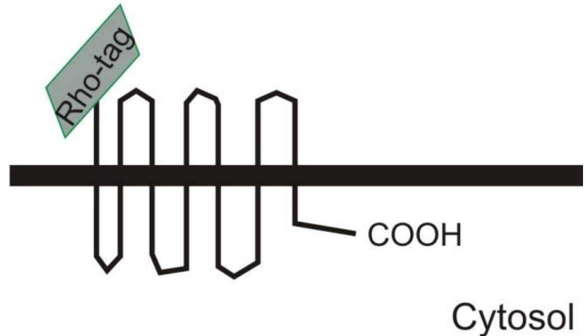


Figure 3.1: Rhodopsin-tag (Rho-tag). The inclusion of the first 20 amino acids of rhodopsin at the N-terminal ends of OR has been shown to promote the cell-surface expression of ORs.

To screen all of the 74 ORs with 66 odourants, due to the large number of ORs and odourants it was not possible to check all of them with the 66 odourants one by one. So we categorised chemical components into 7 different groups (2.3) and then we selected those ORs that were activated by pools of odours.

To analyse the responses of ORs to the odourants, Ca-imaging was used. As described in section 2.5, calcium imaging of the transfected HEK293 cells was done by the use of columns an inverted microscope (Olympus; IX70) using a 10x objective (CPlanFL N Olympus). In experiments, Fura-2 Acetoxymethylester (Fura-2/AM) was used. Regardless of the presence of calcium, Fura-2 emits at the wavelength of 510 nm. Once Fura-2 had bound to free intracellular Ca^{2+} in cell plasma, the excitation wavelengths alternated between 340 and 380 nm and correlated to the amount of intracellular Ca^{2+} (Neuhaus et al. 2009). According to this feature, the analysis of the 340/380 nm excitation ratio for Fura-2 allows us to quantify intracellular calcium levels (Fig. 3.2).

In Figure 3.2 transfected HEK293 cells with OR10Q1 are shown as an example, during screening with pentadecalactone. With increasing Ca^{2+} concentration fluorescence intensity is changed. In this case when the cells are stimulated with pentadecalactone, Ca^{2+}-concentration will increase in the cells. The increasing of Ca^{2+}-concentration was recorded as colour changing by Cell-R software. Different levels of Ca^{2+}-concentration are distinguished by colour from dark blue (basic level of internal Ca^{2+}-concentration) to red (highest level of Ca^{2+}-concentration) by the cell-R software. At the end of the measurements, cells responded to ATP (as a marker of cell vitality) strongly with an increase of internal Ca^{2+}.

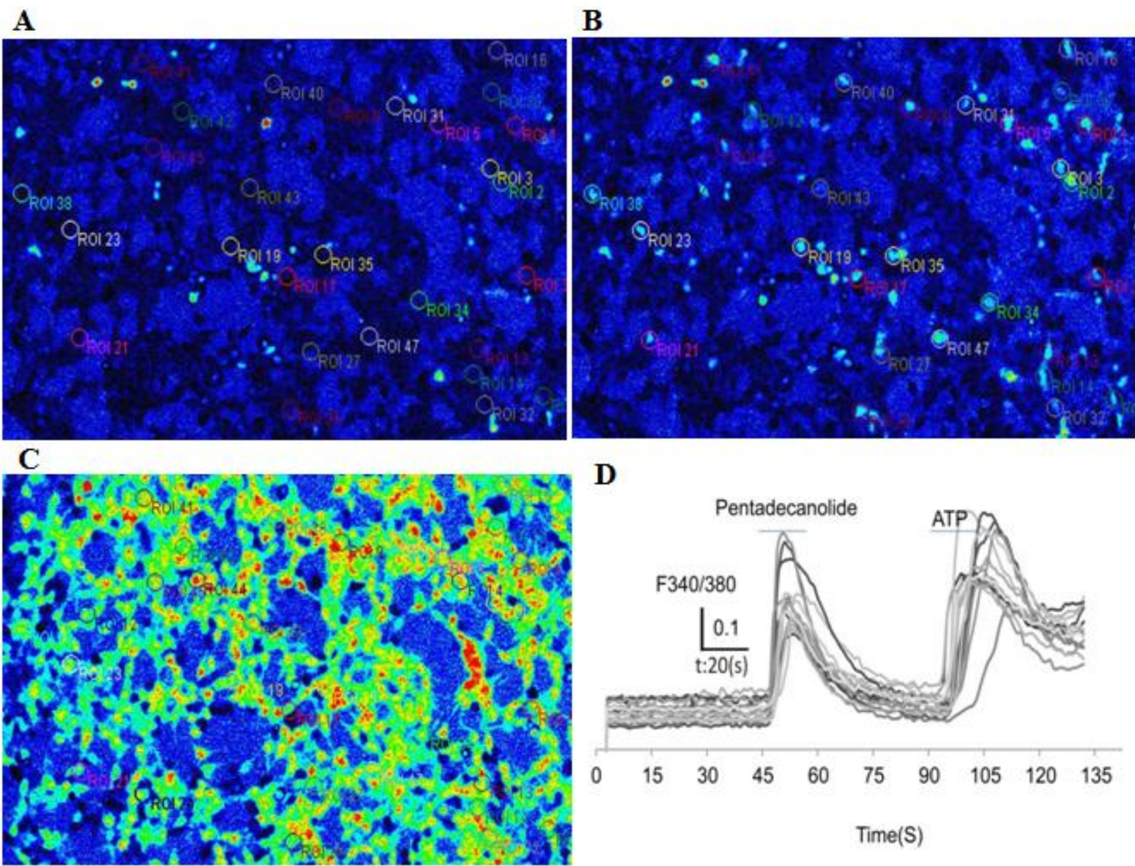


Figure 3.2: Ca-imaging measuring. OR10Q1-transfected HEK293 cells responses to pentadecalactone. (A) Transfected HEK293 cells incubated with Fura-2 after application of Ringer's solution. (B) Transfected HEK293 cells during stimulation with 200 μM pentadecalactone. As is shown by Cell-R software, colours of cells were marked as having changed from blue to green-yellow. Ca^{2+}-concentration is distinguished by colour from dark blue (basic level of internal Ca^{2+}-concentration) to red (highest level of Ca^{2+}-concentration) by the Cell-R software. (C)

The viability of the cells was controlled using 250 µM ATP. D) Response diagrams were calculated using Microsoft Excel.

As a first step, transfected HEK293 cells with ORs were monitored for increases in intracellular calcium during sequential measuring with the 7 odourant mixtures containing 100 µM of each odourant. Of the 74 ORs tested, 38 responded to one or more mixtures and were suitable for analysis; the other 36 ORs were excluded from further analysis (Tab. 3.3).

Table 3.3: ORs respond to odourant groups (OGs). ORs responded to around one to five different odourant groups. G1, G2, G3, G4, G5, G6 and G7 represented as musk, amber, ketone, acid, alcohol, aldehyde and mix groups respectively. Olfactory receptors with three stars (***), two (**) and (*) were kindly screened by Ha Truong (Truong 2012), Atefeh M. Rozbahani (Rozbahani 2012) and Zoha Zaker (Zaker 2012) respectively during their master thesis.

ORs\OGs	G1	G2	G3	G4	G5	G6	G7
OR1B1							
OR1S1							
OR1E3p							
OR2F1							
OR2L8							
OR2J1							
OR2S2							
OR2AG1***							
OR4X1							
OR4X2							
OR4C16**							
OR4E2							
OR5D13							
OR5H6							
OR5AL1P**							
OR5L1**							
OR5R1							
OR6J1							
OR6Q1							
OR7C2							
OR8B4							
OR8K3							
OR8G1							
OR8J2P**							
OR8D2							
OR10A6**							
OR10X1**							
OR10C1**							
OR12D1P**							
OR12D2**							
OR13C7p**							
OR51G1							
OR51B2							
OR51Q1**							
OR51F1							
OR51J1**							
OR52H1							
OR52R1							
OR52N4**							
OR52B4							
OR1N1							
OR1S2							
OR2T10*							
OR2G6*							
OR2G3***							
OR2L13***							
OR2T6***							
OR2A5*							
OR2V1*							
OR4C11*							
OR4K14							
OR4P4***							
OR4S2							
OR4C6***							
OR4A47*							
OR4C5*							
OR4F15							
OR4F21							
OR4K1							
OR4K2*							
OR4K5*							
OR4C3*							
OR4M2							
OR4N2							
OR5AS1*							
OR5L1							
OR5M9							
OR5I1							
OR6C1							
OR10AG1							
OR10Q1							
OR51A4							
OR52E8*							
OR52N5*							

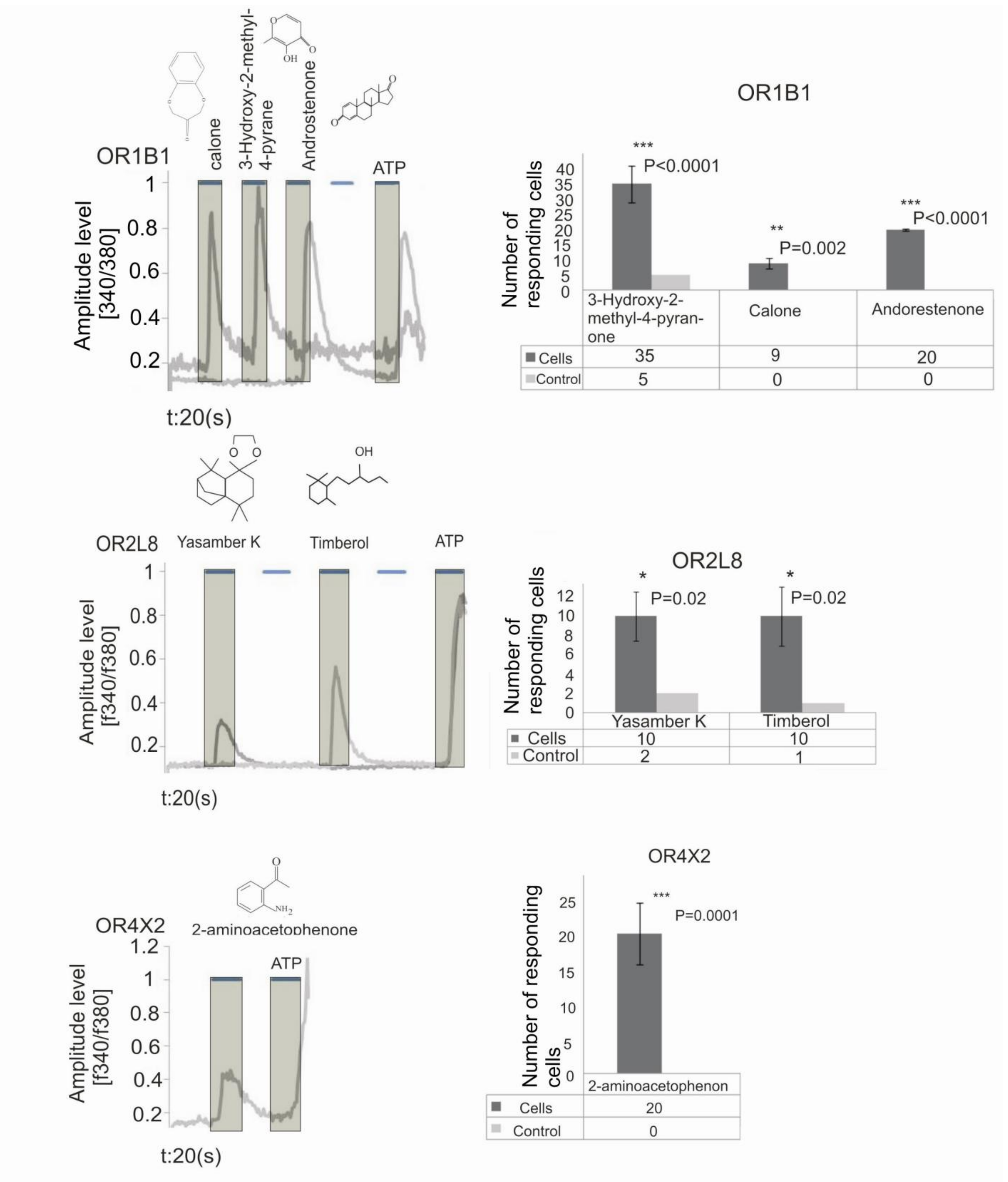
3.3 Deorphanization of olfactory receptors

Out of the 74 olfactory receptors, 38 (51.3%) showed a response to at least one mixture at 200 μM concentration. We then applied the 66 odourants individually at 200 μM to the mixture-responsive ORs. Following the strategy for deorphanization (2.7), at the end of the procedure 18 human ORs (24%) showed a significant response ($p<0.05$) to at least one of the 66 odourants related to specific anosmia (Tab. 3.4). Positive response was determined by the timing of the response, the strength of the response (more than twofold higher than the noise amplitude of the baseline), and the shape of the response curve (sharp rise in curve with gradual recovery). 20 receptors did not show any significant results, and were excluded from the next step of experiments.

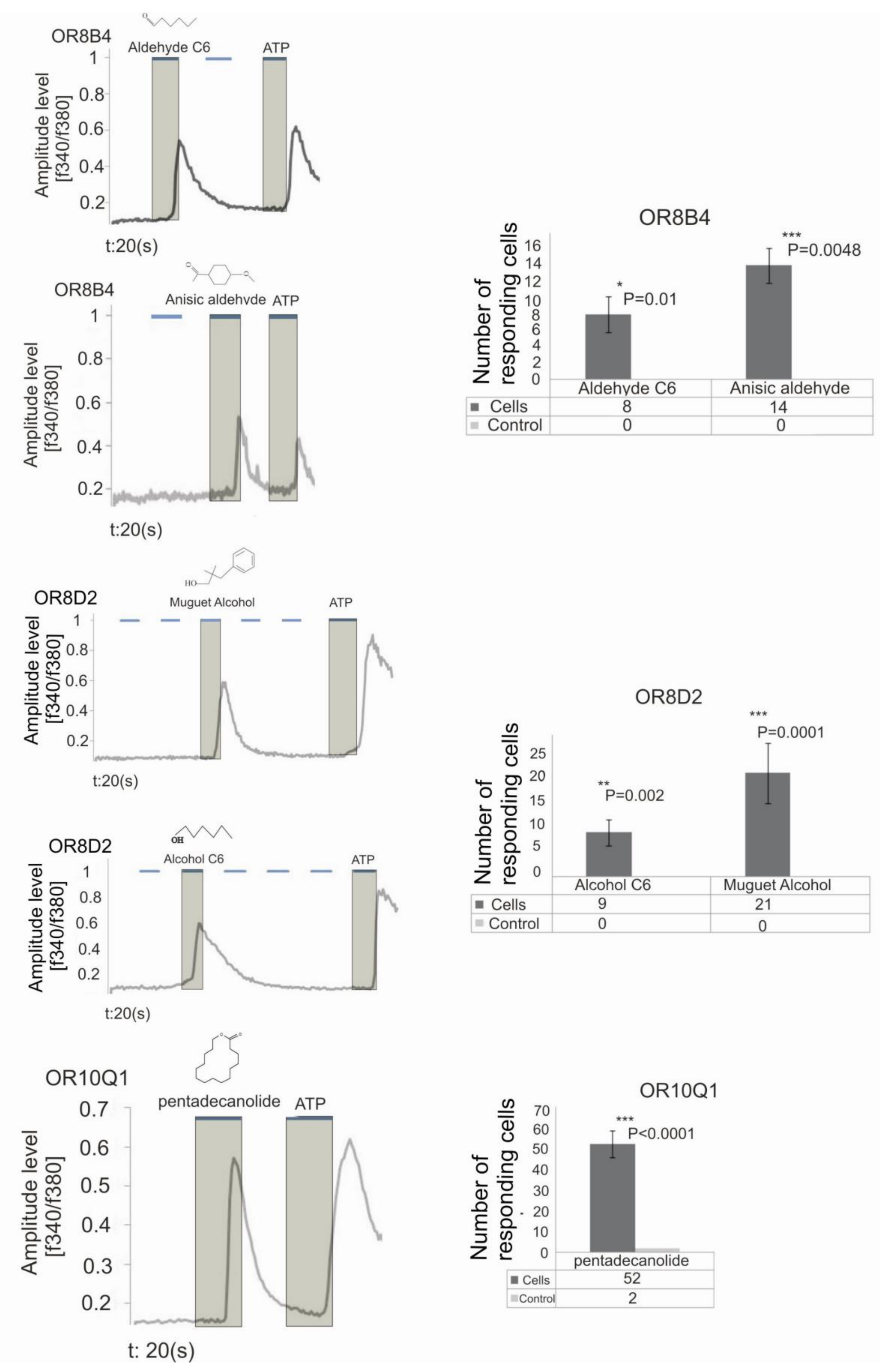
HEK293 cells were transfected with OR1B1, OR2L8, OR4X2, OR8D2, OR8B4 and OR10AQ1. It was observed that these ORs responded to ketone, amber, ketone, alcohol, aldehyde and musk odourant groups respectively. According to the strategy for deorphanization, all olfactory receptors responding to odourant groups were screened with odourants in subgroups. After the final step of deorphanization it was observed that OR1B1 was significantly ($P< 0.05$) activated by Calone, androstenone and 3-hydroxy-2-methyl-4-pyran-one which all belongs to the ketone group. However, although OR1B1 responded to other groups of odourants further investigation did not show any significant activation of this receptor by single substances of other mixtures. The same strategy was used for the screening of OR2L8 with the amber group as a unique group that activated OR2L8. Ca-imaging results showed that the Ca^{2+} concentration increased in transfected cells with OR2L8 during the screening of the cells with Timbrol and Yasamber as individual odourants in the amber group. Also, OR4X2 responded to ketone group. In the following screening procedure by Ca-imaging, it was found that OR4X2 could be the receptor

for 2-aminoacetophenon ($P<0.05$). OR8D2, as a further deorphanized olfactory receptor, responded during group screening to the amber and alcohol groups, but during the subgroup screening only two odourants of the alcohol group, Muguet alcohol and alcohol C6, significantly activated OR8D2 ($P<0.05$). No reaction was observed when individual odourants of the amber group were applied to this receptor ($P>0.05$). OR8B4 responded significantly to Anisic aldehyde and aldehyde C6 as two odourants of the aldehyde group ($P<0.05$). 12 ORs were activated by the musk odourant mixture. However, only three of them responded to individual musk odourants in the screening of single substances. One of these was OR10Q1 which significantly responded to Cyclopentadecanolide ($P<0.05$). In addition to the musk group, OR10Q1 also responded to the amber group mix but the application of single amber odourants did not produce any response.

Ca-imaging graphs of responding ORs including OR1B1, OR2L8, OR4X2, OR8D2, OR8B4 and OR10Q1 which, as mentioned above, are shown in detail in Figure 3.3.



OR1B1
calone
3-Hydroxy-2-methyl-4-pyrane
Androstenone
ATP
Amplitude level [340/380]
t:20(s)
OR1B1
Number of responding cells
*** P<0.0001
** P=0.002
*** P<0.0001
3-Hydroxy-2-methyl-4-pyran-one
Calone
Andorestenone
Cells 35 9 20
Control 5 0 0
OR2L8
Yasamber K
Timberol
ATP
Amplitude level [f340/f380]
t:20(s)
OR2L8
Number of responding cells
* P=0.02
* P=0.02
Yasamber K
Timberol
Cells 10 10
Control 2 1
OR4X2
2-aminoacetophenone
ATP
Amplitude level [f340/f380]
t:20(s)
OR4X2
Number of responding cells
*** P=0.0001
2-aminoacetophenon
Cells 20
Control 0



OR8B4
Aldehyde C6
ATP
Amplitude level [f340/f380]
t:20(s)
OR8B4
Anisic aldehyde
ATP
Amplitude level [f340/f380]
t:20(s)
OR8B4
Number of responding cells
P=0.01
P=0.0048
Aldehyde C6
Anisic aldehyde
Cells
8
14
Control
0
0
OR8D2
Muguet Alcohol
ATP
Amplitude level [f340/f380]
t:20(s)
OR8D2
Alcohol C6
ATP
Amplitude level [f340/f380]
t:20(s)
OR8D2
Number of responding cells
P=0.002
P=0.0001
Alcohol C6
Muguet Alcohol
Cells
9
21
Control
0
0
OR10Q1
pentadecanolide
ATP
Amplitude level [f340/f380]
t: 20(s)
OR10Q1
Number of responding cells
P<0.0001
pentadecanolide
Cells
52
Control
2

Figure 3.3: Significant responses of olfactory receptors to individual odourants related to specific anosmia. Every curve in line charts represents intracellular calcium increases of a single cell by the stimulation with a particular individual odourant at 200 µM. In line charts, cells responded at the end also to ATP, which evidenced for cell viability. To find out if the individual odourants significantly activated the receptor, we counted all responded cells at final stage of three times measuring and tested for significance by using Chi-square test (Column chart). P-value was calculated by number of transfected cells (approximately 2000 transfected cells in three times of measuring) vs. number of cell responses in two groups (in test samples and control samples). Bars indicate the SEM ($P^{*}<0.05$, $0.001<P^{**}<0.0001$ and $P^{***}<0.0001$ according to Chi-square test). First diagram shows responses of OR1B1 to Calone, androstenone and 3-hydroxy-2-methyl-4-pyrane**.** Expressed OR1B1 induced totally 20, 35, 9 cell responses to Calone, androstenone and 3-hydroxy-2-methyl-4-pyrane respectively (column chart) in comparison to 0, 0, 5 cells response in controls; p-value< 0.05 (2000 cells were screened during three times of measuring). Line chart show responses curves in one measurement. Second diagram shows responses of OR2L8 to Timbrol and Yasamber**.** A total of 10 HEK293 cells expressing OR2L8 responded to both odourants (column chart); p-value< 0.05 evaluated significant response. Line chart show response curves in one measurement. Third diagram shows responses of OR4X2 to 2-aminoacetophenone**.** Expressed OR4X2 induced totally 20 cell responses to 2-aminoacetophenone (column chart) in comparison with cell response in controls; p-value< 0.05 (2000 cells were screened during three times of measuring). Line chart show response curves in one measurement**.** Next diagram shows the responses of OR8B4 to Anisic aldehyde and aldehyde C6**.** Expressed OR8B4 induced totally 14 and 8 cell responses to Anisic aldehyde and aldehyde C6 respectively (column chart); p-value< 0.05 evaluated significant response. Line chart show responses curves in one measurement. OR8D2 responded to Muguet alcohol and alcohol C6**.** Expressed OR8D2 induced totally 9 and 21 cell responses to alcohol C6 and Muguet alcohol respectively (column chart); p-value< 0.05 evaluated significant response. Line chart show responses curves in one measurement. Las diagram shows responses of OR10Q1 to Pentadecalactone**.** Expressed OR10Q1 induced a total of 52 cell responses to Pentadecalactone (column chart) in comparison with 2 cells response in controls; p-value< 0.05 evaluated significant response (2000 cells were screened during three times of measuring). Line chart show response curves in one measurement. Control HEK293 cells were transfected with all cofactors and pCI plasmids without ORs.

Among the total 18 deorphanized receptors, 6 receptors belong to the CNV group and 12 ORs are known as SPG (Tab. 3.4). Despite the other receptors not producing a significant response to single odourants from one or more odourant mixtures, our positive responses represent the discovery of a large number of OR agonists.

Table 3.4: 18 olfactory receptors responded to 32 odourants in related with specific anosmia. SPGs receptors are shown in black and CNVs receptors in green. CAN; carbon atom number.

Groups	Odorants	CAN	OR1B1	OR2L8	OR4X2	OR4C16	OR5L1	OR8B4	OR8D2	OR10A6	OR10C1	OR12D2	OR52B4	OR4E2	OR4P4	OR4K2	OR4K5	OR4C3	OR5I1	OR10Q1
Ketone	3-Hydroxy-2-Methyl-4-Pyran	6	■																	
Ketone	calone	10	■																	
Ketone	Androstenon	19	■																	
Amber	Yasamber K	17		■													■			
Amber	Timbrol	15		■																
Ketone	2-aminoacetophenon	8			■															
Lactone	Aldehyde C18	9				■	■													
Ketone	B-Damascone	13					■											■		
Aldehyde	Anisic aldehyde	8						■												
Aldehyde	Aldehyde C6	6						■												
Alcohol	Alcohol C6	6							■	■										
Alcohol	Muguet alcohol	11							■	■										
Alcohol	Cinamyl alcohol	9								■										
Ketone	Hedione	13									■									
Ketone	2-butanone	4									■									
Acid	Acetic acid	2										■								
Acid	Isovaleric acid	5										■								
Acid	Propionic acid	3										■								
Ketone	1-Octan-3-One	8											■							
Mix	2-4-6 trichoroanisole	11											■	■						
Mix	Sandranol	14											■							
Mix	Geosmin	12											■							
Acid	N-Hexanoic acid	6													■					
Musk	Globalide	15													■					
Aldehyde	Banzaldehyde	7														■				
Aldehyde	3-phenyl propyl aldehyde	9												■		■				
Aldehyde	Isobutyr aldehyde	4														■				
Amber	Cedramber	16															■			
Acid	Isocaproic acid	6																■		
Aldehyde	Octanal	8																	■	
Alcohol	Graniol	10												■						
Musk	Pentadecanolide	15														■				■

The failure of a specific OR to respond to any of the tested odourants may reflect a failure of the OR to express in our assay method rather than a lack of sensitivity to the tested odourants. In our further analysis we only selected receptors that responded to at least one of the tested odourants, and were therefore rated functional in our assay.

Each odourant mixture stimulated a subset of olfactory receptors and some mixtures activated more receptors than others. Noticeably, 33% (6/18) of the ORs were activated by mixtures

containing ketones, 22% (4/18) with aldehydes and 16% (3/18) of the ORs responded to the alcohols and musk groups, whereas only 11% (2/18) of receptors responded to the amber and “mix” groups. Regarding the number of odourants per mixture, 8 ketones and 6 aldehydes activated 5 SPGs and 1 CNV and 2 SPGs and 2 CNV receptors respectively, while just 3 odourants from the mix group activated only 2 SPGs receptors and 2 odourants from the musk group activated 3 CNVs receptors. 22% (9/40) and 17% (7/40) responded to ketone and aldehyde odourants. 10% (4/40) of responses were evoked by the amber and mix groups. Carboxylic acids and alcohols constitute 15% (6/40) of all activating odourants. Only 7% (3/40) of the responses were induced by the musk group.

3.4 Ca-imaging and CRE-luciferase: similarities and differences

Despite numerous studies on olfactory receptors, many vertebrate ORs are still orphan. One reason could be that the recombinant expression system requires additional components that allow the expression of enough OR protein at the cell membrane. In addition, it is supposed that for odourants to stimulate cellular pathways they need cofactors to associate with the cAMP pathway (therefore, assays like CRE-luciferase have been developed). We tried to confirm the Ca-imaging results by a CRE-luciferase assay. By Ca-imaging, 6 ORs (OR1B1, OR2L8, OR4X2, OR8B4, OR8D2 and OR10Q1) were deorphanized as receptors for particular odourants (Tab. 3.4). Similar experiments for these 6 ORs were carried out by the CRE-luciferase assay and all were stimulated with their ligands identified by Ca-imaging results. Experiments with the CRE-luciferase assay showed a difference in ligand-responsiveness as measured by $G\alpha_{15}$-mediated Ca-imaging and $G\alpha s$-mediated cAMP elevation. OR1B1, OR4X2, OR8D2 and OR8B4 deorphanized by Ca-imaging but did not produce similar responses by CRE-luciferase assay.

OR10Q1 gave positive responses in the Ca^{2+} assay and also gave positive responses in the cAMP assay and OR2L8 produced a weak answer by the cAMP assay (Fig. 3.4).

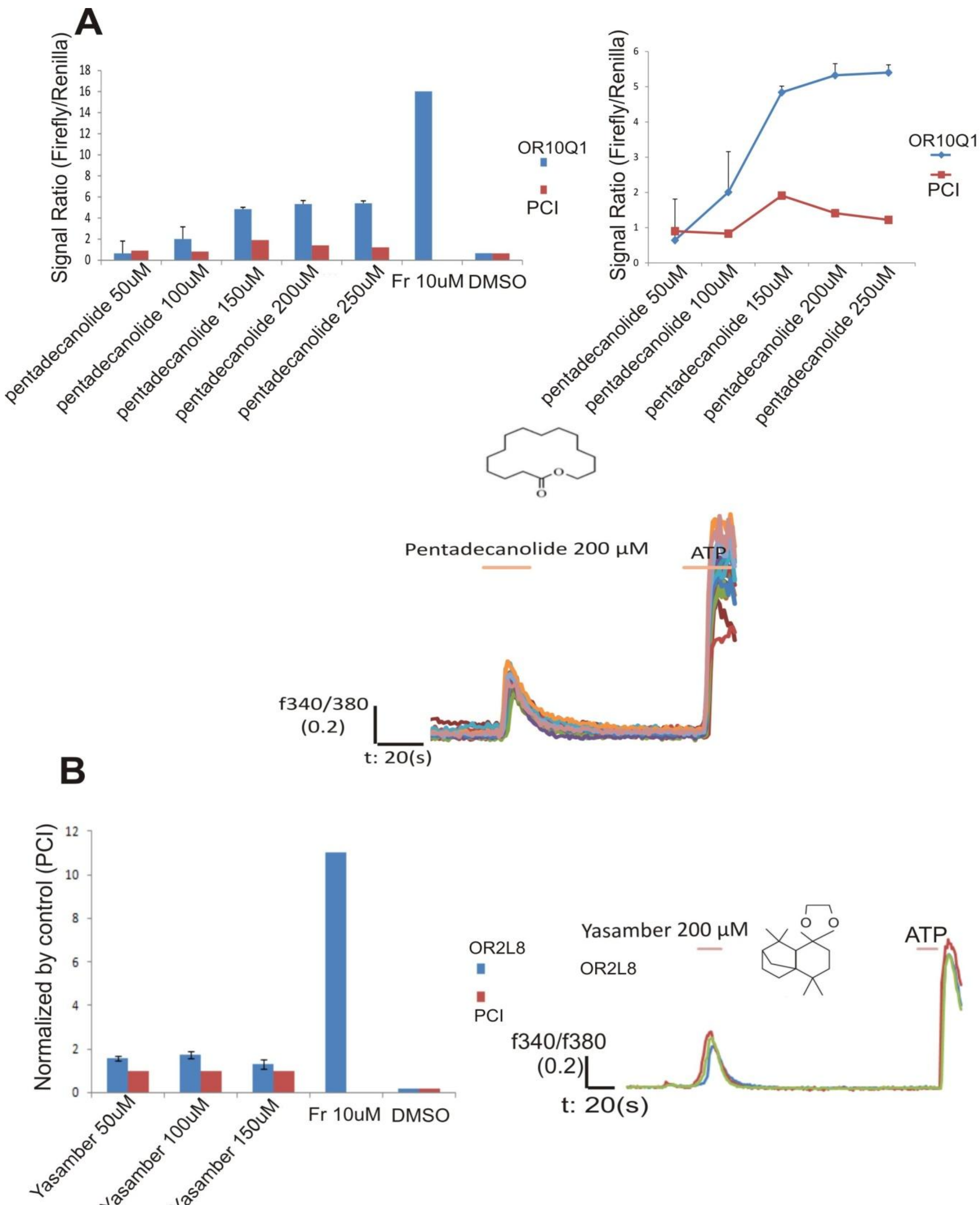


Figure 3.4: Activation of OR10Q1 and OR2L8 by Ca-imaging and CRE-Luciferase. (A) Column and line charts show dose-response curve of OR10Q1 to pentadecalactone in concentrations of 50 µM, 100 µM, 150 µM, 200 µM and 250 µM. pCI vector transfected cells and DMSO (0.2%) were used as negative controls and Forskolin (Fr) was used as a positive control with 10 µM concentration. CRE-Luciferase results were reported as ratio of firefly to renila. Line chart in middle show responses of OR10Q1 to pentadecalactone by Ca-imaging. (B) Column

chart shows weak response of OR2L8 for Yasamber in concentrations of 50 µM, 100 µM and 150 µM. DMSO was used as negative control and Forskolin (Fr) used as a positive control with 10 µM concentration. CRE-Luciferase results were reported according to normalization of receptor responses to DMSO as control. Line chart in right shows responses of OR2L8 to Yasamber by Ca-imaging.

3.5 Cell surface expression of olfactory receptors

Measuring the activation of ORs upon odourant stimulation is important for studying odour-coding by the ORs. One critical step after expression of OR proteins is trafficking of proteins to the plasma membrane. For evaluating the cell-surface expression and measuring the functional activation of ORs, cofactors plasmids including RTP1, Myr-Ric8A, HSC70 and $G\alpha_{15}$ were expressed in HEK293 cells transiently. With the use of fluorescent immunocytochemistry in live cells, we measured cell surface expression level of ORs. As shown in Figure 3.5, level of cell expression was observed to vary according to ORs.

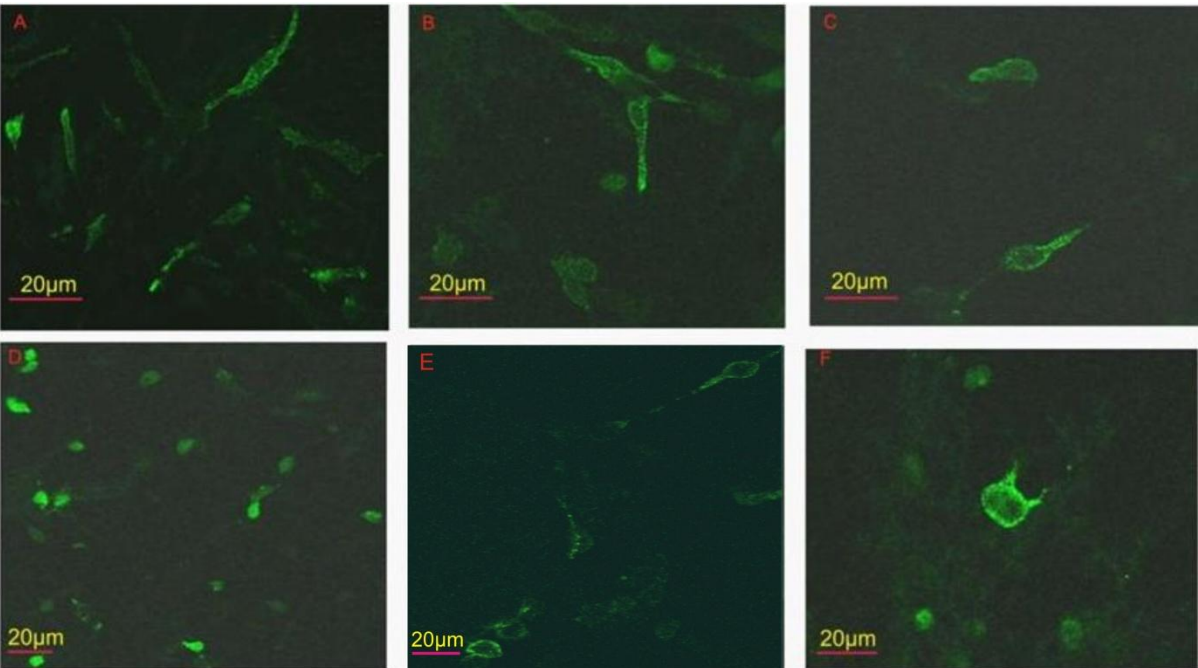


Figure 3.5: Image of live cell-surface staining for olfactory receptors. Expressed OR proteins were visualised by immunofluorescence microscopy using an antibody against Rho-tag. As shown, cell expression of ORs could be seen in cell surface by using antibody Rho-tag. Confocal microscopy of HEK293 cells transfected with A) OR2L8 B) OR10Q1 C) OR1B1 D) OR8B4 E) OR4X2 F) OR8D2.

3.6 Broadly-narrowly tune of olfactory receptors

In olfaction research, due to the lack of an agreed metric scale for measuring odourant similarity, there is no quantitative scale to rate receptors as broadly or narrowly tuned but traditionally it has been measured in terms of a receptor's number of agonists and also the similarity of those agonists to each other (Saito et al. 2009). According to current study, of 18 SPGs and CNVs activated by mixtures, 66.6% (12 of 18) responded to only one mixture containing structurally related odourants. In subgroups, 4 of the 18 deorphanized receptors (22.2%) responded to only one odourant and the other 66.7% (12 of 18) responded to up to three odourants, whereas only 2 of 18 (11.1%) responded to more than 3 odourants and none responded to all odourants in the mixture (Tab. 3.4). In most cases, the odourants recognised by olfactory receptors had related chemical functional groups. Some examples are OR1B1, which selectively responded to three odourants from the ketone mixture, including 3-hydroxy-2methyl-4pyran, Calone and androstenone. OR8D2 responded to alcohol C6 and Muguet alcohol, and two odourants from the alcohol mixture or OR2L8 responded to Yasamber and Timbrol, two odourants from the amber group. In general we confirmed in this study that a single OR can recognise multiple odourants. Out of 18 ORs, 14 ORs responded to more than one odourant. Similar results are shown in other studies where single olfactory neuron or olfactory receptors in recombinant systems can respond to multiple odourants (Hatt et al. 1999; Malnic et al. 1999; Saito et al. 2009; Nara et al. 2011).

Our results show that some SPGs and CNVs are narrowly tuned to recognise a relatively low number of odourants that are categorised in a particular group according to chemical structure. In addition, the results show that there are some single odourants (8/32) that can be recognised by multiple receptors. Yasamber is detected by OR2L8 (from the SPG group) and OR4K5 (from the CNV group). Also, Muguet alcohol and alcohol C6 are detected by OR8D2 and OR10A6.

3.7 Distance between odourants and response variability in the olfactory system

To measure the distance between two odourants, we used different metrics by counting carbon atom numbers (CANs) and functional group. To estimate the response pattern in correlation with CAN, the odourants were restricted to vary only in CAN with all other features such as functional group fixed. With regard to CANs, we classified the deorphanized odourant into 9 groups. It can be seen that among these groups odourants with 8-9 atom carbon numbers constitute the largest group, with 9 responses. Odourants with low CANs (between 2 and 5) and high CANs (between 16 and 19) showed minimum responses to olfactory receptors (Fig. 3.6). According to our results, 40% (16/40) of CANs of all the reactive odourants were between 6 and 9.

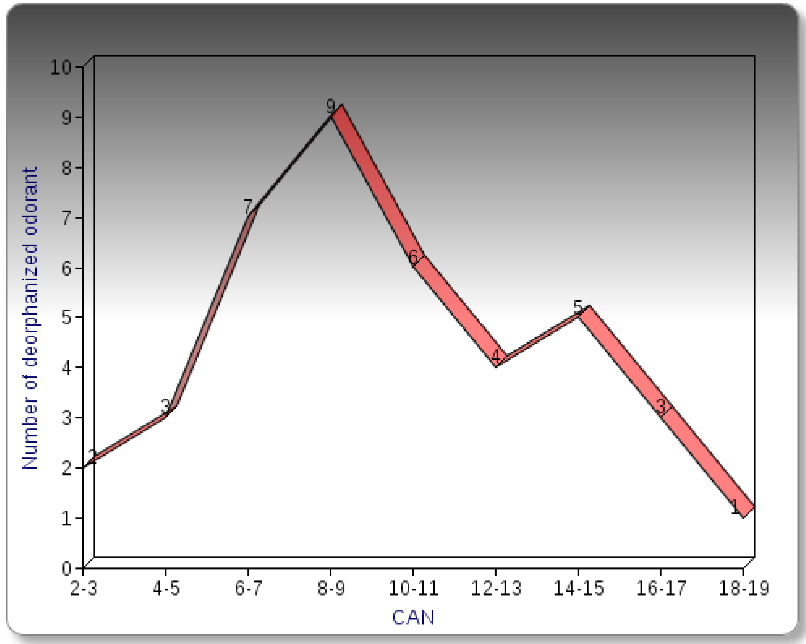
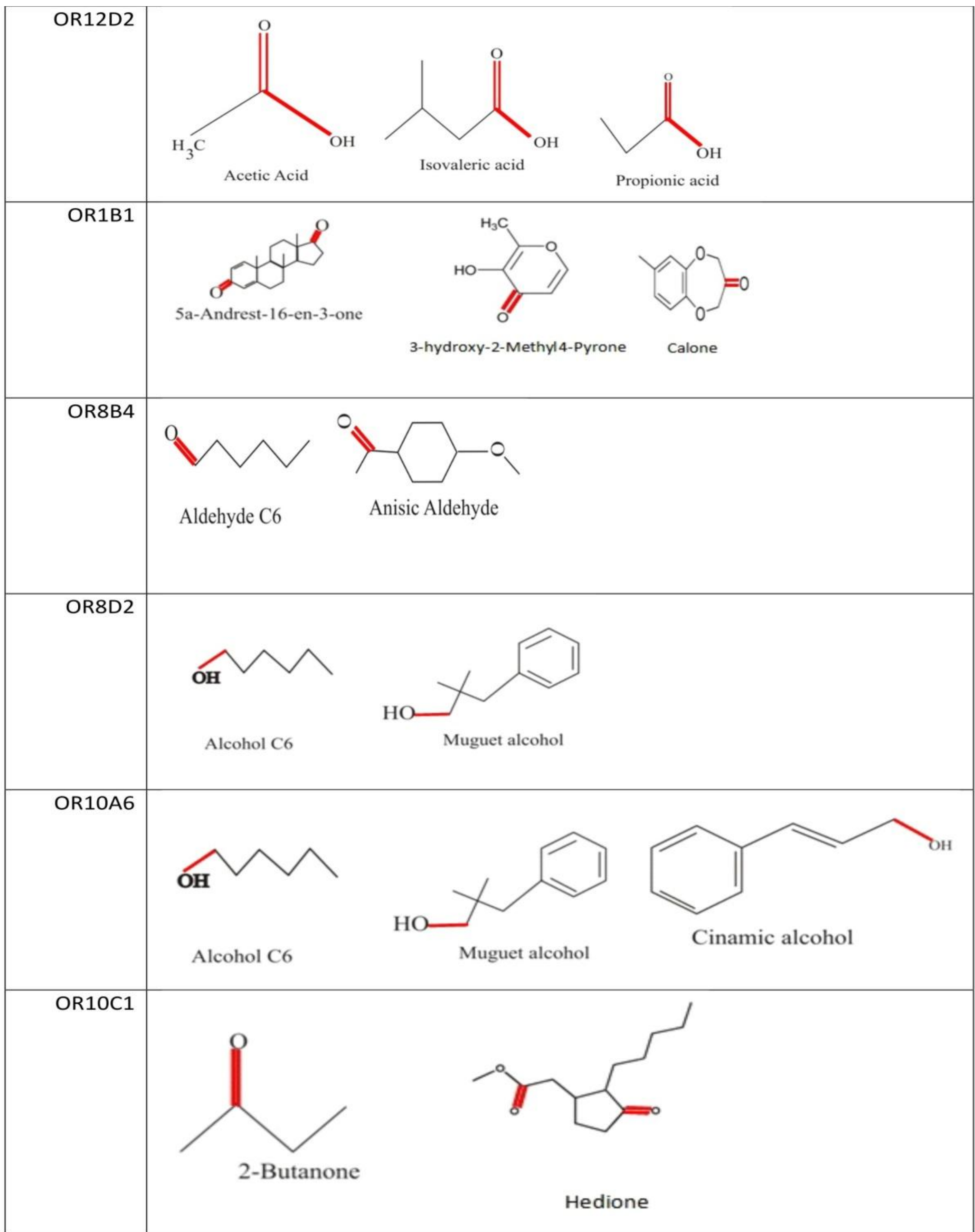


Figure 3.6: Correlation between number of deorphanized odourant and carbon atom numbers. Odourants with less than 5 or more than 12 carbon atom numbers activated low numbers of ORs in comparison with odourants containing between 5 and 12 CANs.

Also, to find a discernible structural feature that might be recognised by the OR, we considered structural features of multiple odourants that are recognised just by one single OR. As can be seen in Table 3.4, some of the receptors responded to odourants with a close number of carbon atoms. For example, OR2L8 or OR4K5 responded to odourants with between 15 and 17 CANs and OR4E2 showed some signal with odourants with between 9 and 11 CANs and OR8B4 between 6 and 8. No responsive receptors recognised odourants of all the lengths tested (i.e. C2–C19); these results were consistent with previous observations (Sato et al. 1994; Malnic et al. 1999). However, it should also be noted that there are ORs responding to odourants with greatly different CANs. For example, OR1B1 responded to 3-hydroxy-2mytyl-4pyran with 6 CANs and also responded to androstenone with 19 CANs.

The other factor that could be observed as a connection between olfactory receptors and odourants are chemical functional groups. The functional groups of the odourants as reported in previous studies are also important in recognition of ORs (Haddad et al. 2008). In our study none of the ORs recognised odourants belonging to all five groups (alcohols, acids, ketones, aldehydes, ambers) of test odourants and 61.1% (11/18) of ORs recognised odourants of only one class. Ketones constitute most parts of this odourant class with 6 subgroups and the other groups including acid, amber, aldehyde and alcohol responded with the same number of 3 odourants (Tab. 3.5).

Table 3.5: ORs responded to odourants with similar functional groups. Red bands show similar functional groups. It can be seen that ORs mostly responded to odourants with different chemical structures but the same functional groups.



The remaining ORs recognised odourants that belonged to two or three chemical classes. This diversity in the recognition properties of ORs is likely to be of central importance to the olfactory system's ability to detect and discriminate a wide variety of structurally diverse odourants.

3.8 Similar odourant structure, similar response?

As shown in Table 3.5, most of the ORs responded to odourants with similar functional groups but not with similar chemical structures. To understand the relation between structure similarity and similar response pattern, we selected OR10Q1, OR4K2, OR4P4 and OR1B1. According to our data file, OR10Q1 and OR4K2 responded to Pentadecanolide and OR4P4 and OR1B1 responded to Globalide and androstenone respectively. We measured OR10Q1, OR4K2 and OR4P4 with other musk odourants (Pentadecanolide, Civetone, Muscone, Globanone and Globalide) with a similar base of chemical structures. But ORs which responded to musk odourants did not show any significant responses to other musk odourants with similar chemical structures (Fig. 3.7).

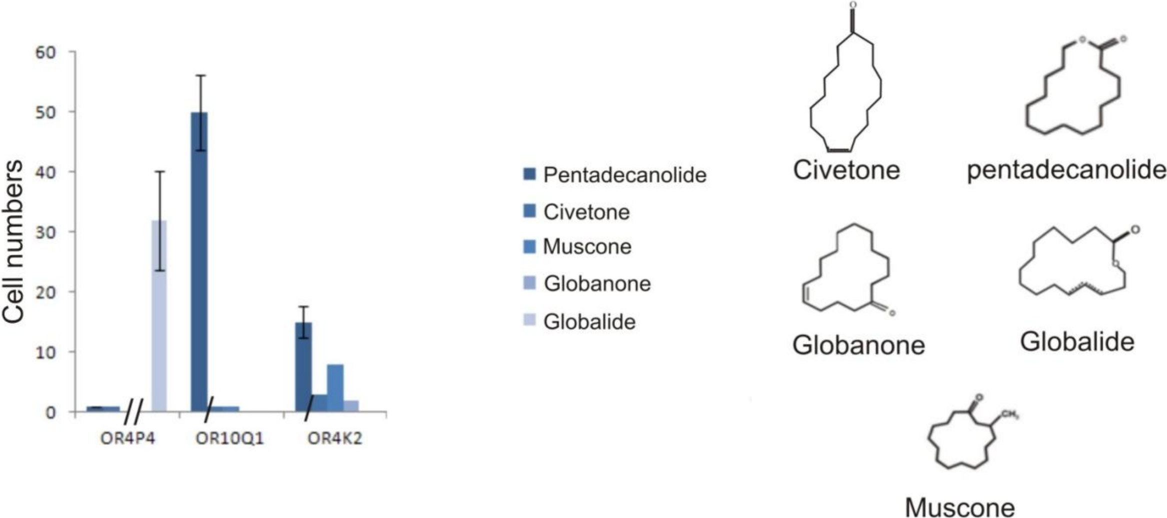


Figure 3.7: Musks with similar chemical structures do not produce the same responses. Civetone is closely related to muscone, the principal fragrant compound found in musk, because both compounds are macrocyclic ketones. Pentadecanolide and globalide are known as lactone musks, and muscone, civetone and globanone are in the ketone musk group. Structurally related odourants were individually tested *in vitro* by Ca-imaging for their ability to activate OR4P4, OR10Q1 and OR4K2. The column chart shows the number of cells that responded to different musks during measuring by Ca-imaging. Approximately 2000 cells were screened during three times of measuring.

As shown in (3.3), OR1B1 is a deorphanized receptor for androstenone. To understand the relation between structure similarity and similarity in response pattern, we screened OR1B1 with testosterone as a chemical substance with close structural similarity to androstenone. Ca-imaging results showed that OR1B1 responded to testosterone as well as to androstenone (Fig. 3.8). Testosterone is a steroid hormone from the androgen group and is found in mammals. It is the principal male sex hormone and an anabolic steroid.

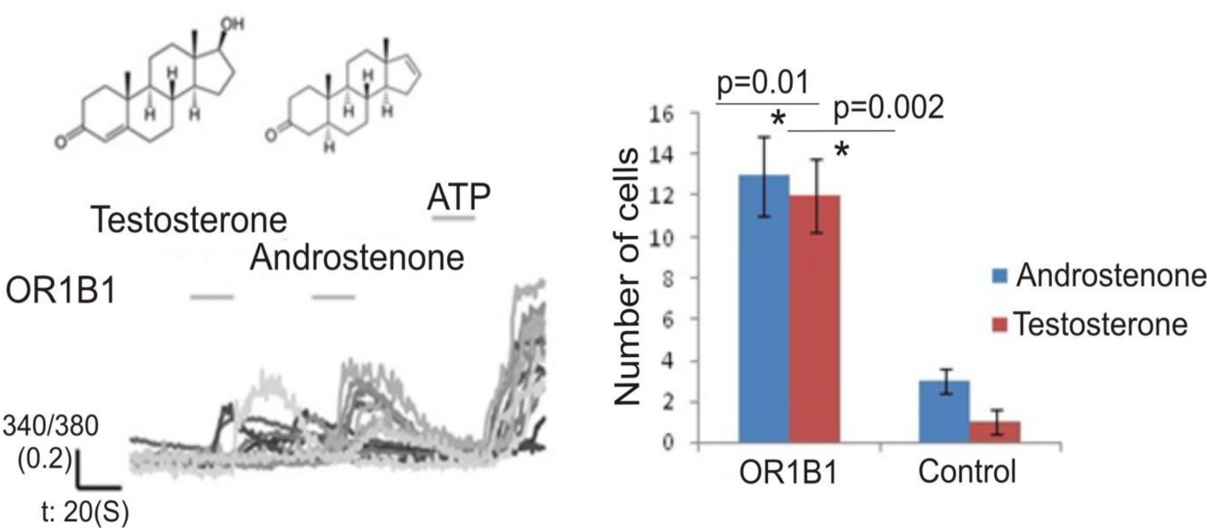


Figure 3.8: Steroid hormones with similar chemical structure produced similar responses by Ca-imaging technique. OR1B1 responds to androstenone and testosterone (200 µM). In this study it was shown OR1B1 also responded to Calone and 3-hydroxy-2-methyl-4-pyrane (200 µM). In the second approach to compare responses between similar chemical structures testosterone was used as an androgen with a similar chemical structure to testosterone. OR1B1 induced in total 13 and 12 cell responses for androstenone and testosterone respectively (column chart) in comparison with 3 and 1 cell response in controls, p-value< 0.05, evaluated a significant response. Control HEK293 cells were transfected with all cofactors and pCI plasmids without ORs. The line chart shows response curves in one measurement of androstenone and testosterone. The Screening was performed three times on a total of around 2000 cells.

3.9 Relation between odour specificity and receptor sequence

Using the similarity of amino acid properties, we constructed a dendrogram of 74 ORs which were screened in this study to show the distribution of ORs with known ligands among OR classes and families (Fig. 3.9).

In our study, we deorphanized a considerable number of ORs and, next, wanted to determine if we could match odour specificity and sequence of the ORs. One common hypothesis is that ORs that are activated by the same odourant will have similar protein sequences or amino acids in a potential binding pocket. To test this hypothesis we constructed a dendrogram based on the protein sequence of the TM3-TM6 region, a particularly variable region in ORs that has also previously been proposed to be involved in OR- odourant interactions (Man et al. 2004).

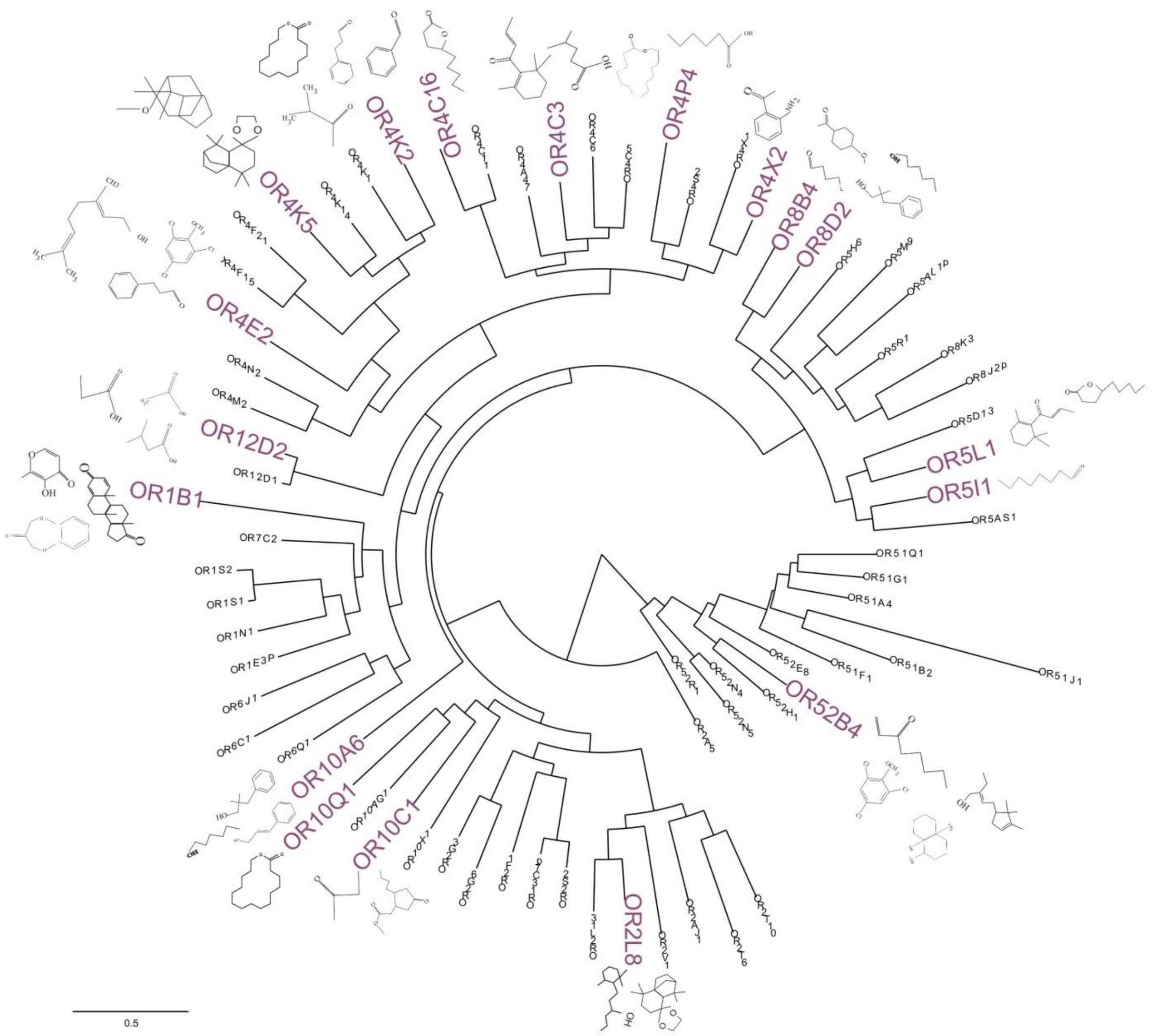


Figure 3.9: Phylogenic tree of 74 olfactory receptors which are known as SPGs or CNVs. Deorphanized ORs are shown in purple with the chemical structure of ligands beside the OR names Variable regions of the ORs were compared using the Phylogeny.fr online program (Dereeper et al. 2008).

In this dendrogram, we mapped the newly identified ligands (Fig. 3.10). The dendrogram in Figure 3.9 compares the TM3-TM6 regions of 18 ORs that responded with 32 odourants. For some of our new ligands, we found that the same odour activates closely related receptors from the same clade but for others, receptors from different clades are activated. For example, OR4P4 and OR4C3 are in the same clade and are activated by acids. It seems these sequences do not necessarily correspond to the binding site of the ORs.

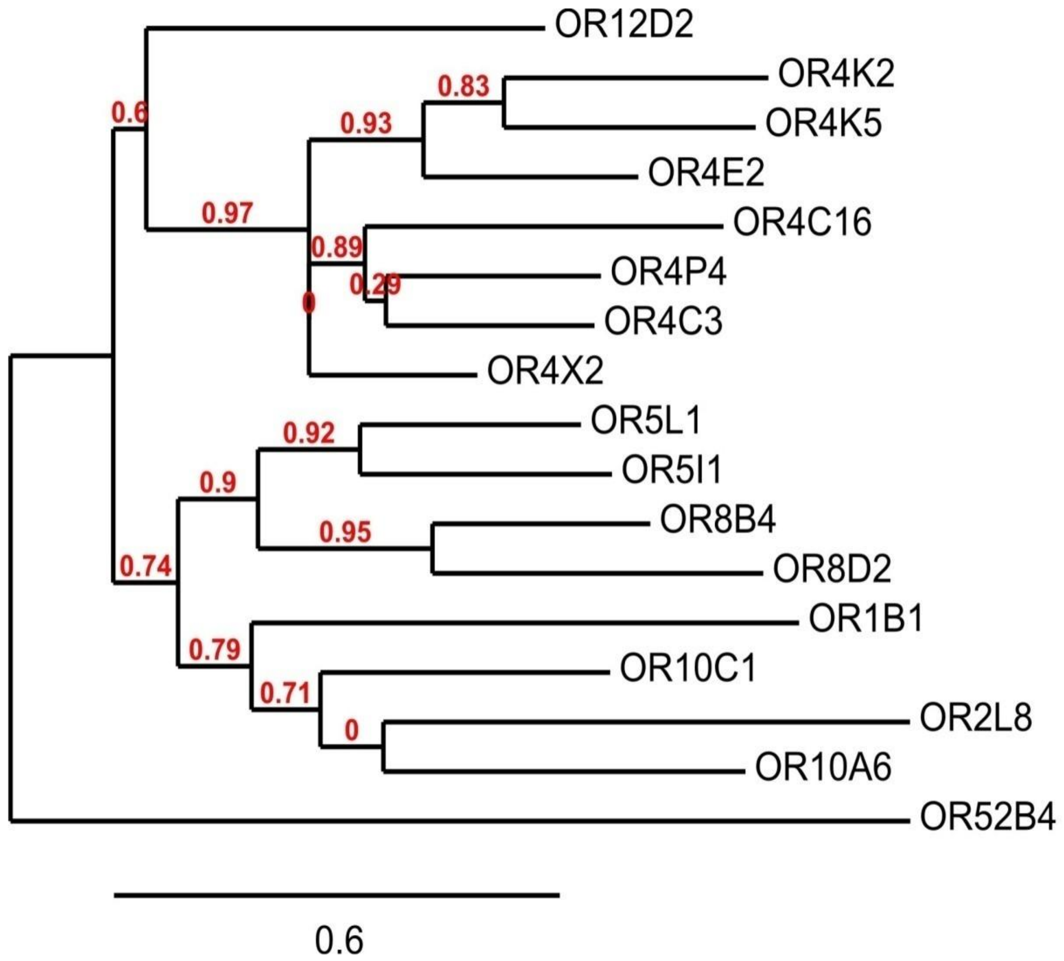


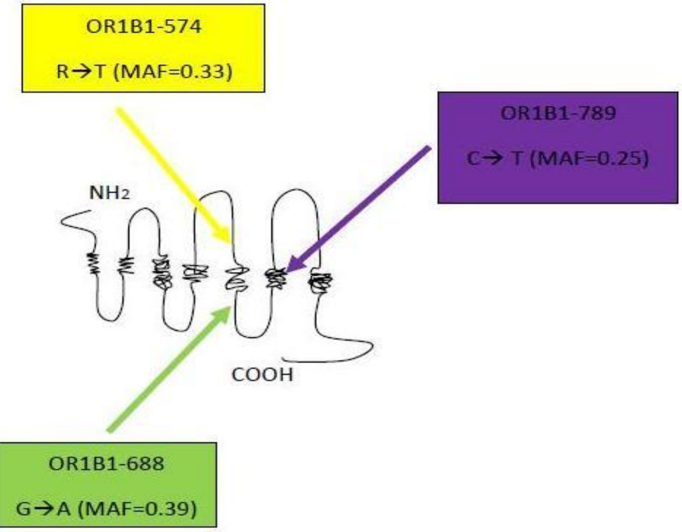
Figure 3.10: Dendrogram of TM3-TM6 as a particularly variable region in deorphanized odourant receptors. TM3-TM6 sequence identity among the 18 ORs that recognised odourants in relation to specific anosmia. Red numbers are branch support values as a likelihood ratio based on the confidence that these branches are neighbours. Variable regions of the ORs were compared with using the Phylogeny.fr online program (Dereeper et al. 2008).

3.10 Single nucleotide polymorphism and odourant responses: functional analysis of OR1B1 and OR10Q1-mutants

Human olfactory perception differs enormously between individuals, with large reported perceptual variations in the intensity and pleasantness of a given odour. Androstenone (5a-androst-16-en-3-one), an odourous steroid derived from testosterone, is variously perceived by different individuals as offensive (‘‘sweaty, urinous’’), pleasant (‘‘sweet, floral’’) or odourless

(Wysocki & Beauchamp 1984; Bremner et al. 2003). The mechanistic basis of variation in odour perception between individuals is unknown. As it has previously been shown that androstenone perception is dependent on genetic variation in human odourant receptor genes (Keller et al. 2007), we tried to investigate whether genetic variation in OR1B1 could be effective in odourant responses. Here we show that a human odourant receptor, OR1B1, is activated in vitro by androstenone and testosterone.

A search for polymorphisms in OR1B1 in SNP databases identified 3 non-synonymous SNPs in this receptor, occurring at frequencies greater than 25%. We refer to the most common allele of this receptor, named OR1B1-574, OR1B1-688 and OR1B1-789. Three different common variants of this receptor contain non-synonymous single nucleotide polymorphisms (with MAF > 0.25), resulting in three amino acid substitutions that severely impair function *in vitro* (Fig. 3.11).



OR1B1 -WT	MMSFAPNASHSPVFLLLGFSRANISYTLLFFLFLAIYLTTILGNVTLVLLISWDSRLHSPMYYLLRGLSVIDMGLSTVTLPQLLA	85
OR1B1-574	MMSFAPNASHSPVFLLLGFSRANISYTLLFFLFLAIYLTTILGNVTLVLLISWDSRLHSPMYYLLRGLSVIDMGLSTVTLPQLLA	85
OR1B1-688	MMSFAPNASHSPVFLLLGFSRANISYTLLFFLFLAIYLTTILGNVTLVLLISWDSRLHSPMYYLLRGLSVIDMGLSTVTLPQLLA	85
OR1B1-789	MMSFAPNASHSPVFLLLGFSRANISYTLLFFLFLAIYLTTILGNVTLVLLISWDSRLHSPMYYLLRGLSVIDMGLSTVTLPQLLA	85
OR1B1 -WT	HLVSHYPTIPAARCLAQFFFFYAFGVTDTLVIAVMALDRYVAICDPLHYALVMNHQRCACLLALSWVVSILHTMLRVGLVLPL	168
OR1B1-574	HLVSHYPTIPAARCLAQFFFFYAFGVTDTLVIAVMALDRYVAICDPLHYALVMNHQRCACLLALSWVVSILHTMLRVGLVLPL	168
OR1B1-688	HLVSHYPTIPAARCLAQFFFFYAFGVTDTLVIAVMALDRYVAICDPLHYALVMNHQRCACLLALSWVVSILHTMLRVGLVLPL	168
OR1B1-789	HLVSHYPTIPAARCLAQFFFFYAFGVTDTLVIAVMALDRYVAICDPLHYALVMNHQRCACLLALSWVVSILHTMLRVGLVLPL	168
OR1B1 -WT	CWTGDAGGNVNLPHFFCDHRPLLRASCSDIHSNELAIFFEGGFLMLGPCALIVLSYVRIGAGILRLPSAAGRRRAVSTCGSHLTM	253
OR1B1-574	CWTGDAGGNVNLPHFFCDHRPLL*ASCSDIHSNELAIFFEGGFLMLGPCALIVLSYVRIGAAILRLPSAAGRRRAVSTCGSHLTM	253
OR1B1-688	CWTGDAGGNVNLPHFFCDHRPLLRASCSDIHSNELAIFFEGGFLMLGPCALIVLSYVRIGAAILRLPSAAGRRRAVSTCGSHLTM	253
OR1B1-789	CWTGDAGGNVNLPHFFCDHRPLLRASCSDIHSNELAIFFEGGFLMLGPCALIVLSYVRIGAAILRLPSAAGRRRAVSTCGSHLTM	253
OR1B1 -WT	VGFLYGTIICVYFQPPFQNSQYQDMVASVMYTAITPLANPFVYSLHNKDVKGALCRLLEWVKVDP	318
OR1B1-574	VGFLYGTIICVYFQPPFQNSQYQDMVASVMYTAITPLANPFVYSLHNKDVKGALCRLLEWVKVDP	318
OR1B1-688	VGFLYGTIICVYFQPPFQNSQYQDMVASVMYTAITPLANPFVYSLHNKDVKGALCRLLEWVKVDP	318
OR1B1-789	VGFLYGTIIWVYFQPPFQNSQYQDMvASVMYTAITPLANPFVYSLHNKDVKGALCRLLEWVKVDP	318

Figure 3.11: OR1B1 schematic picture with amino acid changes indicated with consensus sequence of different mutated receptor. Nucleotides with high prevalence of MAF were selected for mutation. The table shows sequence alignments of OR1B1-WT (wild type), OR1B1-574, OR1B1-688 and OR1B1-789. Transmembrane Segments (TMs) are indicated in red and point mutations in OR1B1 are highlighted in green. (*) is terminated amino acid.

We screened for androstenone and testosterone-mediated stimulation with Ca-imaging and constructed odourant receptors with each of the SNPs, through this approach, we found that OR1B1-574 was not able to reproduce responses to androstenone and testosterone with a concentration of 200 µM (Fig. 3.12).

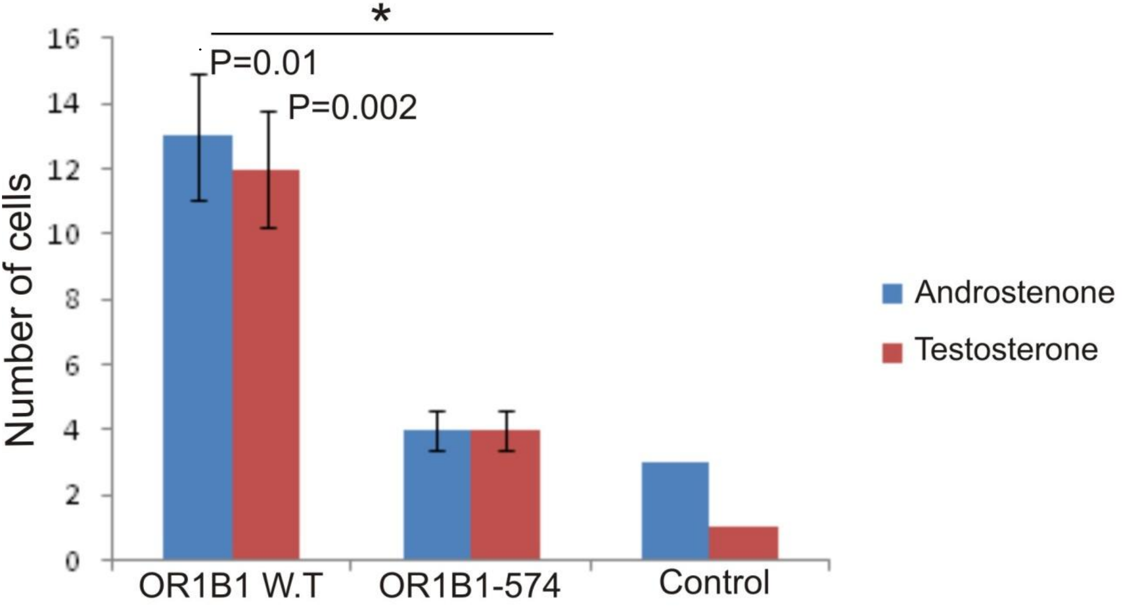


Figure 3.12: Responsiveness of OR1B1-574 to androstenone and testosterone in comparison to OR1B1 WT as examined by Ca-imaging measurements on receptors heterologously expressed in HEK293 cells. Cell responses were quantified with unspecific activity of androstenone and testosterone in controls. HEK293 cells as controls were transfected with all cofactors and pCI plasmids without ORs. Both odourants were applied for 20 seconds with a concentration of 200 µM. Bars indicate the SEM (* $p<0.05$ according to Chi-square test). Control cells respond to androstenone and testosterone (3 and 1 cells respectively). The Screening was performed three times on a total of around 2000 cells.

OR1B1-688 and OR1B1-789 did not show any significant differences responses in comparison with OR1B1 WT (Fig. 3.13).

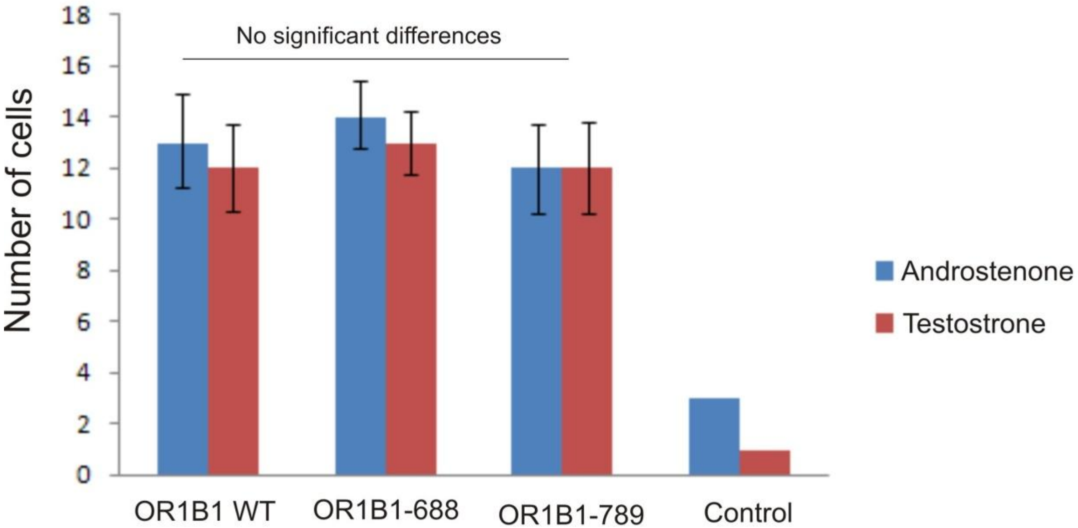
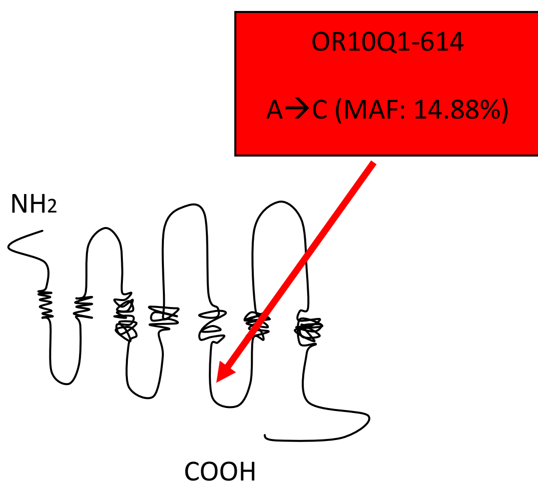


Figure 3.13: No significantly different responses of OR1B1-688 and OR1B1-789 to androstenone and testosterone in comparison to OR1B1 WT as examined by Ca-imaging measurements on receptors heterologously expressed in HEK293 cells. Cell responses were quantified with unspecific activity of androstenone and testosterone on nontransfected cells with ORs as controls. Both odourants were applied for 20 seconds with a concentration of 200 µM. Bars indicate the SEM. Control cells respond to androstenone and testosterone (3 and 1 cells respectively).

Pentadecalactone is known as an odourant with a specific anosmia of 12% in humans (Amoore 1972). With the observation of the Mendelian inheritance pattern of pentadecalactone within families by Whissell-Buechy and Amoore (Whissell-Buechy & Amoore 1973), it is supposed that this specific anosmia is due to an inheritable defect in one of the olfactory receptor proteins (Amoore 1968).

With regard to Amoore's hypothesis, we tried to investigate whether genetic variation in OR10Q1 as a new deorphanized receptor for Pentadecanolide could be effective in odourant perception or not. A search for polymorphisms in OR10Q1 in SNP databases identified one non-synonymous SNP in this receptor, occurring at frequencies greater than 0.05%. We refer to the most common allele of this receptor, named OR10Q1-614. A common variant of this receptor contains non-synonymous single nucleotide polymorphisms (with MAF of 14.88%), resulting in amino acid with substitution of Cys instead of Arg. To estimate the role of this SNP, it was

targeted for site directed mutagenesis by PCR driven overlap extension and cloning into an expression vector. Mutation of this frequent SNP was considered to affect responsiveness of the receptor to pentadecalactone (Fig. 3.14).



OR10Q1-WT	MPVGKLVFNQSEPTEFVFRAFTTATEFQVLLFLLFLLLYLMILCGNTAIIWVVCTHSTLRTPMYFFLSNLSFLELCYTTVVVPLMLS	87
OR10Q1-614	MPVGKLVFNQSEPTEFVFRAFTTATEFQVLLFLLFLLLYLMILCGNTAIIWVVCTHSTLRTPMYFFLSNLSFLELCYTTVVVPLMLS	87
OR10Q1-WT	NILGAQKPISLAGCGAQMFFFVTLGSTDCFLLAIMAYDRYVAICHPLHYTLIMTRELCTQMLGGALGLALFPSLQLTALIFTLPFCG	174
OR10Q1-614	NILGAQKPISLAGCGAQMFFFVTLGSTDCFLLAIMAYDRYVAICHPLHYTLIMTRELCTQMLGGALGLALFPSLQLTALIFTLPFCG	174
OR10Q1-WT	HQEINHFLCDVPPVLRLACADIRVHQAVLYVVSILVLTIPFLLICVSYVFITCAILSIRSAEGRRRAFSTCSFHLTVVLLQYGCCSLVY	263
OR10Q1-614	HQEINHFLCDVPPVLCLATADIRVHQAVLYVVSILVLTIPFLLICVSYVFITCAILSIRSAEGRRRAFSTCSFHLTVVLLQYGCCSLVY	263
OR10Q1-WT	LRPRSSTSEDEDSQIALVYTFVTPLLNPLLYSLRNKDVKGALRSAIIRKAASDAN	318
OR10Q1-614	LRPRSSTSEDEDSQIALVYTFVTPLLNPLLYSLRNKDVKGALRSAIIRKAASDAN	318

Figure 3.14: OR10Q1 schematic picture with amino acid change indicated and consensus sequence of different mutated receptor. Nucleotide with high prevalence of MAF was selected for mutation. The table shows sequence alignments of OR10Q1-WT (wild type) and OR10Q1-614. Transmembrane segments (TMs) are indicated in red and point mutation in OR10Q1 is highlighted in green.

OR10Q1-variants were transiently expressed in HEK293 cells and then functionally characterised by Ca-imaging. A mutant of OR10Q1 was stimulated with pentadecalactone, but did not show any difference in comparison to the response of OR10Q1-WT (Fig. 3.15).

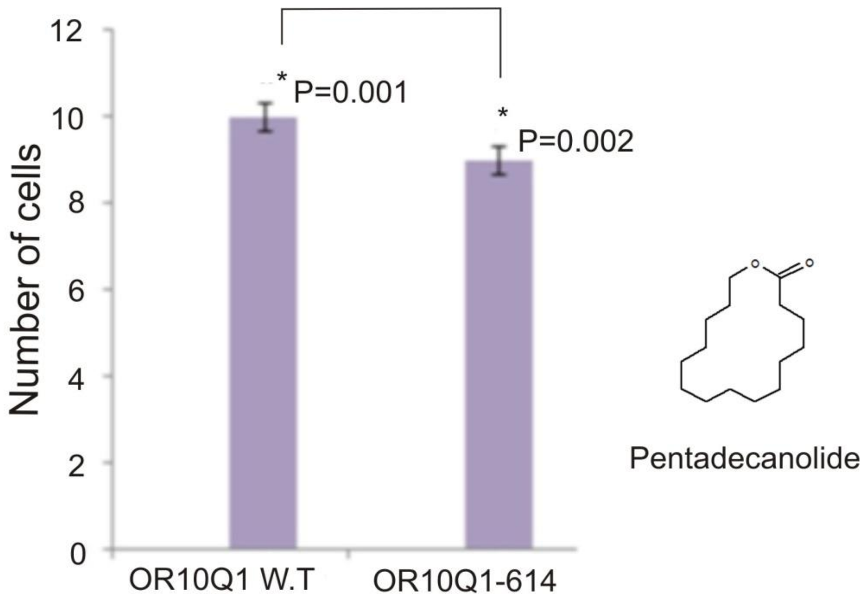


Figure 3.15: Similar responses of OR10Q1-614 to pentadecanolide in comparison with OR10Q1 WT as examined by Ca-imaging measurements on mutated and WT receptor heterologously expressed in HEK293 cells. Odourant was applied for 20 seconds with concentration of 200 µM. Bars indicate the SEM (* $p<0.05$ according to Chi-square test). Control cells did not respond to pentadecanolide (not shown). There was no significant different response between OR10Q1 WT and OR10Q1-614. Approximately 2000 cells were screened during three times of measuring.

4. Discussion:

4.1 Large scale investigation of ORs including SPGs and CNVs

It is known that the OR genes repertory is one of the most genetically diverse regions in the human genome and it contains thousands of deletions or duplications of DNA segments greater than 1 Kb in size (CNV), which are present in some individuals but not in others, and also a large number of single nucleotide polymorphisms (SNPs), some of which lead to the inactivation of OR genes (i.e. segregating pseudogenes) (Menashe et al. 2003).

Menashe et al. (2003) showed the genetic basis of human olfactory variations by focusing on a subset of olfactory receptor genes with widespread mutations that disrupt their coding region and destroy their function. Because these genes coexist with their intact counterparts in the human population (and are thus called segregating pseudogenes, or SPGs), it seems they are promising candidates for explaining human variance in odour detection and to find a relation between genetic variation and anosmia as a kind of phenotype variation in human odourant perception. In this study, we focused on a large-scale analysis of genetically polymorphic odourant receptors.

As described in several studies, there are links between genetic variation in OR and odour perception. Specific anosmia is thought to arise from mutations in olfactory receptor genes; however, no mutation in humans has as yet been linked to specific anosmia. It is suspected that variations in the genes that encode olfactory receptors, which function on the front line of odour recognition, may explain the vast differences seen in humans' ability to detect odours. In several examples it has been demonstrated that variation in OR genes by SNPs is a major reason for this kind of difference (Keller et al. 2007; Hasin-Brumshtein et al. 2009). Also, it is suggested, SPGs have donor potential for variation in human odour perception. With regard to these properties, we selected 40 orphan SPGs (Tab. 3.1) and 34 of the most abundant CNVs (Tab. 3.2) and tested

them with odourants related to anosmia. By a calcium imaging technique we identified 32 agonists for 18 human ORs including SPGs and CNVs. Our results show that, out of 40 SPGs, 12 ORs (30%) and, out of 34 CNVs, 6 ORs (18%) were activated by odourants related to specific anosmia.

In part of the study, OR1B1, OR2L8, OR4X2, OR8D2 and OR8B4 were deorphanized as SPGs with minor allel frequency (MAF of SNPs occurred as a point mutation in a highly conserved amino acid) of 33%, 22%, 16%, 50% and 26% respectively for odourants including 3-hydroxy-2-methyl-4pyran, Calone, androstenone, testosterone, Yasamber, Timbrol, 2-aminoacetophenone, Anisic aldehyde, aldehyde C6, alcohol C6 and Muguet alcohol. Among all odourants, androstenone is one of the best characterised chemical components. Androstenone (5a-androst-16-en-3-one) is reported as an odourous steroid derived from testosterone, and is variously perceived by different individuals as offensive (‘‘sweaty, urinous’’), pleasant (‘‘sweet, floral’’) or odourless (Wysocki & Beauchamp 1984; Bremner et al. 2003). Depending on the study, between 11% and 75% of the population is unable to detect the odour of androstenone (Bremner et al. 2003). This variation in the ability to perceive androstenone might suggest that androstenone perception is in part determined genetically. As some family studies have shown, androstenone thresholds are more similar among identical twins compared with fraternal twins (0.95 and 0.22, respectively) and concordance for the ability to smell androstenone is reported to be considerably higher among identical than fraternal twins (100% and 61%, respectively) (Wysocki & Beauchamp 1984). In addition, Keller (2007) showed that a human odourant receptor, OR7D4, is selectively activated *in vitro* by androstenone and the related odourous steroid androstadienone (androsta-4, 16-dien-3-one). Our results showed that among all of the 74 ORs with genetic variations, only OR1B1 responded to androstenone. OR1B1, as a segregating

pseudogene, displays both functional and nonfunctional alleles in human which makes it as excellent candidate to explain variation of androstenone perception in human population.

A search for polymorphisms in OR1B1 in SNP databases determined SNPs in this receptor, with three occurring at frequencies greater than 25%. We investigated the ligand specificity of OR1B1-574, OR1B1-688 and OR1B1-789 receptor variants in recombinant expression systems with androstenone as a suitable ligand for OR1B1-WT. OR1B1-574 is a mutant form of OR1B1 that converts the active gene of OR1B1 to pseudogene (Arginine change to termination amino acid) with a frequency of 33%. OR1B1-688 and OR1B1-789 are missense SNPs. OR1B1-688 and OR1B1-789 did not show any significant difference in evoke responses to androstenone and testosterone in comparison with OR1B1-WT; however, OR1B1-574 showed that mutations on SNP in position 574, which change amino acids into extracellular loop 2 and convert OR1B1 to pseudogene, severely impair OR1B1 function. It should be noted that OR1B1 responded to androstenone and, in addition, also responded to 3-hydrtoxy-2-methyl-4-pyran and Calone, thus cannot be regarded as a specific receptor for androstenone, but the other 73 ORs did not respond to this odourant. This result could provide a link between polymorphism in the OR1B1 gene (as SPGs) and phenotypic variation. Also, our results demonstrated that one odourant can activate more than one receptor. So, under the assumption of a multi-receptor response to androstenone, androstenone hyposmia may reflect total lack of, or a reduced number/ density of, particular olfactory receptors. Our results, however, do not rule out specific androstenone hyposmia as a helpful key to consider the genetic basis of odour discrimination.

Despite ω-cyclopentadecalactone (Pentadecanolide) being known as an odourant related to specific anosmia, no olfactory receptor(s) were presented as a deorphanized receptor for the detection of pentadecalactone. Since the early studies (Whissell-Buechy, Amoore 1973) it has

been shown that the incidence of specific anosmia to pentadecalactone within families follows a simple Mendelian inheritance pattern and the reason of specific anosmia explained as an inheritable defect in one of the olfactory receptor proteins. Here we presented OR10Q1 as one OR in the CNV group that responded to ω-cyclopentadecalactone as an odourant in the musk group. The comparative analysis indicates that the genetic variations of OR10Q1 and OR4K2 as deorphanized receptors for pentadecanolide are different in comparison with specific anosmia rates of pentadecanolide in the human population (Tab. 4.1). Among all of the 76 olfactory receptors, including SPGs and CNVs, three olfactory receptors in the CNV group responded to the musk group. With the exception of OR4P4 that responded to Globalide with a 40% deletion rate in the human population, OR10Q1 and OR4K2 responded to Pentadecanolide. It could be concluded that at the very least segregating pseudogenes do not play an effective role in the phenotype variation of Pentadecanolide and Globalide musky odourants. Also, affinity of OR10Q1 to Pentadecanolide was deliberate with mutagenesis by overlap extension PCR. The mutated OR10Q1 was produced according to a unique single nucleotide variation with MAF> 10%. We investigated the ligand specificity of OR10Q1-540 (mutated variant) and OR10Q1 (wild type) receptor variant *in vitro* with Pentadecanolide as a suitable ligand for OR10Q1. The mutated variant does not show any significant difference in comparison to OR10Q1-WT.

There is limited knowledge about most odourants in terms of different kinds of anosmias. This lack of data about anosmia leads to some difficulties finding a relation between genetic variation and odourant perception in different populations. However, some particular odourants (isovaleric acid, 1-pyrroline, Trimethylamine, Isobutyraldehyde, 5a-androst-16-en-3one and ω-pentadecalactone) were determined by Amoore (1977) as primary odourants. Among the identified primary odourants we determined some ORs response to isovaleric acid,

Isobutyraldehyde, 5a-androst-16-en-3one and ω-pentadecalactone. With regard to Amoore´s theory about "specific anosmia and the concept of primary odourants" which suggested that the perception of a particular odourant could be related to the perception of corresponding primary odourants (Amoore 1977), we compared the genetic variation rates and the possible perceived variations (Tab. 4.1).

Table 4.1: Genetic variation rates of olfactory receptors in comparison with anosmia occurrence.

ORs	Genetic variation occurrence	Ligands	Primary odour	Anosmia occurrence (Amoore)
OR1B1	33%	5a-androst-16-en-3one	Urinous	47%
OR4K2	54%	Isobutyraldehyde	Malty	36%
OR10Q1	0.6%	ω-pentadecalactone	Musky	12%
OR12D2	37%	Isovaleric acid	Sweaty	3%

The comparative analysis indicates that the genetic variation of OR1B1 may play a role in the urinous anosmia while the genetic variations of OR4K2 (54%), OR10Q1 (0.6%) and OR12D2 (37%) are different in comparison with the specific anosmia rates of musky (12%), malty (36%) or of sweat odour (3%). However, this analysis is based on the hypothesis of the theory "specific anosmia and the concept of primary odourants".

With regard to our results and those of previous studies (Nara et al. 2011; Malnic et al. 1999) showing that most odourants are recognised by more than one OR, it is difficult to state that just one olfactory receptor could determine phenotype of odour smelling in a population. For

example, Doron Lancet (Olender et al. 2012) showed highly different haplotype variation of OR2L8 between Asian, African and Europian populations (Fig. 4.1). According to our results, OR2L8 responded to Ysamber k and Timbrol.

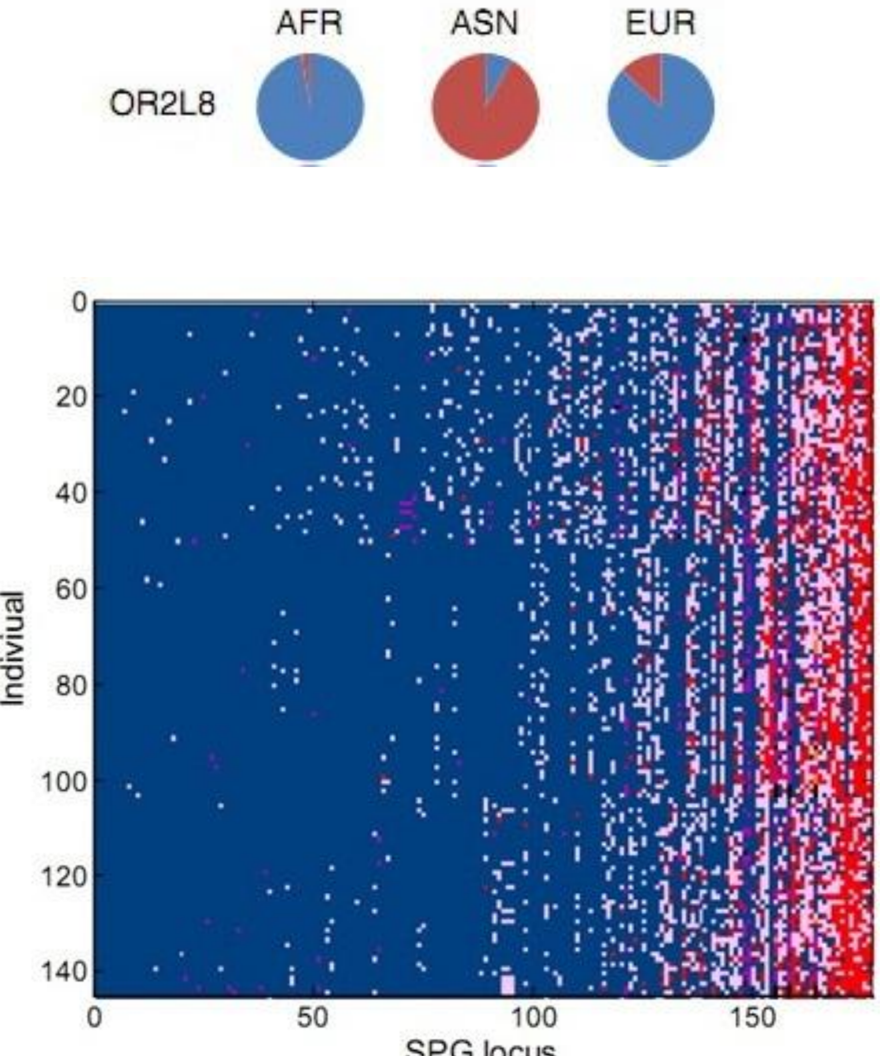


Figure 4.1: Genetic variations of olfactory receptors in the human population (Olender et al. 2012). The top schematic picture shows haplotype allele frequencies for the OR2L8 gene which shows the inter-population variability. The second picture is of personalised OR repertoires in 145 individuals. Blue - homozygotes for an intact allele; red - homozygotes for a disrupted allele; pink - heterozygotes.

The present study is in agreement with previous studies (Malnic et al. 1999; Kajiya et al. 2001; Saito et al. 2009) and indicates that different ORs can respond to the same odourant. According to our results, 25% (7 of 32) of odourants responded to more than one OR (Tab. 4.2).

Table 4.2: Odourants that responded to more than one OR.

Odourants	Olfactory receptors
YsamberK	OR2L8 OR4K5
Aldehyde C18	OR4C16 OR5L1
β - Damascone	OR5L1 OR4C3
Alcohol C6	OR8D2 OR10A6
Muguet alcohol	OR8D2 OR10A6
2-4-6 trichloroanisole	OR52B4 OR4E2
3-phenylpropyl aldehyde	OR4E2 OR4K2

In relation to the above point, that odourants are detected by a collection of ORs, and the findings of the Lancet group (2012) showing that everyone has a special combination of ORs with vast inter-individual variability (Fig. 4.1), it could be considered that the olfactory system

produces an enormous potential for phenotype variation for odourant perception in terms of different kinds of anosmias. Also, it seems that the interpreting of SNPs in individual ORs cannot lead to a prediction plane about the conception of odourants in the human population.

According to the aim of our large-scale investigation about the ORs with genetic polymorphism, we provided more deorphanized receptors to get a better understanding of the relation between olfactory receptors and chemical components. In our study we screened approximately 20% of human repertory olfactory receptors as SPGs and CNVs, so it could be expected that in the future the number of olfactory receptors that respond to similar odourants would be increased. Also, the failure of a specific odourant receptor to respond in this assay must be interpreted with caution because it may reflect a failure of the odourant receptor to be functional in the assay rather than a lack of sensitivity to the tested odour.

To conclude, with regard to the total number of deorphanized olfactory receptors, it should be noted that despite starting with roughly similar numbers of SPGs and CNVs, we identified agonists for over two times more SPGs than CNVs. These results are in agreement with current knowledge that every human individual is characterised by a different combination of such segregating pseudogenes, which makes a genotypic diversity in human population, and it is indicative of the important role of SPGs in genetic variation in relation to different anosmias. With regard to recent findings about specific combinations of intact and inactive alleles with CNV and SPGs in individual humans, which leads to each person having highly personalised barcodes of functional olfactory receptors (Olender et al. 2012), and in connection with our deorphanized receptors and different odourants between SPGs and CNVs, makes it easier to explain the variation patterns of odourant conceptions.

4.2 Broadly and narrowly tuned olfactory receptors

Some olfactory receptors are "generalists" which bind a variety of ligands and reveal broad recognition abilities and large plasticity of their binding cavity (Charlier et al. 2012) while others are reported as "specialists" that are narrowly tuned to a small number of ligands (Keller et al. 2007). The results of this present study showed that 77% (14/18) of our receptors are broadly tuned and do not respond only to one special odourant or chemical component. The sense of smell allows us to perceive volatile chemicals present in our environment. The almost unlimited numbers of odourant molecules have to be accurately understood by the human nose. To this end, our sense of smell has to adapt to concept wide variety of odourants and chemical components by limited numbers of active ORs. We perceive odours through a combinatorial code involving less than 400 receptors. Then, as this study showed, many of our receptors should probably be broadly tuned and do not respond only to specific odourants. Charlier (2012) explained a "multimodal way of binding" as the main characteristic that makes some ORs broadly tuned. As Charlier (2012) explained, the plasticity of the binding cavity of OR allows different ligands to interact with various residues and odourant receptors could be adapted to different chemical structures. So, broadly tuned ORs make it possible to explain the perception of unlimited odourants by limited numbers of functional receptors.

It should be noted that ORs are presented as responsible for specific odourants (4/18) and could respond to more chemical components but because of the small number of odourants that we used in our study we were not able to show other possible odourants.

4.3 Scent and Chemistry

The structure-odour relationship is complicated and some description of odour like smell or quality of odour is usually unpredictable from its molecular structure (Rossiter 1996). It is suggested that an olfactory receptor detects a part of an odourant molecule, rather than the entire shape of a molecule (Araneda et al. 2000). Our data, in accordance with this view, shows that most odourants respond to one OR have the same functional groups. It seems that chemical functional groups and carbon atom numbers (CAN) are more important in the detection of odourants by an OR rather than the similarity of chemical structures. Also, it is known that functional groups roughly determine the specifications of odourants but it should be noted that only the presence of functional groups cannot explain the odour of molecules.

We classified our observation about the potential link between individual ORs and perceived odour characteristics into three different groups. First, ORs that only responded to one odourant: OR4X2, OR4C16, OR5I1 and OR10Q1 responded to 2-aminoacetophenone, aldehyde C18, Octanal and Pentadecalactone respectively. Second, ORs recognised odourants that share special chemical descriptors such as functional groups (Tab. 3.5) or odour quality like OR2L8 and OR4K5 that only responded to the amber odourant group or OR5L1 that responded to odourants with fruity properties. In our study, 64% (9/14) of ORs that responded to more than one odourant have similar functional groups or the same odour quality. Carbon atom numbers (CAN) are known to be an important odourant descriptor from a number of studies (Araneda et al. 2000; Haddad et al. 2008). 35% (5/14) of ORs that responded to more than one chemical component, responded to odourants with similar CAN. OR4E2 responded to odourants with CAN between 9 and 11; OR2L8 and OR4K5 responded only to odourants with CAN between 15 and 17. With reference to Figure 3.6, odourants with a low or high number of CAN (6<CAN<12) consist of a

low number of deorphanized odourants. It should be noted that most of the known odourants such as anosmia like musks, ambers or androstenone have high numbers of CAN. Third, ORs responded to odourants that do not share a common descriptor. OR52B4, OR4P4, OR4K2 and OR4C3 are located in this category. With the exception of OR12D2 we could not find any other OR that only responds to odourants with similar chemical shapes. But it should be noted that all ligands of OR12D2 belong to the acid carboxylic group and share the same functional groups (Fig. 4.2).

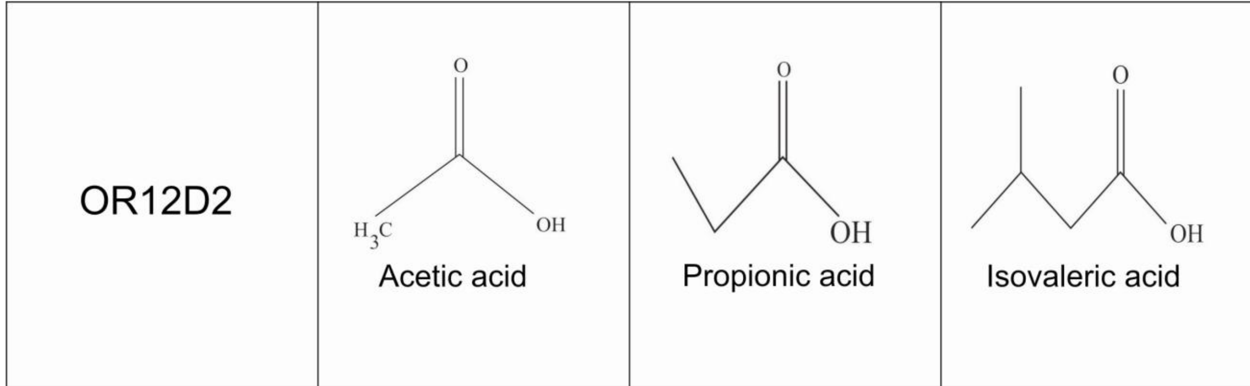


Figure 4.2: OR12D2 responded to structurally related odourants. Among all of the 18 deorphanized ORs just OR12D2 responded to structurally related odourants with similar functional groups.

In a parallel study we screened all of the ORs that responded to one of the musk odourants (OR4P4, OR4K2 and OR10Q1) with other similar odourants in the musk group (Fig. 4.3). But in this case we could not detect any significant relation between chemical structure similarities and responses of OR in the musk group.

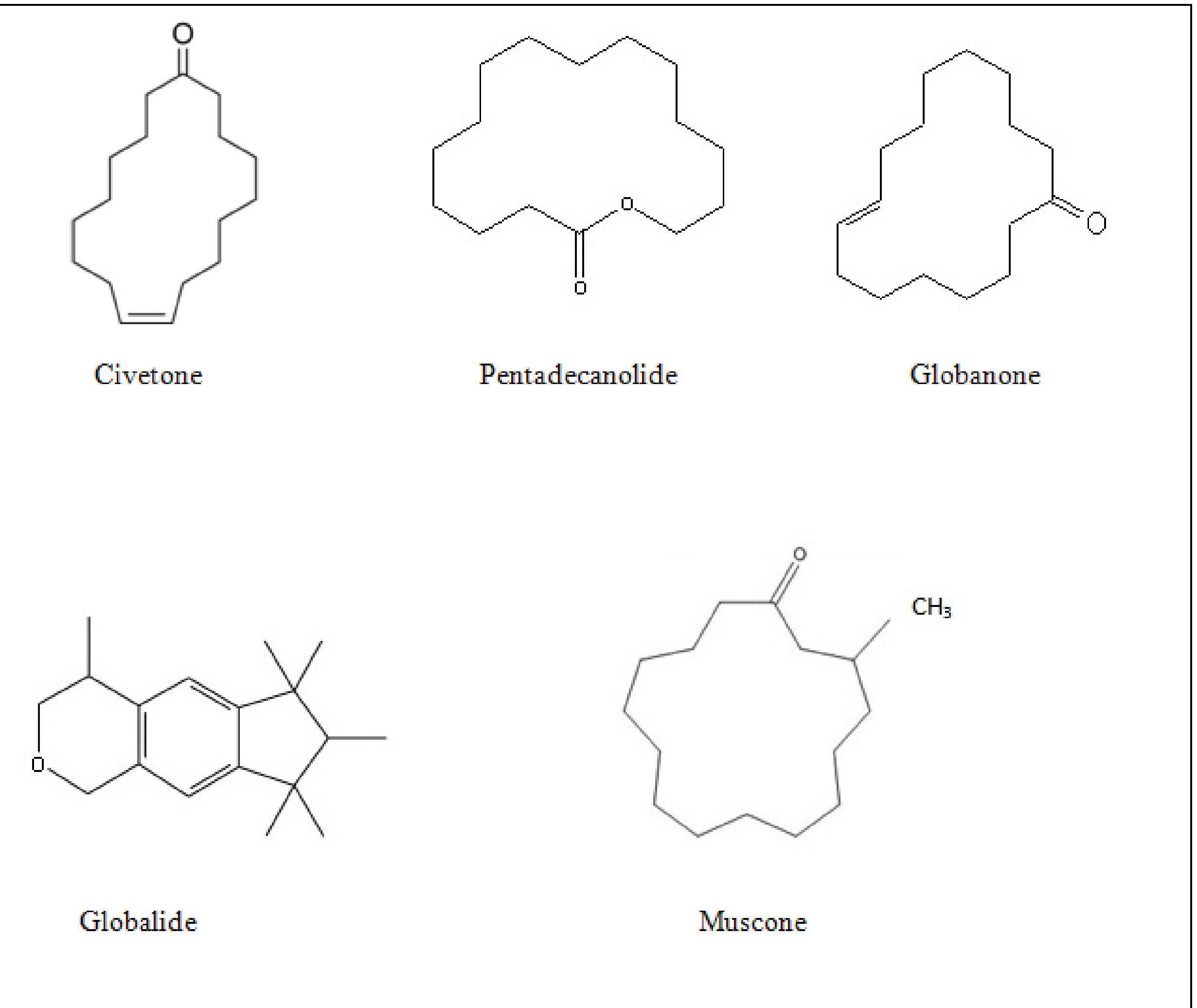


Figure 4.3: Structural formulae of the five musk odourants.

With regard to our results, although the third group that responded to unrelated odourants cannot be excluded, in most cases these findings are in agreement with the idea that individual ORs might have reacted and responded to particular odour descriptors like chemical groups, odour qualities (odourant perception as minty, fruity, woody or musky) or CAN in comparison to the entire shape of odourant chemical structures. Also, among the odourant descriptors, it seems that functional groups are more important than carbon atom numbers.

In our study we showed that one OR can recognise multiple odourants with related functional groups while one odourant is recognised by different receptors. For example, OR8B4 and OR8D2 are activated by Muguet alcohol, and alcohol C6 elicited a response in OR8D2 and OR10A6 (Tab. 3.4). Also, in comparison with previous studies, some deorphanized receptors have ligands in common with the present study. Aldehyde C6, alcohol C6, Graniol and Octanal are known as ligands for OR2W1; Graniol is also reported as a ligand for OR2M7 (Saito et al. 2009). OR51E2 and OR51L1 are known to be receptors for propionic acid and n-hexanoic acid, respectively (Saito et al. 2009). In addition, OR11H4, OR11H6 and OR11H7 were identified in a genetically based study as receptors for isovaleric acid (Menashe et al. 2007). Although in our study there were not many odourants that activate more than one OR but in combination with previous deorphanization results (Malnic et al. 1999) it is obvious that the relation between ORs and odourants is complex and that one odourant could activate with more than one receptor, according to the hypothesis known as “combinatory code”, which allows for the perception of unlimited odourants by different combination of ORs.

4.4 5α-androst-16-en-3one (urinous) and ω-pentadecalactone (musky) molecules

Previously 6 odours including sweaty, spermous, fishy, malty, urinous and musky were presented as primary odours by Amoore (1977). 5a-androst-16-en-3one and ω-pentadecalactone were presented as reference odourants for urinous and musky odourants respectively. These two odourants have molecular weights in the same general range and a similar orientation of functional groups and also have the same molecular thickness. But the androstenone molecule is longer and narrower, whereas the pentadecalactone is shorter and wider. It is suggested that these

differences is origin of differentiate between urinous and musky odourants in the human olfactory organ (Amoore 1976). In our experiments we found different receptors to be responsible ORs for androstenone and pentadecalactone. The separation of responding ORs for these two primary odourants could be part of a possible discriminatory mechanism.

Also, in a parallel study, we showed that OR1B1 (which deorphanized for androstenone) responded to testosterone as a steroid hormone from the androgen group. Androstenone is a steroid and a metabolite of testosterone. Testosterone and androstenone have similar general chemical structures with the same number of CAN (Fig. 4. 4). Interestingly, testosterone is secreted partly by adrenal glands. The HORDE data base shows the expression of OR1B1 in adrenal glands. Also, it has been shown that OR1B1 is expressed in prostate and brain tissues (Flegel et al. 2013).

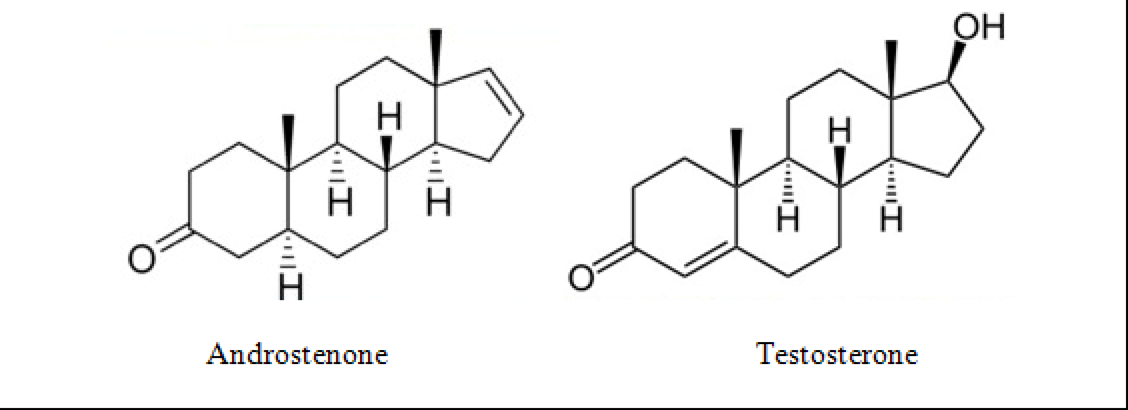


Figure 4.4: Androstenone and testosterone. Two steroidal chemical components with a similar general shape of chemical structure and CAN responded to the same OR.

Summary

Olfactory receptors (ORs) belong to heptahelical G-protein coupled receptors as the largest group of membrane receptors in the human genome and they have evolved to detect a wide range of chemical structures. The first step in odour transduction in the olfactory system is mediated by olfactory receptors. Perception of odourants shows diversity within different human populations. It has been proposed that widespread phenotypic diversity in human olfaction is, partly, related to genetic variation in OR genes. In some examples it has been demonstrated that variation in OR genes by single nucleotide polymorphism (SNP) is at least partially responsible for this kind of difference. Segregating pseudogenes (SPGs) are known as genes that due to a disruptive SNP segregate in populations between intact genes and pseudogenes. This divider mutation can introduce a stop codon, or alter a highly conserved amino acid that is important for the proper functioning of the protein.

In this study, to gain more insight into the relation between olfactory receptors and chemical components, we carried out a large-scale investigation of ligand-receptor interactions for the human olfactory receptors as copy number variations (CNVs) and SPGs by a broad range of odourants related to anosmia with functional analysis and site-directed mutagenesis.

In this study OR1B1, OR2L8, OR4X2, OR8D2 and OR8B4 were deorphanized as SPGs with minor allel frequency (MAF of SNPs that convert active genes to pseudogene) of 33%, 22%, 16%, 50% and 26% respectively. Our data indicate that OR1B1 was activated significantly by 3-hydroxy-2-methyl-4pyran, Calone, androstenone and testosterone. OR2L8 had statistically significant responses to Yasamber and Timbrol and OR4X2 showed significant response to 2-aminoacetophenone. OR8D2 responded to anisic aldehyde and aldehyde C6 and OR8B4 to alcohol C6 and Muguet alcohol.

OR1B1 as SPGs display both functional and nonfunctional alleles in humans which make it a suitable candidate to explain variation of androstenone perception in human populations. Androstenone is a steroid and a metabolite of testosterone. Testosterone and androstenone have a similar general chemical structure with the same number of CAN. The relation between SNPs and odourant responses were studied by point mutations. OR1B1-574 as a mutation form of OR1B1 that converts the active gene of OR1B1 into a pseudogene, as was expected severely impairs OR1B1 function. OR10Q1 as one OR in the CNV group responds to ω-cyclopentadecalactone as an odourant in the musk group. The mutation form of OR10Q1 was produced according to unique single nucleotide variation with MAF> 10%. We investigated the ligand sensitivity of OR10Q1-540 (mutated variant) and OR10Q1 (wild type) receptor variants *in vitro* with Pentadecanolide as a suitable ligand for OR10Q1. The mutated variant did not show any different significant response in comparison with OR10Q1-WT.

We deorphanized 18 ORs including SPGs and CNVs and showed that there is no simple, direct relation between molecular features and the ability to respond to receptors. In our study, three groups were classified by OR activation: first, those ORs that only responded to one odourant;, second, ORs that responded to more than one odourant according to special odourant descriptors; and third, ORs that responded to more than one odourant but did not follow any special rules.

It was hypothesised that similar functional residues between TM3 and TM6 may recognise the same or similar odourant ligands. So, we made multiple sequence alignments between all of the TM3-6 in deorphanized olfactory receptors. However, for some of the ligands, we found that the same odour activates closely related receptors from the same clade but for others, receptors from different clades are activated. It can be stated that by comparing the amino acid sequences of OR, no accurate prediction about OR response to odourants can be performed.

Although segregating pseudogenes seems to be a promising factor for genetic diversity in olfactory receptors, we could not show the role of individual SPGs in the variation of odourant perceptions, especially when similar odourants could activate more than one OR. The lack of one of them probably cannot interrupt the procedure of odourant perception. Also, according to our results, in addition to SPGs, CNVs responded to odourants related to specific anosmia. For instance, musk odourants only responded to ORs in the CNV group.

Zusammenfassung

Olfaktorische Rezeptoren (ORs) sind heptahelicale G-Protein gekoppelte Rezeptoren und bilden im menschlichen Genom die größte Gruppe von Plasma Membran Rezeptoren. Der erste Schritt zur Wahrnehmung von Duftstoffen wird von ORs vermittelt. Dabei ist zu beachten, dass beim Menschen jeder Duftstoff unterschiedlich bewertet werden kann. ORs können eine große Breite von chemischen Strukturen wahrnehmen. Es wird angenommen, dass die phänotypische Diversität in der Wahrnehmung von Duftstoffen mit den genetischen Variationsmöglichkeiten der OR-Gene zusammenhängt. So konnte für einige ORs gezeigt werden, dass schon eine kleine Änderung der OR-Genstruktur durch einen Einzelnukleotid-Polymorphismus (SNPs) mitverantwortlich für eine andersartige Wahrnehmung von Duftstoffen ist. Segregierende Pseudogene (SPGs) sind Gene, die funktional oder auch nicht funktional sein können. Eine Solche Mutation kann durch ein Stop-Codon oder eine Veränderung einer hoch konserviertes Aminos, welche für die Funktionalität des Proteins verantwortlich ist, bedingt sein.

Um mehr über den Zusammenhang zwischen ORs und deren chemischen Liganden zu verstehen, haben wir in dieser Studie Liganden-Rezeptor Interaktionen für humane ORs untersucht, von denen Kopienzahlvariation (CNVs) oder SPGs bekannt sind. Dabei werden Liganden benutzt, von denen bekannt ist, dass sie von einigen Menschen nicht wahrgenommen werden können.

In der Arbeit wurden die ORs OR1B1, OR2L8, OR4X2, OR2D2 und OR8B4 als SPGs mit einer Minor Allele Frequency von 33%, 22%, 16%, 50% und 26% deorphanisiert (MAF der SNPs, welche intakte Gene zu pseudogenen konvertieren). Unsere Daten zeigen, dass OR1B1 signifikant durch 3-Hydroxy-2-Methyl-4-Pyran, Calon, Androstenon, und Testosteron aktiviert werden kann. OR2L8 hingegen wurde durch Yasamber und Timbrol aktiviert, und OR4X2 signifikant durch 2-Aminoacetophenon. OR8D2 konnte durch Anis-Aldehyd und Aldehyd-C6

aktiviert werden. OR8B4 wurde durch Alkohol-C6 und Muguet-Alkohol aktiviert. OR1B1 als SPG, veranschaulicht beides, die Funktion und die Dysfunktion von Allelen im Menschen, welche im Zusammenhang mit der Wahrnehmung von Androstenon stehen. Androstenon ist ein Steroid und eine Metabolit von Testosteron. Testosteron und Androstenon haben die gleichen molekularen Grundstrukturen mit der gleichen Anzahl an Kohlenstoffatomen. Der Zusammenhang zwischen SNPs und Duftstoffwahrnehmung wurde bezüglich Punktmutationen analysiert. OR1B1-574 ist eine Mutation von OR1B1, welche das aktive Gen zu einem Pseudogen konvertiert, da dieser Abschnitt funktional von Bedeutung für OR1B1 ist. OR10Q1, ein OR aus der CNV Gruppe, antwortet auf Omega-Cyclopentadecalacton, ein Duftstoff aus der Moschus Gruppe. Die Mutation in OR10Q1 beruhte auf einer SNP Variante, deren MAF 10% betrug. Wir haben die Liganden-Sensitivität für OR10Q1-540 (Mutante) und OR10Q1 (Wild Typ) *in vitro* mit Pentadecanolid als möglichen Liganden identifizieren können. Die mutierte Variante zeigte keine signifikanten Antworten im Vergleich zum Wild Typ.

Es wurden 18 ORs deorphanisiert, die SPGs und CNVs aufwiesen. Dabei zeigte sich, dass es keinen einfachen oder direkten Zusammenhang zwischen den molekularen Strukturen und aktiven Rezeptoren gibt. In unserer Studie haben wir drei Gruppen von ORs zusammengestellt: Die erste Gruppe umfasst ORs welche nur auf einen Liganden geantwortet haben. Die zweite Gruppe von ORs hat auf mehrere Duftstoffe abhängig von der molekularen Struktur geantwortet. Die dritte Gruppe hat auf mehrere Duftstoffe geantwortet, unabhängig von der jeweiligen Struktur.

Es wird angenommen, dass transmembranäre Abschnitte von TM3 bis TM6 an der Interaktion beteiligt sind. Aus diesem Grunde haben wir die Sequenzen der TM3-6 in ORs vergleichen. Wir konnten zeigen, dass einige Duftstoffe weitere ORs aktivieren, welche eine sehr ähnliche

Struktur zum bereits deorphanisierten Rezeptor aufweisen. Allerdings konnten wir auch zeigen, dass Duftstoffe, die einen bestimmten OR aktivieren, auch nicht Struktur- verwandte ORs aktiveren konnten. Nur mit einem Protein-Sequenz Vergleich deorphanisierter ORs ist es deshalb nicht möglich weitere Liganden für orphane ORs zu finden.

SPGs scheinen eine vielversprechende Möglichkeit zu sein, genetische Diversitäten in ORs zu untersuchen, jedoch konnten wir hier keine SPG spezifische Rolle identifizieren. Übereinstimmend mit unseren bisherigen Ergebnissen, antworten auch CNVs auf Duftstoffe, welche mit bekannten Anosmien im Zusammenhang stehen. So zum Beispiel aktivieren Duftstoffe aus der Moschus Gruppe nur ORs aus der CNV Gruppe.

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Abbreviations

AC	adenylyl cyclase
ATP	adenosine-5'-triphosphate
cAMP	cyclic adenosine monophosphate
CAN	carbon atom number
CNG	cyclic nucleotide-gated channel
CNV	copy number variation
DGV	database genomic variants
DLR	dual-luciferase reporter
DMSO	dimethyl sulfoxide
Fig	figure
GEF	guanine nucleotide exchange factors
GPCR	G-protein-coupled receptor
HEK	human embryonic kidney cells
HGNC	human gene nomenclature committee
HORDE	human olfactory data explorer
InsP	inositol 1, 4, 5-trisphosphate
MAF	minor allele frequency
OB	olfactory bulb
OE	olfactory epithelium
OEP	overlap extension PCR
OR	olfactory receptors

OSN	olfactory sensory neuron
PLAP	placental alkaline phosphatase
PLC b2	phospholipase C beta-2
REEP	receptor expression enhancing protein
Rho	rhodopsin
RTP	receptor transporting protein
SEAP	secreted embryonic alkaline phosphatase
SEM	standard error mean
SNP	single nucleotide polymorphism
SPG	segregating pseudogenes
Tab	table
Tm	temperature
TM	trans membrane
TR	taste receptors
TRPC6	transient receptor potential channel 6
VR	vomeronasal receptors
WT	wild type

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Curriculum Vitae

Personal information

Name: Kaveh Ashti Baghaei

Home address: Semper st 113, 44801

Bochum-Germany

Telephone: +491729174532

E-mail: kaveh.ashtibaghaei@RUB.de

Date of Birth: 7 Dec. 1982

Place of Birth: Tehran

Nationality: Iran

Academic History

2009/ 2013: PhD project at the department of Cell Physiology

under supervision of Prof. Dr. Dr. Dr. Hatt

Title: Large scale analysis of olfactory receptors

with highly genetically variations in relation with specific anosmia.

2010/2011 Scholarship for Teaching Assistantship from DAAD

2009/2013 Member of International Graduate School of Biosciences

Member of the RuhrUniversität Bochum Research School

2008/ 2005:	Master of Science in Cellular and Molecular -Microbiology, Azad University- Karaj, Iran. Thesis title: "Isolation, Cloning and sequencing of Helicobacter pylori cagA gene in Escherichia coli".
2006/ 2009	Research assistant in Research Center for Gastroenterology and Liver Diseases, Shahidbeheshti University,Tehran, Iran.
2005/ 2001:	Bachelor of Science in Cellular and Molecular Biology, Azad University- Tehran, Iran.

School Training

1996-2000	High school at Jalal Al Ahmad Tehran/ Iran
1994-1996	Guidance school at Emam Tehran/ Iran
1989-1994	Elementary school at shajari Tehran/ Iran

Acknowledgements

First of all, I would like to thank my supervisor Prof. Dr. Dr. Dr. Hanns Hatt for giving me the opportunity to work on this project. I am grateful for his scientific and personal support and guidance.

My appreciation also goes to my second supervisor Prof. Dr. Stefan Wiese for supervising my doctoral work.

Special thanks go to my direct supervisor Dr. Gunter Gisselmann for his scientific support and for being a mentor during my study.

For their technical assistance, I am grateful to Farideh Salami, Jasmin Gerkrath, Franziska Mossler, Andrea Stoeck, Thomas Lichtleitner, and Ute Muller. For administrative support, I am very thankful to Ulrike Thomes.

Thanks go to the ‘Room 132’: Caroline Flegel, Desiree Massberg, Leopoldo Beltrán, Stavros Manteniotis, Dominik Holatz and Jonas Kuklan for their friendship and support, fruitful discussions and ideas for problem solving. We had a lot of fun!

My appreciation also goes to Truong Thi Hong Ha, Atefeh Rozbahani Mohammadi and Zoha Zaker for their collaboration as master students.

To the Department of Cell Physiology as a whole, for all the helpful discussions, and warm working atmosphere I am extremely grateful to all those involved.

My best and big thanks go to my parents Farzaneh and Mohammad and my lovely brother Dr. Maziar for all they did, they are doing and they will do.

Last, but not least, to Anahid for her love that brings me always happiness and tranquility regardless to conditions. I wholeheartedly thank you “und einfach ich liebe dich”.

This work was financially supported by the DFG and the Ruhr-University Research School.