REVIEW

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What we don't know about olfaction*

Part 1: from nostril to receptor

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SUMMARY

The sense of smell is still mysterious in many ways, despite the advances of the past few years. This review gives a broad overview of the state of the field by examining each step of the process of signal transduction from odorant to brain. Each subject section was searched individually in Pubmed, ISI Web of Science and Google Scholar as well as materials known to the author. The results are sequentially presented in order of anatomical progression of the signal. The review finds many surprising and interesting theories, facts and methods worthy of further research.

Keywords: smell, olfactory perception, olfactory receptor, neuron olfactory mucosa, olfactory tract, odorant receptor, olfaction disorders

INTRODUCTION

To begin to study the human sense of smell is to realise just how much we still don't know about some very basic processes of life. Without dismissing the advances of researchers in this field thus far, how is it that in this day and age we still do not understand the structure of the olfactory receptor? Without this and hundreds of other facts we are operating with one hemisphere tied behind our backs.

To understand a process is to have a working, predictive, model of it; but as the statistician George Box said, "all models are wrong, but some are useful" [(1)]. I attempt here to present the results of a review of the literature on olfaction science in a clear and systematic manner, to codify the areas where there is need for more work and those where our models are useful enough.

This review concentrates mainly on the molecular basis of the sense of smell, although the current understanding of the olfactory bulb is sketched, space considerations force this to be an overview at best.

METHODS

A systematic review of the published literature in olfaction was undertaken. The search strategy is included in appendix A. Additional information was obtained from papers personally known to the author and from publications cited by the above.

OVERVIEW

From the point of view of a molecule of, say, Chanel N°5 as it enters the nasal cavity the odorant has to traverse the cavity to the olfactory area in the olfactory niche and surrounding structures. The small, volatile, hydrophobic molecule [(2)] must dissolve into the overlying mucus and perhaps bind with a class of general-purpose binding molecules known as **Odorant Binding Proteins** (OBP). This complex moves through the mucus layer to the cilia of the **Olfactory Sensory Neuron** (OSN) where the odorant will interact with one of a range of 200 - 350 or so expressed **Olfactory Receptors** (OR). Each OR is linked to a G-protein which when activated causes an increase in intracellular cAMP and therefore activation of the transmembrane cAMP activated Cationic channel **CNG** with depolarisation as the end result. Each of these OSNs pass axons through the cribriform plate to one of two **Glomeruli** specific to that receptor in the Olfactory bulb. There is some further processing before second and third order neurons pass back along the olfactory tract to their relative lateral (primary), intermediate and medial (secondary) olfactory areas of the **rhinencephalon**.

GLOSSARY AND LIST OF ABBREVIATIONS

CNG = cAMP activated cationic channel; Homolog = A gene related to a second gene by descent from a common ancestral DNA sequence. The term, homolog, may apply to the relationship between genes separated by the event of speciation (see ortholog) or to the relationship betwen genes separated by the event of genetic duplication (see paralog); OBP = Odorant binding Protein; Ortholog = Orthologs are genes in different species that evolved from a common ancestral gene by speciation. Normally, orthologs retain the same function in the course of evolution. Identification of orthologs is critical for reliable prediction of gene function in newly sequenced genomes. (See also Paralogs.); OR = Olfactory Receptor; OSN= Olfactory Sensory Neuron (also called Olfactory receptor neuron: ORN); Paralog = Paralogs are genes related by duplication within a genome. Orthologs retain the same function in the course of evolution, whereas paralogs evolve new functions, even if these are related to the original one.; QSAR = Quantitative Structure-Activity Relationship

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OLFACTORY MUCUS (Figure 1)

The olfactory system proper can be said to begin with the mucus layer overlying the olfactory epithelium of the olfactory cleft and superior turbinate. About 10-40 μm thick in humans [3] and produced by Bowman's glands in the lamina propria of the olfactory mucosa [4] and elsewhere [5], it contains the secreted Odorant Binding Proteins (OBP).

The role of the olfactory mucus is not well defined; it has been suggested that it functions as a kind of separation column to fractionate the odorant particles [6], which is said to agree with some computational models of molecule deposition during the turbulent air flow of sniffing [7]. The presence of a liquid layer undoubtedly has a function as it is conserved even in the "inside-out" structure of the insect olfactory organ, the sensilla [8].

Cometto-Muniz et al. [9] have provided some evidence of a quantitative structure-activity relationship (QSAR) based on solvation energies predicting the pungency (trigeminal activation) thresholds of a wide range of volatile organic compounds, except for acetic acid, "implying that a key step in the mechanism for threshold pungency involves transfer of the inhaled substance from the vapor phase to the receptive biological phase."

Perireceptor events

The as-yet ill-understood processes acting on the odorant molecules before they reach the cell membrane and the olfactory receptor are called peri-receptor events.

Odorant Binding Proteins

Odorant Binding Proteins and their related Pheromone Binding Proteins are secreted proteins of the lipocalin family and are able to bind numerous odorants of diverse chemical structures, with a higher affinity for aldehydes and large fatty acids [10]. They have a molecular weight of about 20 kDa and are present in concentrations of between 0.1-1 mM [5]. Recent work suggests that these molecules may function best as dimers, thus creating a central "binding pocket" for the ligand [11].

Like the Olfactory Mucus layer, the OBP is conserved amongst terrestrial animals and likewise the precise role of OBPs is controversial. Steinbrecht has summarised their putative role as likely to be one or more of: solubiliser, biocarrier, scavenger, cofactor or deactivator [5]. Breer [12] added the possibility of specific filtering: "pre-selecting" the compounds which interact with the receptor.

Using a sophisticated sensing device called a surface plasmon resonance chip (SPR), Vidic [13] and colleagues have recently shown another role for OBP-1F in the binding of the odorant helional to the rat OR 1740. The OBP was found to specifically bind to the olfactory receptor in the absence of any odorant, was released on binding of the specific ligand, and most importantly, modified the dose response curve of the ligand binding from a bell shape (where the highest and lowest concentrations gave a minimal response) to a S-shape saturation curve. This gives a linear relationship between receptor activation and odorant concentration over a range of concentrations, rather than having at least two values of concentration for every level of activity.

Because even the entrance to the binding site of the receptor is not known, it has been suggested that the OBPs play a role in trafficking hydrophobic odorants to the cell membrane where they dissolve and diffuse laterally to the receptor, accessing the site through the membrane [14]. The avidity and general broadly tuned nature of the OBPs has also attracted much interest from the biosensor and pharmacology communities as a possible nanoscale binding agent [15].

Biotransformation enzymes

The role of OBP as enzyme is unlikely, but the olfactory mucosa has long been known to be a highly metabolically active tissue. One of the confounders of *in vivo* experimentation is the potential for multiple unknown chemical changes to the odorant before it reaches the receptor.

For a sense to "scan" the environment there must be a continual updating of the data, which means, for smell, a fast and efficient way of clearing "old" molecules.

Since the early '80s, interest has been directed at Cytochrome P-450 in the olfactory epithelium, Dahl et al. showed there was, weight-for-weight, as much C-P450 in the mucosa as in the liver [16]. Later in that decade a specific C-P450 which they called Cytochrome-P450olf1 was discovered in rats (and cows) by Nef et al. [17]. It seemed to be part of a subfamily whose

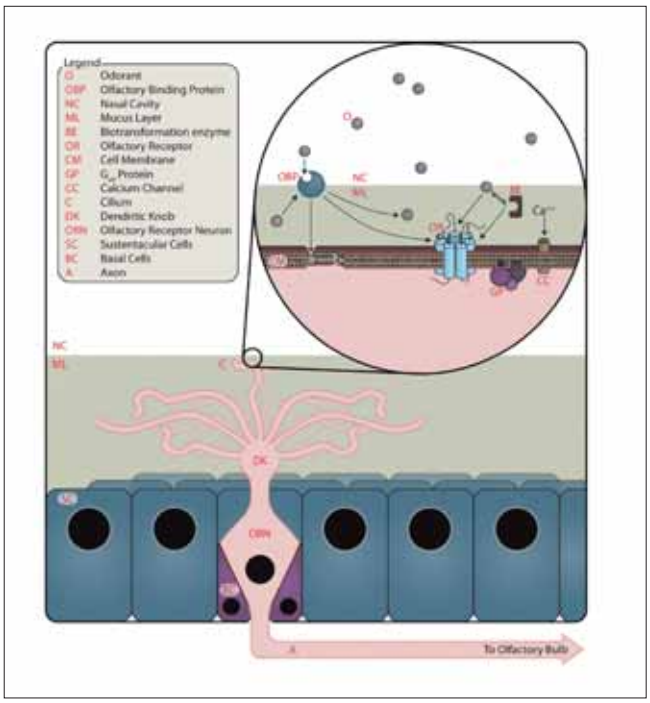

Figure 1. Schematic diagram of Olfactory Receptor neuron with inset showing receptor and perireceptor events.

"members are known to catalyze the conversion of numerous hydrophobic compounds" including many odorants. Since then, the genomic revolution has allowed several "nasal mucosa-predominant" enzymes to be found [18]. Still others have been shown to have expression levels commensurate with those in the liver.

These enzymes may exist in a complex network of interconversion between receptor agonist/partial agonist/antagonist/enzyme catalyst /inhibitor which may further allow discrimination between sterically similar odorants, similar to a mechanism proposed in 1950 by Kistiakowsky [19].

OLFACTORY EPITHELIUM (Figure 1)

The olfactory epithelium is the one place where the central nervous system is directly exposed to the external environment, albeit behind a layer of mucus and some torturous airways. Lying under the mucus layer of the olfactory cleft and superior turbinate as well as parts of the middle turbinate and septum is the olfactory epithelium. This is specialised sensory epithelium containing the mature and immature **Olfactory Sensory Neurons (OSN)**, some mucus-producing goblet cells and supporting (sustentacular) cells. It is attached to bone via a lamina propria that is characteristically thick and contains the Bowman's glands previously mentioned and the axonal processes of the neurons [20].

Each OSN is a bipolar neural cell with multiple (10-50) fine cilia (about 0.3 microns in diameter) projecting off a single dendritic knob within the mucus layer. These cilia are covered in only one of about 350 types of olfactory receptors [21] and it is on their surfaces that the odorant molecules are detected (by means of the olfactory receptors discussed below). The body and nucleus of the cell is within the olfactory mucosa layer and the axons of the OSNs project upwards through the cribriform plate to the olfactory bulb. The axons are wrapped in a special form of Schwann cell, the olfactory ensheathing glia, which is thought to allow the constant of the neurons; one of only 2 places this has been demonstrated in the adult human CNS [22], the other being the hippocampus [23].

OLFACTORY RECEPTORS

Once the olfactant molecule has traversed the olfactory mucus, it must interact with the olfactory receptor on the surface of the OSN, causing intracellular changes that initiate the depolarisation of the nerve.

The Nobel Prize for medicine was awarded in 2004 to Richard Axel and Linda Buck "for their discoveries of odorant receptors and the organization of the olfactory system" [24].

Structure and crystallography

The olfactory receptors are known to be part of the class A (or Class 1) (rhodopsin-like) superfamily of G-protein-linked receptors. These are associated with and act via the olfactory G-protein Golf through an as-yet unknown mechanism [25]. They are known to have seven transmembrane domains with an extracellular N-terminus and the C-terminus intracellularly [26]. The receptors have not been crystallised yet and much about their structure remains unknown. Homology mapping (deriving the structure based on similarity to the sequence of other known proteins) suggests the seven transmembrane domain shape but the true structure remains elusive and because of this there is still much speculation about the exact properties of odorants which the receptors are detecting [14]. The prevailing model is the "Odotype" or weak shape but there are several other theories discussed below.

Each receptor is said to be "tuned" to a set of odorants, some more broadly so than others [27]. This is its "functional specificity", the range of molecules that it detects. It is commonly accepted that it is the pattern of activation amongst the suite of receptors that is used by the brain to recognise the molecule as a particular "smell" [21].

Ever since Axel and Buck's [28] description of the OR superfamily, attempts have been made to understand the structure and therefore the mechanism behind their function. The Holy Grail is an accurate prediction of which olfactants will activate which receptor and to what extent. Two strategies have been the experimental exposure of the receptor to a wide range of possible odorants and the computer modelling of the receptors and their ligands. Of late, the two have come together to each inform the other, with interesting results.

Functional specificity: in vivo and in vitro

In the last few years, big strides have been made in identifying the ligand specificity and recognition of the ORs. Although "tuning" of receptors to a certain range of compounds was assumed, it was not until the mid-90s that this was shown to be the case.

Zhao et al.'s functional expression of the rat OR-I7 in rat mucosa [29] with analysis of the neural response (both in mucosa and when dissociated) to a panel of seventy-four odorants showed the receptor to have a fairly specific response to octyl aldehyde and the C_7 to C_{10} aliphatic aldehydes.

Araneda et al. [30] went on to use electro-olfactogram recordings in rats transfected with the receptor to determine a wider receptive range of OR-I7. Using octyl-aldehyde as a template for a range of chemical substitutions, all trigger molecules were all found to be within 7-12 ångströms in length. Additionally, to be detected by the OR, a molecule needed to have an aldehyde carbonyl, although there was a wider tolerance of various functional groups at the tail. The next OR investigated was in 1999 with the cloning of MOR-23 from dissociated OSNs with known functional response to lyral [31]. Both of these experiments demonstrated the precision detection of molecules by a single OR and the "fussiness" of a receptor in identifying and activating a response.

It stood to reason that if there were molecules that activated a receptor, there were probably molecules that prevented that activation, but it was not until 2004 that the first solid evidence of inactivation by competitive antagonism was shown. Oka et

al. [32] demonstrated that the chemical MIEG (methyl isoeugenol) prevented activation of the OR mOR-EG by eugenol, although Araneda [30] had noted a partial antagonism in 2000.

Functional specificity: in silico

The use of computer modelling to predict the tuning of receptors is yet to reach its full potential. The techniques have used the known genetic structure of the receptors to work out, almost from first principles, the constraints on the recognition of odorants. The two techniques have been to compare the genes of receptors to each other (sequence) and to compare them to GCPRs whose 3D shape is known (structure) to give an educated guess at the shape of the unknown.

From sequence

Early methods of mapping the binding site relied on the knowledge of the genetics of the receptor: looking for conserved regions or residues, sequence analysis or correlated mutation analysis [33]. Known as homology mapping, examining the linear genetic code for changes between receptor genes, one might be able to gather clues about the receptors and the roles of their amino acids.

Pilpel and Lancet [34] looked at hypervariable regions amongst 197 paralog (closely related genes duplicated within one genome) ORs identifying seventeen residues which were presumed, because they were so variable between each receptor, to have a role in the unique character of each: i.e. its affinity for a particular odorant set and therefore to be present in the binding pocket.

Man et al., in 2004, used a clever technique of comparison between residues conserved between orthologs and variable amongst paralogs in the mouse and human genomes to predict twenty-two binding site residues over all but the first TM segment [35].

From structure

Modelling the receptor and ligand-docking behaviour has been limited by the fact that only two GPCRs have ever been crystallised (bovine rhodopsin [36] and β2-adrenergic GPCR [37]). Rhodopsin is still the model that all homology modellers have used, despite it having some striking differences in function to that of the olfactory G-protein (non-reversible activation, for instance).

Various attempts using homology modelling based on Rhodopsin [35] as well as *ab initio* folding [38] have had some success in guiding experimental efforts (see below), but it was not until the work of Lai et al. in 2005 [39] that the experimental results of Araneda [30] were used to refine the computer models of ligand-receptor interaction. These models found an exit (and possible entry) path and ligand stability within the receptor to be crucial for predicting activation of the neuron. Khafizov et al. [40] in 2007 looked at 3D models of 29 mouse ORs which had binding affinity (although not activation) data and predicted binding pocket residues for further investigation, based in part on Katada et al.'s work in determining the important residues in mOR-EG. Several assumptions and omissions in all these models mean that this may be a fertile area for research in the future as greater computing resources become available. A notable omission from all of these models is the function of the Odorant Binding Protein (OBP) as discussed above. There is new evidence for allosteric modulation of GCPR function [41] by molecules which do not bind at the receptor binding site, which is yet another part these mysterious proteins may play.

Functional specificity: in combination

Published in 2005, an elegant set of experiments by Katada et al. [42] combined Ca^{++} imaging in a human embryonic kidney cell (HEK 293) expression system, molecular modelling based on the crystal structure of bovine rhodopsin and site-directed mutagenesis to map a putative odorant binding site on the mOR-EG receptor. Building a model of the receptor, they looked at several sites that were predicted to be in the binding pocket and changed the amino acids at that site. The change in response of the new mutant receptor to the odorant was measured. For instance: varying the polar serine at position 113 (thought to be on the 3rd transmembrane domain) to a smaller, nonpolar, alanine stopped the Ca^{++} response to eugenol completely; whereas changing it to a slightly larger and still polar threonine increased the sensitivity of the receptor to the odorant. Similar variation in another serine at the 210 (on the 5th transmembrane domain) produced no such variation in the dose-response curve to eugenol. Then holding the receptor constant they went on to predict the qualitative variation in activity of four different odorants in mutant receptors. Theorising that a valine at the 109 position was preventing the binding of odorants with certain bulkier functional groups; they replaced it with a smaller alanine and a much bulkier leucine. These mutants had the same affinity for eugenol but the valine-to-alanine mutant had a much higher activity when exposed to odorants with bulky side chains. The valine-to-leucine mutant, however, lost all activity when stimulated with 3,4-diethoxy benzaldehyde, which has 2 bulky side groups.

The accuracy of the model in predicting the changes in specificity of mutant receptors lends weight to it being a true reflection of the mechanism of olfactant-receptor interaction.

GENETICS

The entire family of olfactory receptor genes comprises approximately 1% of the genome, about 1500 genes in mice and 900 in humans. Humans are remarkable in that so many of our olfactory genes have become non-functional by the inclusion of errors such as premature stop codons [43]. These errors are much higher than in the mouse or even other primates [44]. Approximately one thousand genes are estimated to be expressed in rodents [45], humans are thought to manage less than three hundred [46].

The genes are mostly found in groups, but some occur singly

and they are found on all human chromosomes apart from 20 and Y [47]. Approximately 42% of the genes are found on chromosome 11 which is also the only one which contains genes for Class I receptors which were, until recently, assumed to be unique to fish and amphibians [48]. OR Genes seem to be very strictly conserved, and are usually coded from one exon [49]. They usually occur in clusters for which some have been shown to be preceded by a 2 kb cis-acting (and perhaps trans-acting [50]) regulatory element upstream, which occurs in mice and humans [51], with similar non-homologous LCR/enhancers in Zebrafish [52]. This "H-element" has been recently shown to have a smaller core-H region of 124bp [52], deletion of which stops the transcription of the nearest 3 out of the 4 OR genes in the cluster. It is hypothesised that there may be several overlapping transcription controllers, similar to the H-element.

The evolutionary genetics of the human olfactory receptors are fascinating. A review of the genomes shows that, along with cetaceans [53], the higher primates have lost about half of their expanded mammalian inheritance of ORs [54], which has almost doubled since mammals diverged from the monotremes. It is obvious why marine mammals would depend less on olfaction and the acquisition of trichromatic vision has been posited as the reason for the primate loss [55].

The loss of functional receptors is not uniform amongst individuals. Menashe et al. [56] in 2003 showed that genomic analysis of 189 individuals showed an amazing 178 different patterns of inactivation amongst the receptors. That is to say: 178 different "suites" of active receptors, where one may have receptors {ABCDEF… etc.} active, another suite might have {_B_DEFG…} active where A and C were inactive. If the perception of an odorant is dependent on the relative strength of activation of a range of receptors (its receptor profile) this means that almost no two noses are the same! Although the noses will correctly identify an odorant as the same, the "quale", experience of "what it is like" to the smeller will differ [57].

INDIVIDUAL EXPRESSION OF RECEPTORS IN OLFACTORY MUCOSA

Zonal expression

Nothing is known about the locality of expression in humans. In rats, receptors are expressed over the surface of the epithelium in a zonal pattern [58]. Several zones have been described with bilateral symmetry in the two nasal cavities and are organized along the dorsal-ventral and medial-lateral axes. Although any receptor may occur in any of the four zones, certain receptors are more likely to occur in "their" zone. The exact mechanism and reason for this is not yet known.

One receptor – One neuron rule

Each neuron stochastically [59] expresses one of the two thousand alleles (2 for every gene) available to it as its one and only receptor. This is maintained by a feedback loop of unknown form [51], but is similar to the allelic inactivation in the immune system [60]. What is known is that the transcription of the gene region is not sufficient (such as happens with a pseudogene), the product must be an active OR to prevent another OR gene from being expressed [51]. The OSN switches genes at a low frequency until it expresses one that forms a fully functional OR: the so-called "serial monogamy" model [61]. Disruption of the downstream signal transducers G_{olf} [62] or the ion channel CNG [63] does not make the OSN switch receptor transcription so it is unlikely that this is how the OSN tells it has a functional OR, ie: it is not the signals from an odorant which are the OSN's method of determining whether a gene expression protein is a functional receptor or not. It may be a signal arising from the OR's other role as axonal pathfinder that initiates this feedback.

Once an OSN has selected a functional OR gene allele, this is irreversible, but this choice can be reset on nuclear transfer to another OSN [64]. Unlike immunoglobulins, the gene does not undergo DNA rearrangements to force the cell to express only that receptor but seems to be selected and fixed by some intrinsic cellular pathway.

Some recent work has emerged to challenge the one neuron-one receptor doctrine [65], but only in Drosophila. In fact, it seems Drosophila may require at least 2 receptors per neuron to have any olfactory function at all [66].

Surface expression of receptors

The difficulty of getting expressed receptors to the cell membrane has hindered analysis of ligand specificity and agonist / partial agonist / antagonist relationships. Recent work has shown that association with β2-adrenergic receptor [67], a protein called Ric-8B, "a putative guanine nucleotide exchange factor for G_{olf}" [68] or any of three subtypes of purinergic receptor (P2Y1R, P2Y2R, and A2AR) [69] increases the expression of ORs on the surface of model cell systems. There is some supporting evidence that at least some of these co-expressions may be important *in vivo* [67].

Dual role of receptors

Olfactory receptors occur both on the cilia or axonal region of the OSN, where they are exposed to the olfactants, and on the dendritic extensions to the olfactory bulb [70]. They appear to not only detect the olfactants but also in guide the neuron to its appropriate glomerulus in the olfactory bulb (see Part 2: neuron to brain and beyond). It may be the axonal expression or activation of the OR which is the initial step in the feedback loop described above. This makes a certain logical sense, as it is this connection that confirms that the axon has a "meaningful" receptor: one that the brain recognises and can interpret.

ACTIVATION OF RECEPTORS

Conformational change

Although activation of a receptor is presumed to be by a conformational change in the protein, the details of this are not known. A common mechanism with other GCPRs is assumed

[14] and the general models are stated here. Studies of Rhodopsin suggests that activation results in the transmembrane helices moving as rigid bodies relative to each other within the membrane [71].

The intracytoplasmic loop between TM3 and TM4 contains a highly conserved DRY (aspartic acid-arginine-tyrosine) motif [14] which some site-directed mutagenesis experiments [72-73] have shown plays an important role in the activation process of GPCRs. It is hypothesised that this motif is exposed to the intracellular domain on activation of the receptor and interacts with the G-protein to initiate the signal cascade [38].

Electron tunnelling

A novel mechanism of olfactory molecule recognition has been proposed by the biophysicist Turin [74]. Somewhat controversially, the receptor is suggested to use quantum effects to perform a biological "electron tunnelling spectroscopy" upon the odorant molecule. Instead of shape, the receptor detects the vibrational frequencies of the intramolecular bonds of the odorant. A recent model published by a group of physicists working at UCL has given some support to the role of electron movement within the receptor as the means of both ligand recognition and receptor activation [75]. Referring to what they call the "swipe card model of smell", Brookes et al. combine vibrational and shape theories of molecular recognition. Just as a swipe card must have the correct shape to fit into the card reader but also the correct information on the "magnetic strip" to open a lock, so too must the molecule fit both shape and vibrational characteristics to activate the OSN [75].

The physics are complex and the theory controversial but the mechanism may account for several oddities of smell perception.

Time scales in receptor activation

There are some experimental constraints to receptor-ligand interaction, all of which point to a very fast reaction somewhere on the order of 1 millisecond.

In 2005, Bhandawat and colleagues [76] published an attempt to quantify the unit activation of the G-Protein linked receptor in isolated frog OSNs. Extrapolation of a linear relationship between response time and odorant exposure had a time intercept near zero on a millisecond scale. Other data also supported this "receptor dwell time" at being on the order of 1ms. In a set of experiments published in 2008, Wesson et al. [77] looking at response to novel odour presentation in head-fixed rats, showed that a response was initiated within 80–160 ms from time of odorant exposure (timed from beginning of inhalation). Subtracting time taken from transmission from initiating event (estimated at 2-12 ms depending on the distance of the glomerulus from the bulb) gives a time as small as 70 ms for the entire process of transduction *in vivo*. This process must consist of time taken to travel through the nasal cavity, partition into the nasal mucus, diffuse to the receptor, bind and activate it, as well as the time taken for the subsequent intracellular machinery to work.

Simple physics gives an estimated time of diffusion through the mucus as 25-40 ms (depth of mucus is 5-20 μm in rats [78], diffusion constant D is 10-4 cm^2/s), this leaves 30 to 45 ms to accomplish all other steps.

It is known cAMP reaches peak intracellular values within 25 ms of odorant stimulation in isolated rat OSNs [79]. This leaves very little time: 15-20 ms for inhalation, binding and activation of the OR as well as activation of the downstream adenylate cyclase and ion channels and supports the millisecond range activation times implied in earlier work.

Part 2:

The initial molecular events of the sense of smell initiate the signal within the neuron: the second part of this review will follow the olfactory signal from activation of the receptor and generation of neural depolarisation to the olfactory bulb and beyond. It will be published in a next issue of Rhinology.

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APPENDIX A:

Search strategy

Numerous queries were made to the ISI web of science, Pubmed and Google Scholar from January to 29 September 2009. All headings and many subheadings were entered in conjunction with topic: olfaction. Further information was obtained from publications known to the author and co-workers as well as subject and term-specific searches using Google and websites above.