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Molecular biology of olfactory receptors

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Overview

In order to elicit an olfactory response, a substance has to partition into the gas phase and diffuse into the nose. Such odorant molecules, usually low-molecular-mass hydrophobic compounds, encounter the ciliated endings of sensory neuronal dendrites, which protrude into a mucus layer at the surface of the olfactory epithelium in the nasal cavity. Embedded in the membranes of such cilia are olfactory receptor (OR) proteins, which recognize odorants and elicit a transduction cascade that underlies the nerve cell response. The sensory axons project to the olfactory bulb in the brain, where they converge into synaptic structures called glomeruli. The specific convergence patterns of olfactory axons, which depend on OR expression, provide a model system for neuronal network development. Here, initial processing of odour information occurs, which is followed by additional analysis in higher olfactory brain centres.

Chemical detection in a probabilistic receptor repertoire

Most biological recognition systems have evolved towards an optimized specificity for endogenous ligands, such as hormones, neurotransmitters and enzyme substrates. Perhaps the most peculiar aspect of the olfactory system is that it has to bind, and uniquely recognize, a vast array of ligands, most of which are xenobiotic in origin. By analogy with the immune system, it was proposed that the olfactory pathway must have evolved a large repertoire of potential ORs,

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most of which may have arisen without *a priori* specificity towards particular odorants [1]. A probabilistic receptor affinity distribution model has been developed, which relates the number of different receptor types required to ensure binding of any odorant with a minimal physiologically significant affinity [2]. This allowed an early prediction that the olfactory repertoire in mammalian species contains approx. 1000 different proteins [1], in good agreement with later experimental estimates [3].

Prior to the discovery of the first ORs, it had been established by electrophysiological recordings that each olfactory sensory neuron may respond to a wide range of odorant chemicals [4]. This raised two alternative explanations: that each neuron expresses a number of different, narrowly tuned ORs; or that a neuron expresses only one OR type, but with an unusually broad specificity. It is currently believed that the latter situation prevails, i.e. that OR expression is 'clonally excluded' (see below). With the number of possible odorants far exceeding that of OR types, it is obvious that each sensory neuron contributes to the recognition of numerous chemical substances. However, each odorant is unequivocally identified through its eliciting a unique 'across-neuron' pattern, as demonstrated clearly when numerous single-unit recordings are analysed [4].

ORs belong to the G-protein-coupled receptor (GPCR) hyperfamily

The earlier demonstration that odorant responses are mediated by GTP-binding proteins and adenylate cyclase activation raised the hypothesis that ORs might belong to the GPCR hyperfamily of proteins [5], all of which share seven hydrophobic transmembrane helices. Such receptors have been implicated in numerous cellular transduction processes, including photoreception, as well as hormone and neurotransmitter binding [6]. Accordingly, the first cloning of candidate OR genes from rat olfactory epithelium was accomplished through the use of degenerate GPCR-conserved sequences as PCR primers [3]. This was then followed by the cloning of such genes in several species, including human [7,8], mouse [9], dog [7] and fish [10]. OR genes from all vertebrate species constitute a superfamily whose members share at least 25% amino acid sequence identity [11].

Analysis of numerous OR sequences from a variety of species clearly confirms their identity as GPCRs (Figure 1a). On the other hand, several features appear to be unique to the OR protein sequences. The most prominent hallmarks are the conserved regions in the second and third intracellular loops (Y. Pilpel and D. Lancet, unpublished work; Figure 1a). These may be implicated as molecular interfaces with the olfactory G-protein.

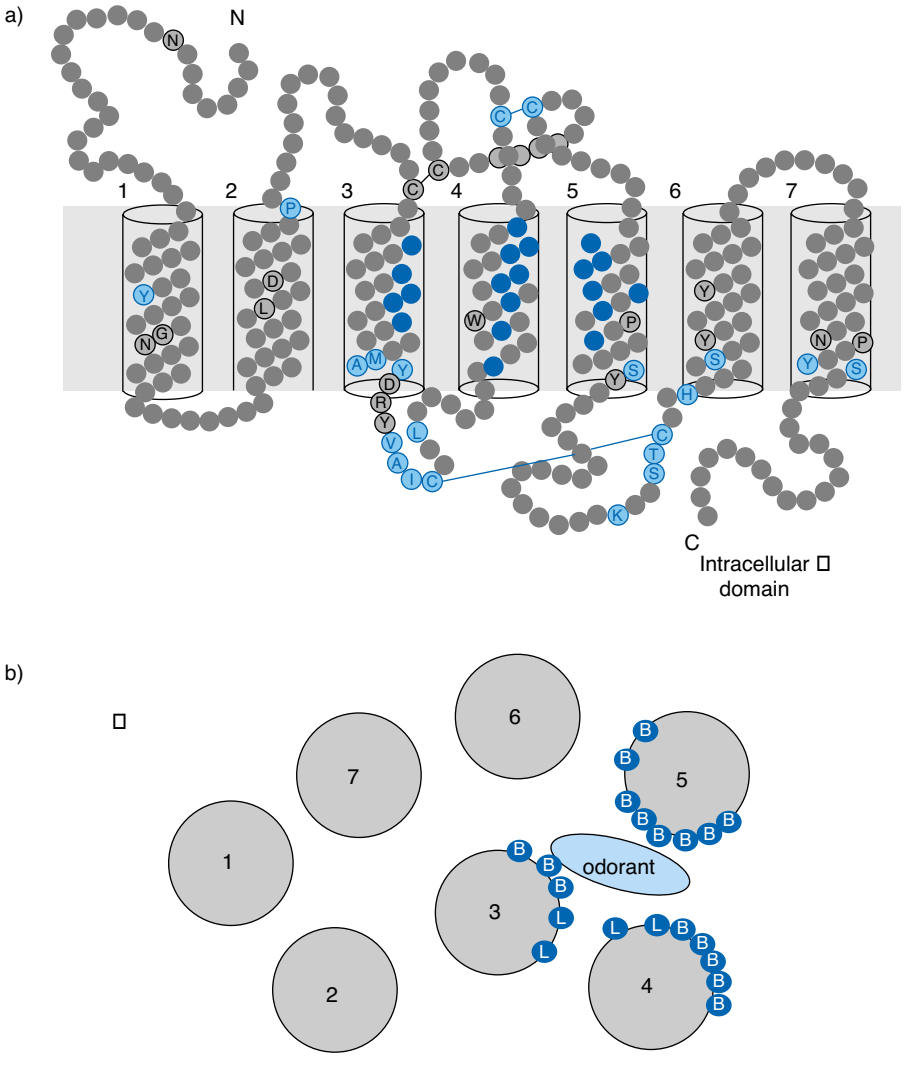

Figure 1. (a) Schematic two-dimensional diagram of the OR protein, and (b) cross-sectional representation of the OR protein in the cell membrane (extracellular view) (a) The 20 variable residues that constitute the putative complementarity-determining region on transmembrane helices 3, 4, and 5 are shown as solid blue circles. These residues are mainly clustered along one side of each of the three variable helices. Highly conserved residues, marked with the single-letter amino acid code, are either conserved in all GPCRs (circled in blue) or unique to ORs (circled in black). These include the conserved glycosylation site at an asparagine residue near the N-terminus (marked by N) and the DRY motif at the end of the third transmembrane helix. Putative disulphide bridges are indicated with a black line (for those unique to ORs [12]) or a blue line (conserved in all GPCRs). (b) Looking down on the transmembrane helices (1–7), the residues comprising the putative complementarity-determining region are shown as dark blue circles. Residues with hydrophobic and hydrophilic side chains are marked B and L respectively. A schematic odorant is shown in the putative binding site.

Odorant complementarity-determining regions

A wealth of pharmacological and site-directed mutagenesis experiments on several GPCRs established that the binding of low-molecular-mass ligands takes place in the plane of the membrane, within the barrel enclosed by the seven helices [6]. While no analogous experimental information is yet available for OR proteins, a computational approach has been developed for these proteins, based on the availability of numerous homologous sequences. Initial analyses indicated that transmembrane helices 3, 4 and 5 harbour most of the variability [3] (Figure 1a), and it was suggested that they take part in odorant binding, subserving a function similar to the immunoglobulin hypervariable domains [5]. However, it remained unknown whether the OR variable residues are clustered in space. In a recent study [12], analysis of hundreds of OR sequences, along with molecular modelling of the receptor structure, revealed a unique pattern of diversity, whereby most of the variable amino acid residues are clustered in a specific region in the interior of the seven-helix barrel (Figure 1b). The set of 20 interior-facing variable residues thus defined was proposed to serve as the complementarity-determining region for odour recognition (Figure 1b).

Evolution of the OR repertoire

In mammalian species, the OR gene superfamily is estimated to consist of several hundred genes [3], with a relatively large percentage of pseudogenes, especially in the human [13]. This gene repertoire, which constitutes the 'olfactory sub-genome' [11], is estimated to encompass ~1% of the entire genome of mammalian species. The open reading frames are found in several chromosomes, arranged in clusters of 10 or more members [8]. Unlike the somatic gene recombination and mutation mechanisms that account for immune diversity, OR repertoire diversity seems to be germ-line inherited, since the OR coding regions are uninterrupted in the genome [3,14]. The OR repertoire, similar to that of other multigene families, is likely to have arisen by a long evolutionary process of gene duplication, followed by germ-line mutations. This process of repertoire expansion is likely to have been driven by the need to enhance the chances of a successful probabilistic odorant binding.

The biochemical cascade in olfactory signalling

While odorant binding to an OR may be considered as an input 'written' in a chemical language, a corresponding output must be generated which translates the information into electrophysiological signals. The relevant signal transduction cascade has been shown to include a stimulatory GTP-binding protein, adenylate cyclase and a cAMP-gated cation channel [15] (Figure 2). Olfactory transduction manifests an aspect of universality by involving the same primary components as many other neural systems with seven-helix receptors, includ-

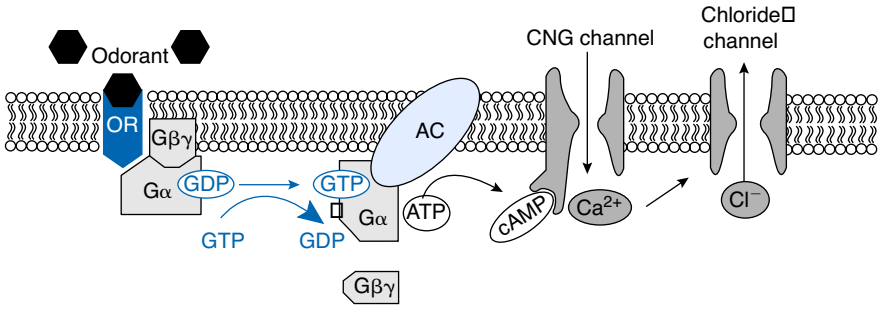

Figure 2. Odorant-dependent signal transduction pathway
Following the binding of an odorant to its receptor (OR), the olfactory G-protein exchanges GDP for GTP. Concomitantly the α subunit (G_α) dissociates from the βγ subunits ($G_{\beta\gamma}$) and activates adenylate cyclase type III (AC), which generates cAMP from ATP. cAMP opens the cyclic-nucleotide-gated (CNG) cation channel for passage of Ca^{2+} ions, resulting in cell depolarization and the generation of an action potential. The current is amplified by activation of a Ca^{2+}-dependent chloride channel.

ing neurotransmitter and visual receptors [16]. On the other hand, it seems to share only with the visual system the involvement of a direct gating of the ion channel by the cyclic nucleotide second messenger [17]. While the overall features of the signal transduction pathway are identical to those of other signalling systems, the specific components are olfactory-unique and highly tissue- and cilia-enriched. The cloned olfactory specific transduction proteins are G_{olf} (a G_s-like protein), adenylate cyclase III and the cAMP-gated channel.

The central role of cAMP and G-proteins in the olfactory pathway was substantiated by several independent approaches. A strong positive correlation exists between the magnitude of adenylate cyclase stimulation and the summated electrophysiological responses [18]. In addition, both cAMP derivatives and guanine nucleotides modulate or mimic odorant responses in epithelial sensory neurons [19] and in cultured olfactory cells [20]. Perhaps the most clear-cut evidence for the primary significance of the cAMP pathway to mammalian olfactory reception has been obtained recently with knock-out mice lacking either the functional cyclic-nucleotide channel [21] or the olfactory G-protein (G_{olf}) [22]. Both mutant strains exhibited a dramatic decrease in the primary electrophysiological response to all odorants tested, and revealed behavioural phenotypes consistent with general anosmia.

The multi-step olfactory transduction cascade has been implicated in signal amplification. Odorant receptors are rather broadly specific, and may typically have rather low ligand affinities. However, when coupled to a system where a single activated receptor may catalyse the generation of thousands of second-messenger molecules, an ultra-high sensitivity may materialize [1,5]. In this, an analogy may be drawn with other sensory pathways, including vision and some taste pathways [23].

An alternative olfactory transduction pathway has been proposed, involving the second messenger inositol 1,4,5-trisphosphate ($Ins P_3$). The evidence was based on rapid kinetic measurements in isolated olfactory cilia [24] and OR-expressing cells [25], showing $Ins P_3$-mediated responses to certain classes of odorant. However, the 'odorant pharmacology' experiments [18], and most convincingly the gene inactivation experiments [21,22], demonstrated that cAMP is very likely to also mediate the responses to the $Ins P_3$-related odorants. It may therefore be concluded that cAMP is a necessary second messenger for all or most odorants in mammals.

Expressed OR proteins and their ligand specificity

For years OR genes constituted 'semi-orphan' receptors: as a group, they were assigned a very likely function in odorant recognition, yet a detailed assignment of odorant specificity to individual OR genes remained largely lacking. This has begun to change rapidly, starting with the first identification of a functional odorant receptor, coded by the *odr-10* gene, in the nematode *Caenorhabditis elegans*, identified in a behavioural mutant incapable of detecting the odorant diacetyl [26]. Odr-10 functionality was further corroborated in a human expression system, where the receptor was shown to activate Ca^{2+} release in response to diacetyl [27].

Mammalian OR gene expression was first described in a baculovirus system [25,28], including the identification and purification of the encoded 30 kDa OR polypeptide. Following the earlier observation of odorant responses in such OR-expressing insect cells [25], the expressed protein was reconstituted in lipid vesicles and binding of similar odorants was observed by photoaffinity labelling [29]. Subsequently, three zebrafish odorant receptors were expressed in a human cell line, resulting in a transient increase in intracellular Ca^{2+} in response to odorants in fish-food extracts [30].

More recently, Zhao et al. [31] used rat nasal epithelium as an *in vivo* OR expression system, employing recombinant adenovirus infection. Electrophysiological recordings showed that, due to the expression of the rat receptor *I7* gene by an increased number of olfactory neurons, a 4–7-fold enhancement was effected in the responses to the odorant octyl aldehyde (octanal), as well as to several structurally related compounds. A carefully documented dose–response curve lends credence to the notion that OR gene expression has been achieved.

Another strategy employed for the identification of functional ORs was mutation analysis and genetic linkage. In a study of two inbred mouse strains differing in their sensitivity to isovaleric acid, genetic linkage was established between odorant sensitivity and markers on chromosomes 4 and 6. It was concluded that the most likely cause of the inability to sense isovaleric acid is the loss of receptor protein(s) residing in the vicinity of these markers. A similar linkage analysis has been conducted in humans, resulting in a tentative assign-

ment of odorant sensitivity traits to distinct OR clusters in the human genome (S. Horn-Saban and D. Lancet, unpublished work).

Patterns of olfactory receptor expression and their transcriptional regulation

The patterns of expression of different odorant receptor genes within the olfactory epithelium of rodents have been examined in a series of *in situ* hybridization experiments [32,33]. A given OR probe was found to identify about 0.1% of the sensory neurons. Given an estimate of 500–1000 OR genes in the rat genome [3], these findings are consistent with the hypothesis of OR clonal exclusion, i.e. each neuron expresses a single receptor gene [34]. Sensory neurons expressing the same receptor or receptor subset appear to be topologically segregated into one of four broad zones extending along the anterior–posterior axis of the nasal cavity (Figure 3). These zones exhibit bilateral symmetry in the two nasal cavities. Within a given zone, however, olfactory neurons expressing a given receptor appear to be distributed randomly rather than spatially localized. Moreover, RNA from cells heterozygous for an OR gene hybridizes with only one of the allele probes [35], suggesting that only one OR allele can be activated in a given neuron. In summary, since each neuron may express only one OR protein from a single allele, the task of odour-quality perception may be reduced to detecting which subset of sensory neurons has been activated [1,33,34].

The functional significance of clonal exclusion was demonstrated recently in an elegant analysis of olfactory-dependent chemotaxis in *C. elegans* [36]. It was shown that nematodes that transgenically expressed the chemoattractive diacetyl receptor Odr-10 in neurons that normally mediate odorant-induced repulsion were repelled by diacetyl. It was thus concluded that the cellular context of the activated receptor, and not the receptor itself, determines the final odour-quality perception and the subsequent behavioural response.

What is the mechanism that controls this complex pattern of odorant receptor expression? A recent study, using a transgenic animal approach, has shown that a 6.7 kb region upstream of the mouse M4 OR coding region is sufficient to direct several aspects of OR-regulated expression [37]. This includes the specificity to olfactory epithelial tissue, the restricted expression in only one of the epithelial zones, and excluded expression in a small randomly disposed subset of the cells within a zone. This is in line with reports of a 4–6 kb 5′ intron that is immediately preceded by a non-coding exon and a putative upstream gene-control region [14,37]. However, the transgenic 6.7 kb upstream region was not sufficient to dictate expression in a specific zone in which a given OR is known to be expressed naturally, and did not include the potential feedback loop that prevents individual sensory neurons from expressing more than one OR gene. Such specific orchestration is likely to arise from a more complex, hierarchical series of regulated transcriptional controls.

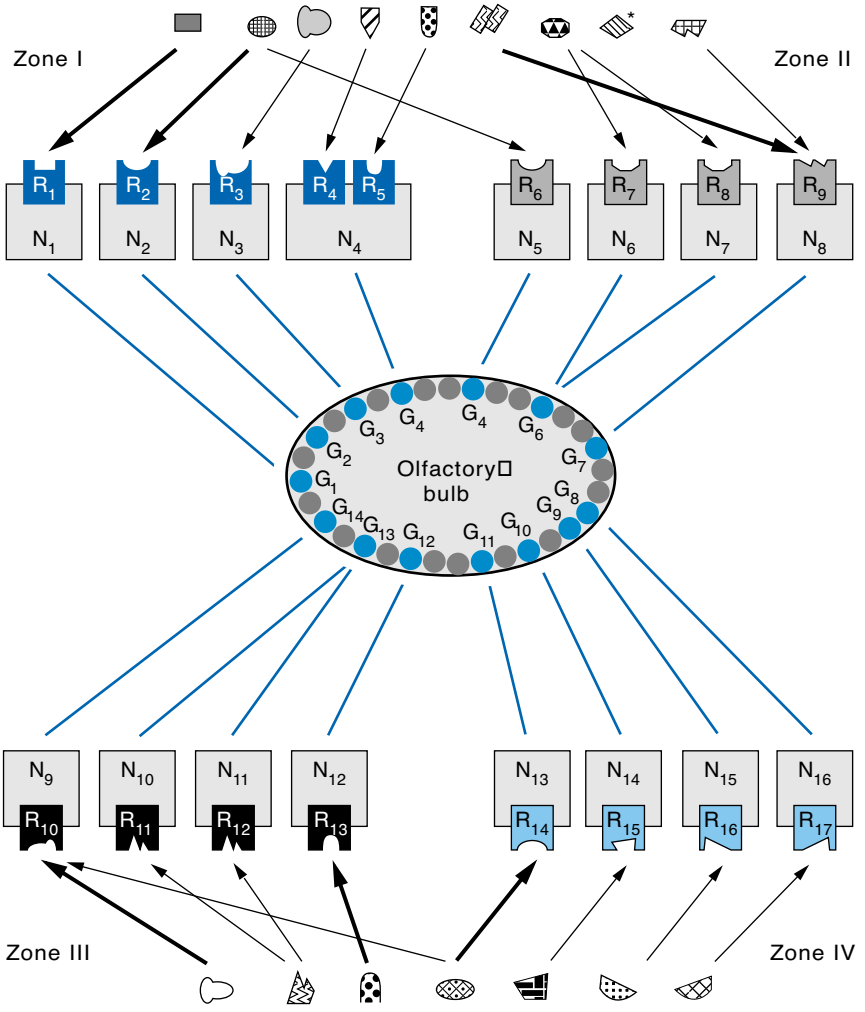

Figure 3. Schematic view of neuronal odour recognition and coding apparatus
OR-expressing neurons are designated by N_i. Each neuron may express one type of OR (in multiple copies), or perhaps very few (e.g. N_4). However, the same OR is expressed by several neurons, scattered randomly in one of four zones of the olfactory epithelium (all ORs expressed at a given zone are shown in the same colour). An array of odour molecules may bind the ORs with different affinities (shown by the size of the arrows) (e.g. R_7 and R_8); conversely, the same odour molecule can bind to different receptors with different affinities (R_2 and R_5). An odorant with no OR binder (*) may not be detected by this system. Neurons expressing identical ORs (N_6 and N_7) project axons to the same glomerulus (G_6) of the olfactory bulb.

An attractive model [32,35,37] is that, during development, spatially restricted transcription factors activate a subpopulation of genes within an active chromosomal OR cluster, so as to define the repertoire of receptor genes that can be expressed within a topological zone. These factors could bind to distant regions (e.g. enhancers), functioning perhaps in co-operation with the

elements located within the proximal promoter. Subsequent selection of the receptor(s) to be expressed in a single olfactory neuron could result from competition for *trans*-acting factors or relief from negative regulation mediated by DNA methylation or inhibitory factors. Finally, a feedback mechanism could serve to restrict expression to a single functional receptor in each neuron [3].

Olfactory bulb glomeruli represent ORs

The OR-expressing neurons project their axons to the olfactory bulb, where they converge on to synaptic complexes termed glomeruli (Figure 3). Each of the roughly 1000 glomeruli receives input from about 10000 sensory neurons. Early analyses of this convergence pattern led to the idea that glomeruli serve as 'functional addresses' [38] and, more specifically, that each glomerulus subserves all the neurons that express a particular OR [34]. This was later confirmed experimentally by showing that all olfactory neurons in the mouse expressing a given OR gene project axons to only two topographically fixed glomeruli in each of the two olfactory bulbs [39]. Further experiments, in which one OR coding region was swapped with another, led to axonal convergence on to a glomerular target different from that of both receptors. It was then concluded that the OR coding region itself carries necessary, but probably not sufficient, information required for axonal guidance. Furthermore, sequence analysis of several ORs identified a specific region of the OR molecule, on the second extracellular loop, that may be responsible for such axonal guidance [40].

What is the logic behind this situation? It appears that an olfactory neuron has to make two major decisions during its lifetime, namely which OR to express and into which glomerulus to project its axon. If the choices are coordinated, a link is formed between ligand specificity and neuronal processing. Observations obtained from 2-deoxyglucose measurements [38] and single-unit recording of neurons from the olfactory bulb [41] support this notion. It was shown that glomerular cells at given bulb loci respond to odorants with well-defined chemical sub-modalities, size and structure, potentially recognized by the same, or similar, ORs. The relationship between OR function and central nervous system addressing allows thousands of similar neurons to converge upon a restricted subset of secondary neuronal targets. This allows signal averaging and noise reduction, as well as the formation of a functionally consistent map in the brain [38].

Summary

- *OR proteins bind odorant ligands and transmit a G-protein-mediated intracellular signal, resulting in generation of an action potential.*
- *The accumulation of DNA sequences of hundreds of OR genes provides an opportunity to predict features related to their structure, function and evolutionary diversification.*
- *The OR repertoire has evolved a variable ligand-binding site that ascertains recognition of multiple odorants, coupled to constant regions that mediate the cAMP-mediated signal transduction.*
- *The cellular second messenger underlies the responses to diverse odorants through the direct gating of olfactory-specific cation channels.*
- *This situation necessitates a mechanism of cellular exclusion, whereby each sensory neuron expresses only one receptor type, which in turn influences axonal projections.*
- *A 'synaptic image' of the OR repertoire thus encodes the detected odorant in the central nervous system.*

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