Olfactory Receptors

From basic science to applications in flavors and fragrances

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The past two decades have seen radical progress in the understanding of the mechanism of olfaction. The discovery of olfactory receptors (ORs) and the recent improvement achieved in their functional expression has opened the way for new applications for the F&F industry.[1] Although the field of molecular olfaction is still in its infancy, the new concepts under development may already be put to use.

In this second part of our review, we will summarize the most significant insights in the field. The different advances will also be put in perspective with the concrete applications they could lead to.

Progress in OR Deorphanization

It took more than six years between the initial discovery of ORs and the first reliable demonstration of the interaction of an OR by an odorant molecule. Different approaches aiming to identify odorant molecule-OR interactions have been developed. Some require working with a whole animal or at least on olfactory tissues.[1] Such approaches have allowed the deorphanization of several tens of mouse and rat ORs but are not applicable to humans.

Expression of human ORs into heterologous cell lines has allowed the identification of ligands for human ORs. However, fewer than 20 human OR deorphanizations have been reported so far. This is due to the difficult functional expression of human ORs. Most of the published studies used a restricted set of test molecules (around 100) and were focused on a limited number of receptors. The most complete deorphanization of human ORs published so far, concerns a library of 93 odorants and 245 ORs.[2] This work led to the identification of a total of 47 activators for 10 receptors. The deorphanization of the whole contingent of human ORs takes time and requires the capacity to manage a large number of molecules as well as a large number of receptors.

For non-olfactory receptors with potential pharmaceutical interest, processes allowing the screening of libraries of hundreds of thousands of molecules have been set up. The so-called high throughput screening requires an efficient automation and a functional assay that is both readily achieved and cost-effective. Assays relying on reporter genes such as the luciferase gene fit these criteria and are convenient for ORs.[1] Such a screening platform devoted to deorphanization of human ORs has been developed by TecnoScent. It allows researchers to perform up to 10,000 assays per day with a single robot unit. This represents a daily screening of libraries of 100 to 500 compounds in parallel on a series of ORs.

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Each odorant-OR couple, once identified, has to be validated. Increasing concentrations of the molecule are then used to stimulate the receptor generating dose-response curves (**see Explanatory Panel**). These dose-responses also allow the characterization of the odorant-receptor couple and therefore the comparison of the different activators for given ORs. To date, more than 100 human ORs have been deorphanized by TecnoScent.

Diversity Among ORs

The early animal data strongly suggested that one OR may interact with several molecules and that most of the molecules can interact with several ORs; this has been fully confirmed by several studies. However, the range of selectivity may vary considerably from one OR to another.

Some human and mouse ORs are activated by more than 40 different odorants. Surprisingly, the ligands can be structurally unrelated. For example, the receptor ORL420 (TecnoScent code number) interacts not only with molecules encompassing an aromatic ring such as 1-phenyl-3-methyl-3-pentanol or anisyl acetate, but also with linear alcohols such as 1-octanol. Other receptors have a more restricted range of selectivity and recognize homogenous families of ligands. The human receptor OR51E1 is specifically activated by carboxylic acids in the C4–C10 range (mainly malodors). The carboxylic group is mandatory since substitution of this group by an amine, an aldehyde, an alcohol, or even an ester results in total deactivation. As a third example, the receptor OR7D4 was found to be activated only by androstenone and androstadienone, two steroid malodors released in human sweat, from a screening library of 66 compounds.[3] TecnoScent has also found very narrowly tuned receptors that respond only to a few close analogues from a library of more than 1,000 molecules.

Explanatory Panel

If one administers various concentrations of an agonist to a receptor, the dose-response curve will go uphill as one moves from left (low concentration) to right (high concentration). Many steps can occur between the binding of the agonist to a receptor and the production of the response. Depending on the odorant used and the response measured, concentration-response curves can have almost any shape. However, in many cases concentration-response curves follow a standard shape, which is a sigmoid, or "S-shaped," curve (see **F-1**). A standard dose-response curve is defined by four parameters: the baseline response (bottom), the maximum response (top), the slope, and the odorant concentration that provokes a response halfway between baseline and maximum (EC50). These parameters allow comparisons of the different activators of an OR.

EC50 is defined quite simply as the concentration of agonist that provokes a response halfway between the baseline (bottom) and maximum (top) response. As shown in **F-2A**, lower EC50 values correspond to an increased potency. This means that a lower concentration of activator is necessary to trigger the OR and, therefore, these agonists are considered better activators. The differences in potency for the agonists of a receptor may reflect their different abilities to bind to the OR. The efficacy may also vary from one agonist to another (**F-2B**). The differences may result from an altered ability of the odorant to induce the conformational change that moves the OR into its active state. Agonists that have a significantly lower Emax with respect to the best activator known for a receptor are called partial agonists. At a given concentration a plateau is obtained, and increasing the activator concentration further does not result in an increase of efficacy.

Example of a concentration-response curve obtained when an olfactory receptor (OR51E1) is activated by a specific agonist (5-norbornenecarboxylic acid) **F-1**

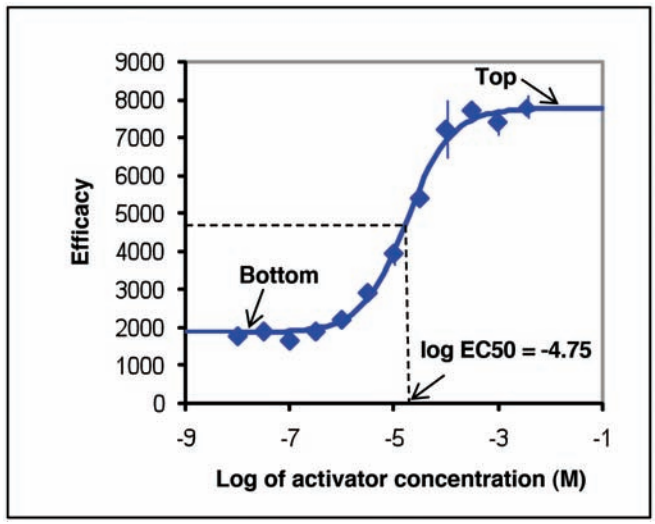
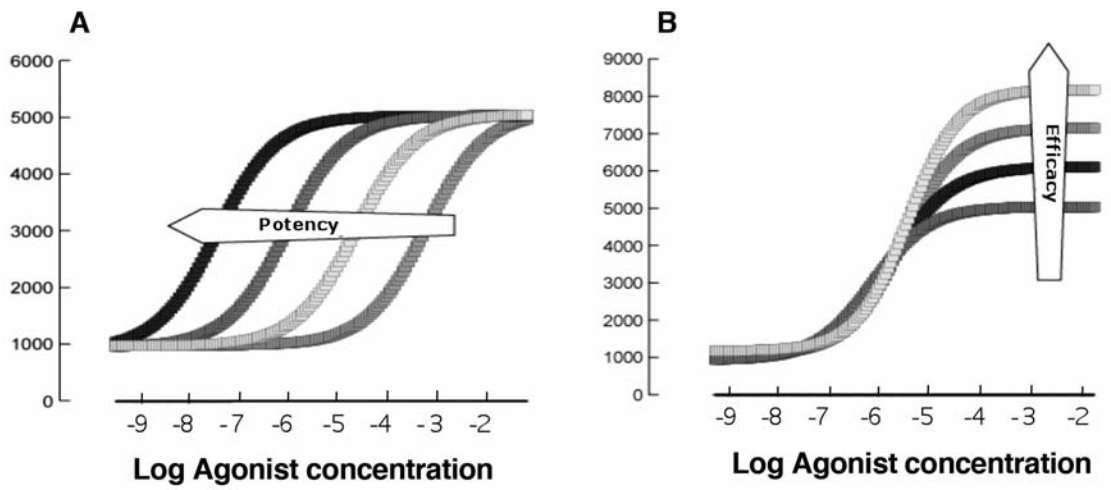


Illustration of different potencies (A) and efficacies (B) of different agonists for a given OR **F-2**



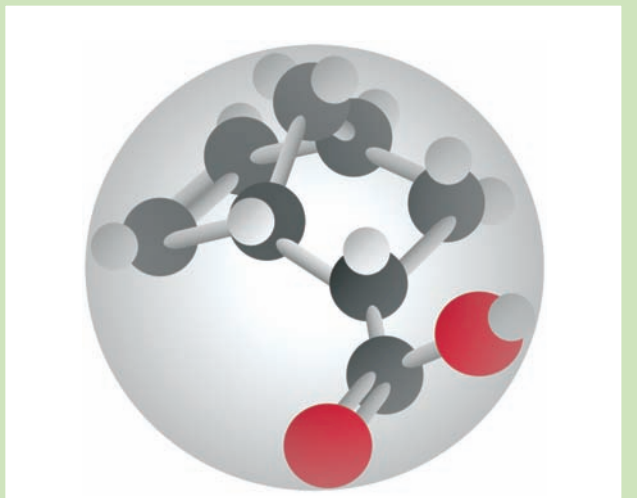
High throughput screening of OR/malodorant/potential blocker combinations would be essential in the search for such blockers and would represent decades of activity of panelists.

The meaning of these different selectivities is still elusive. One might predict that broadly tuned ORs might play a role of more generalist detectors alerting the brain when odorant molecules come into the nasal cavity, whereas narrowly tuned ORs would discriminate odorant molecules. Coincident activation of both types of ORs might possibly be required to allow the perception of an odorant.

Structure-activity Relationship Studies

Structure-activity relationships (SARs) for OR activation consist of comparing the efficacy and the potency (see **Explanatory Panel**, **F-2**) of different molecules having related structures. One published example has focused on the mouse receptor OR-I7, which is known to respond to aliphatic aldehydes.[4] Using octanal as a reference, the authors of this study have shown that an optimal length of the carbon chain is important in conferring a high potency to the ligand. For instance, aldehydes with a chain less than 6.9 Å, regardless of the number of carbon atoms present, may bind to OR-I7 but are unable to activate it. These molecules block the receptor in an inactive state and therefore have an antagonistic effect on the receptor. This work has also shown that the conformation of the molecule is important in triggering the receptor. When shaped in a semi-extended conformation, octanal maintains the receptor in an active state. Cyclic analogs mimicking this conformation are also potent activators of OR-I7, whereas other analogs with a more compact conformation fail to induce activation of the receptor.

Schematic representation of 5-norbornene carboxylic acid enclosed in the spherical binding pocket of OR51E1 **F-3**



A similar structure-activity relationship study has been performed at TecnoScent on the previously mentioned OR51E1, which responds almost exclusively to carboxylic acids. A comparison of linear aliphatic acids has shown that optimal activators have a main chain of four to five carbons. A methyl substitution on this chain reduces the potency of the molecule if it occurs on the second carbon of the chain with respect to the carboxyl. Methyls located further along the chain increase the activity of the molecule with respect to the corresponding linear aliphatic acid. This observation led to the consideration of monocyclic and bridged alicyclic substrates. The optimal activators from this class of ligands correspond to cyclopentane carboxylic acid and to a molecule with a bridged ring, 5-norbornene carboxylic acid. Interestingly, the methylated analogs of this latter molecule failed to trigger the OR. These different observations have led to the refinement of the current model of the binding pocket and to the propoal that OR51E1 has a binding pocket with an almost spherical cavity with a volume of about 120 $Å^3$ that accommodates the hydrophobic moiety of the ligand at one side, and fits the carboxylic function of the active molecules at its opposite side (**F-3**). Linear ligands of the correct size and substitution patterns would have enough conformational flexibility to adopt a suitable shape to fit the pocket; however, bulky ligands with the right volume (120 $Å^3$ in this case) will lead to optimal interactions and therefore to better stimulations.

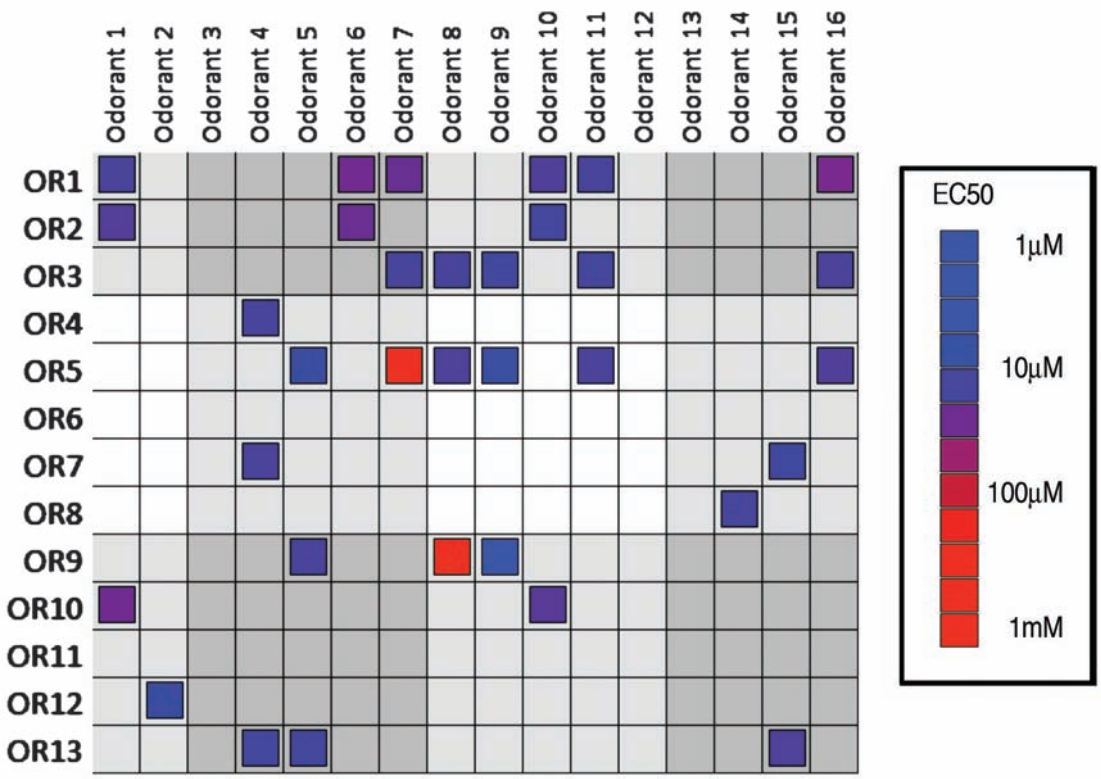
These two examples reveal one reason for such SAR studies. Beyond the identification of new, more potent activators, they may help to identify the minimal common scaffold shared by the odorants that activate a given OR. They also allow, to some extent, the prediction of how a molecule will interact with the receptor and direct the search for activators or inhibitors to a particular group of molecules. One of the emerging interests of SAR studies relies on the generation of the helpful information that they provide in the context of computer-aided three-dimensional modeling of receptor-ligand interactions. Finally, the comparison of SAR studies performed by assessing different molecules belonging to the same odor family on a series of responding receptors might lead to the identification of the set of ORs responsible for the perception of a characteristic odor as illustrated by **F-4**.

Molecular Modeling of ORs

The three-dimensional modeling of a protein helps determine the spatial folding that is adopted by the amino acid chain that composes this protein. The amino acid sequence of a protein may adopt different shapes (secondary structures) such as alpha helix, beta sheet, beta turn, etc., in different parts of the amino acid chain. The whole of these secondary structures forms the ternary structure of the protein which corresponds to its true three-dimensional shape. For receptors, such modeling is instructive since it allows prediction of interaction sites with ligands. Although *ab initio* computation of G-protein-coupled receptors (GPCRs, the family of receptors to which ORs belong; **F-5**) based

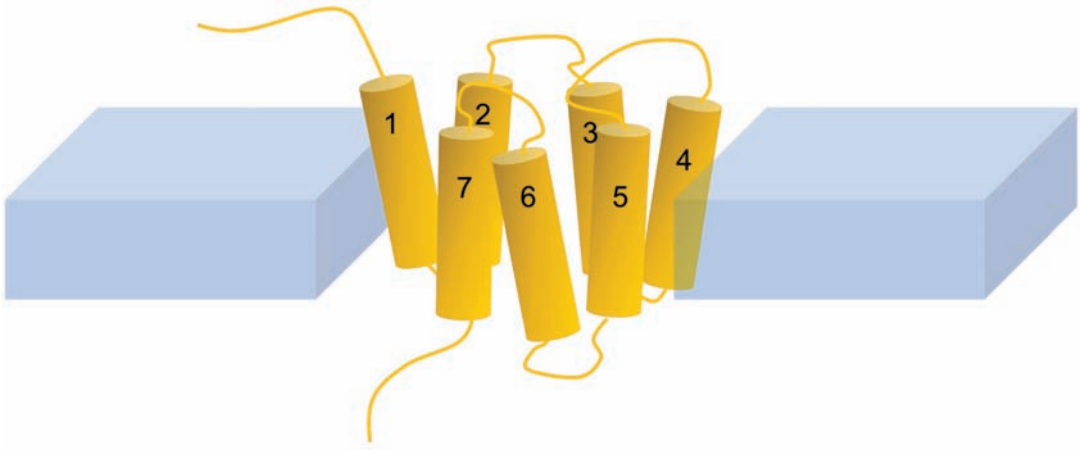
only on their amino acid sequence has been described, the modeling of the three-dimensional may require additional information from complex techniques such as X-ray crystallography or nuclear magnetic resonance (NMR) spectroscopy. Both approaches aim to determine the precise spatial coordinate of each atom constituting a protein. The secondary and ternary structure may be determined from this atomic three-dimensional cartography. These techniques require a sufficient amount of highly purified material. This step of purification constitutes the major hurdle on the path to determining the ternary structure of GPCRs. Despite the high interest of many different GPCRs for pharmaceutical applications and the efforts made to purify them, only a very limited number of three-dimensional determinations have been performed so far. The first corresponds to the photoreceptor rhodopsin, and the second to the β2-adrenergic receptor. Beyond their own interest, these first examples may serve as a template to extrapolate to the structure of other GPCRs, including ORs. All GPCRs have a common major structure relying on seven helices that are inserted into the membrane of the cells (**F-5**). By assuming that the orientation of each of these transmembrane domains is conserved among all the GPCRs, it is possible to compute a virtual model of an OR by replacing the amino acid sequence of the transmembrane domains of rhodopsin or β2-adrenergic receptors by the corresponding domains of the OR. Once the molecular model of the OR is set up, the next step consists in indentifying the region of the receptor that interacts with known ligands. Comparison of ORs from different species has led to speculation that the binding region is most likely constituted by the upper part of the transmembrane domains 3, 4, 5 and 6. Different ligand-docking software may also be of use in predicting how and where the molecule will bind to the receptor. The interest of such modeling lies in the accurate elucidation of the interactions between the odorant molecules and the OR amino acids involved in the binding. From this model, the interaction of new molecules, not yet identified as ligands, may be predicted. Such modeling has been performed for mouse and human ORs. In the case of the receptor mOREG, 10 amino acids spread in the transmembrane domains 3, 4, 5, and 6 have been proposed to interact with the different ligands.[5] This prediction has been validated using modified receptors where the amino acids in these key positions were replaced. Some modifications were predicted to reinforce the interaction of the ligand with mOREG and led to a higher potency when assessed with a functional assay.

Typical activation pattern against a set of human ORs for a group of odorants with a similar odor profile[a] **F-4**



[a]The challenges are: First, to determine why these patterns are interpreted in the same way by the brain, and second, to find the missing receptors for these odorants. There must be more receptors to be identified since odorants 12 and 13 did not activate any of those in the table, yet they possess an odor very similar to those of the other molecules tested.

Schematic representation of a G protein-coupled receptor[b] **F-5**



[b]The barrels correspond to the helices that are inserted into the cell-surface membrane shown in blue. This seven-transmembrane domain-structure is common to all GPCRs, including the olfactory receptors.

Molecular modeling may also help us to understand the basis of OR selectivity for its ligands. A comparative study has shown that the human OR1A1 and OR1A2, which share high similarity (82% of their amino acid sequences are identical), respond differentially to (*S*)-citronellol while responding in a similar way to (*S*)-citronellal.[6] Using molecular modeling, the researchers were able to show that, in spite of their sequence similarities, both receptors bind the alcohol and the aldehyde differently.

Beyond the mechanistic explanation of the interaction between a receptor with a ligand, molecular modeling might be of use in predicting what structure a molecule should have in order to bind to an OR or a group of ORs. In the human nose, ORs exist in a dynamic equilibrium between the active and inactive states. This results in a continuous level of electrical activity in the glomerulus. Volatile molecules such as odorants can affect this balance in three different ways. The molecule could act as an agonist and stabilize the OR in its active state, thus increasing the size of the signal. Alternatively, it could stabilize the OR in the inactive state, thus acting as an inverse agonist, reducing the level of electrical activity. The third possibility is for the molecule to bind to the OR without activating it, but in doing so, to prevent an agonist from reaching the binding site, thus acting as an antagonist. The current knowledge of structure and properties of ORs is insufficient to understand or predict the differences between these binding modes. However, in view of the accumulation of information from SAR studies on the structure of activators and inhibitors of ORs, these data will help to refine the computer-aided modeling of interactions between ORs and odorant molecules. It is therefore conceivable that TecnoScent will be able to give accurate predictions of the structure of potent agonists or antagonists. This approach will be helpful in designing new odorant molecules or receptor blockers.

Antagonist Identification: A New Concept for Fragrance and Flavor Development.

Odor perception relies on the combinatorial activation of ORs resulting in specific activation patterns, or odoprints. One should now also consider agonist molecules that might influence the perception of odors by blocking other receptors. These antagonisms sound very similar to pharmacologists' work on non-olfactory GPCRs, which has led to several drug discoveries. Examples include beta-blockers, which obstruct human beta-adrenergic receptors and are therefore used in the treatment of hypertension and other cardiovascular diseases.

In the field of fragrance and flavor creation, perfumers and flavorists must rely on both their experience and time-consuming trial and error techniques in the development of new molecules and compounds that will dampen the perception of unpleasant odors. Malodor coverage is not fully satisfactory as the active ingredient is added to the undesirable malodor background. Blocking (antagonizing) the receptors that specifically bind to these malodors represents an interesting improvement for various commercial applications. An ideal blocker would have no odor per se, would not affect the bouquet, and therefore would give full creative freedom to perfumers and flavorists. Such blockers are difficult to identify empirically. High throughput screening of OR/malodorant/potential blocker combinations would be essential in the search for such blockers and would represent decades of activity of panelists. Screening of antagonist libraries on ORs using cell-based expression

systems can readily be achieved. It merely consists of adding together the known activator and the potential blocker. A decrease in OR activation reveals the blocking effect of the antagonist. Some OR antagonists have already been identified. For example, undecanal was found in vitro to block the activation of the human OR17-4 (also known as OR1D2).[7] The antagonist effect has also been confirmed *in vivo* in humans.[8] Additionally, this demonstrates the role of OR17-4 in the perception of a lily of the valley odorant. It is also important to note that, in this case, the antagonist is an odorant molecule belonging to the perfumers' palette. Understanding antagonism among fragrance ingredients will help researchers to understand the sensory interactions that perfumers have learned empirically in the past and will, one hopes, aid in the development of algorithms for perfume creation.

Making the Link between OR and Perception

One could question whether the results generated by the very objective *in vitro* approach are well related to the in vivo (i.e. on human) situation. The identification of a receptor activated by an odorant of interest may be the starting point of extensive researches of analogs that activate the OR (for example, for replacing an expensive odorant molecule by a cheaper one) or for seeking potent blockers (in the case of malodors).

Making the link between *in vitro* and *in vivo* remains difficult since no direct experimental approach consisting of erasing the expression of an OR is allowed on humans. However, it is known that the variation among humans in terms of active receptor expression is such that it might be possible to identify a sufficiently large group of subjects lacking a specific functional receptor to enable one to determine the effects of this on perception of odorants that are agonists for that receptor. This phenomenon might be linked to specific anosmia (e.e. the inability to detect a specific odorant), and for several odorants it may affect a significant percentage of the population. Once an OR for an odorant of interest is identified, it is worth recruiting people anosmic to this molecule and analyzing their sequence of the corresponding OR. The OR of these people may be altered and therefore unable to be triggered by the odorant. This approach has been carried out in a study performed on androstenone.[3] This sweat malodor is not perceived by about 30% of the human population. The human OR7D4 was found to respond to androstenone from a test performed on 335 human ORs. It was further shown that a significant percentage of andorstenone non- or weak-smelling people possessed mutations in the sequence of OR7D4 that reduces both the potency and the efficacy of the receptor. The authors of this study therefore conclude that OR7D4 is an important factor for the perception of androstenone, although other, still unidentified ORs must also be involved in this steroid perception. Another way to prove the involvement of an OR in a fragrance perception is to identify antagonists. As exemplified by undecanal (as mentioned before), the fact that a receptor antagonist also blocks the perception of this OR activator is a strong argument in favor of the OR participation to its activator perception. One could hope that it will soon be possible to make reliable predictions on the antagonist structure using modeling. In one study, the number of molecules needed to test as possible antagonists would be much more limited, and their use to determine the involvement of OR in perception should be facilitated.[4]

Conclusions

There still remains much to be done in order to map all human OR/odorant/blocker interactions and to determine how the resultant signal patterns are interpreted by the brain. However, researchers have identified receptors for many commercially important odorants and malodorants and results to date already demonstrate that innovative applications will be possible. The outcome of future work will complement that of perfumers and flavorists and lead to exciting new directions for the industry.

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