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Single olfactory sensory neurons simultaneously integrate the components of an odour mixture

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Abstract

Most odours are complex mixtures. However, the capacities of olfactory sensory neurons (OSNs) to process complex odour stimuli have never been explored in air-breathing vertebrates. To face this issue, the present study compares the electrical responses of single OSNs to two odour molecules, delivered singly and mixed together, in rats *in vivo*. This work is the first aimed at demonstrating that single OSNs simultaneously integrate several chemical signals and which, furthermore, attempts to describe such processes for the whole concentration range over which single OSNs can work. The results stress that complex interactions occur between components in odour mixtures and that OSN responses to such mixtures are not simply predictable from the responses to their components. Three types of interactions are described. They are termed suppression, hypoadditivity and synergy, in accord with psychophysical terminology. This allows us to draw links between peripheral odour reception and central odour coding. Indeed, events occurring in single OSNs may account for the dominating or even the masking effects of odour molecules in complex mixtures, i.e. for the prevailing action of a minor component in the final qualitative perception of a mixture. We conclude that our observations with binary mixtures anticipate the complexity of processes which may rise at the level of a single OSN in physiological conditions. Following this hypothesis, a natural odour would induce a multi-chemical integration at the level of single OSNs which may result in refining their individual odour-coding properties, leading them to play a crucial role in the final performance of the olfactory system.

Introduction

Odours present in the natural environment of animals and humans are mostly complex mixtures of several tens, even hundreds, of compounds which the olfactory system can recognize and discriminate over a remarkably wide range of qualities and intensities. However, all functional approaches of cellular and molecular mechanisms involved in peripheral olfaction in air-breathing vertebrates have been performed using pure chemicals only.

Using such simple stimuli, coherent physiological and molecular data have been obtained showing that functional properties of single olfactory sensory neurons (OSNs) are fully explained by the wide molecular receptive range of their olfactory receptors (ORs). Rat OSNs have been shown to respond to qualitatively distinct odour molecules, their broad tuning allowing overlapping arrays of excited neurons to achieve odour discrimination and intensity coding (Duchamp-Viret *et al*., 1999, 2000). Molecular binding mechanisms underlying these OSN functional properties have been elucidated through studies demonstrating that individual ORs recognize overlapping sets of odour molecules (Kajiya *et al*., 2001; Touhara, 2002). Interaction between a given molecule and a single OSN/OR has been described as inducing functional mechanisms which are both specific and complex. Indeed, the response threshold, kinetic of concentration–response curve (Duchamp-Viret, 1988; Duchamp-Viret *et al*., 1999, 2000), odour–ligand specificity and affinity (Kajiya *et al*., 2001; Touhara *et al*., 1999; Touhara *et al*., 2002), and even the transduction pathway (Fadool & Ache, 1992; Michel & Ache, 1992; Hatt & Ache, 1994; Kang & Caprio, 1997) are specific for each couple.

To our knowledge, no data are available on OSN coding properties of odour mixtures in air-breathing vertebrates. This is why we addressed this issue in rats. To do so, the first step was to use binary mixtures of two pure chemicals. Stimuli were chosen from odour molecules reported as increasing either inositol triphosphate (IP_3) or cyclic adenosine monophosphate (cAMP) (Sklar *et al*., 1986; Breer & Boekhoff, 1991; Noe & Breer, 1998) because the way in which two chemicals may interact in a mixture may depend on whether they employ the same or two distinct transduction pathways. We hypothesized that single OSN electrical responses may result not from the simple summation of the effects of the two components but from the simultaneous integration of several of the molecular signals forming a mixture. This hypothesis was tested using electrophysiological extracellular single-unit recordings *in vivo*, the most appropriate technique to describe OSN responses to odours in conditions as close as possible to the physiological operating system.

Materials and methods

Surgical methods

All experiments were carried out in accordance with the European Communities Council Directive of November 24th, 1986 (86/609/EEC) for the care and use of laboratory animals and all efforts were made to reduce the number of animals used. Adult Wistar rats (250–300 g) were used. Rats were anaesthetized with an intraperitoneal injection of equithesin (mixture of pentobarbital sodium and chloral hydrate) at an initial dose of 3 mL/kg. Anaesthetic was then supplemented as necessary to maintain a deep level of anaesthesia, as determined by

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the depth and rate of the respiratory rhythm of the rat and the lack of withdraw of the leg in response to a moderately intense toe pinch. Rectal temperature was maintained at 37 ± 0.5 °C by a homeothermic blanket (Harvard Apparatus, USA) and surgical wounds of the animals were regularly infiltrated with 2% procaine. For recordings, anaesthetized animals were tracheotomized and secured in a sterotaxic apparatus. Recordings were performed in the Endoturbinate II: endoturbinates are small bones in the nasal passages, lined with the olfactory mucosa. This terminology is well known in anatomical description of the nasal cavity. Each turbinate is numbered, Endoturbinate II is perfectly located. Access to the olfactory mucosa was gained by removing the nasal bones and then gently slipping aside the dorsal recess underlying these bones.

Electrophysiological recordings and data analysis

Single-unit action potentials were recorded using metal-filled glass micropipettes (2–4 MΩ), and electro-olfactograms (EOGs) were recorded with glass micropipettes of 50-μm diameter filled with saline solution. The two signals were led on line through conventional amplifiers to a Data Tape Recorder (Biologic, France) and to a CED-1401 data acquisition system (Cambridge Electronic Design Ltd, UK) connected to a computer. Spike and EOG signals were filtered between 300 and 3000 Hz and between 0 and 30 Hz, respectively. The signal-to-noise ratio of spike recordings was ≥ 10. They were sampled at 15 kHz and 200 Hz, respectively, on the CED-1401 data acquisition system. The single-unit nature of the recorded spikes was first verified during the experiment by triggering the sweep at a level just above the background noise with the aid of a storage oscilloscope. This allowed us to check the characteristics of the polyphasic spike of the cell studied in order to ensure that the same cell was recorded throughout all the experimental procedures. The single-unit activity was then triggered using the facilities offered by the Spike2 language associated with the CED-1401 system. Spikes were detected using their waveform signal over a triggering level, and then by visual inspection of the consistency of the shape of the sorted spikes on the computer screen. The response determination has been precisely described in a previous paper (Duchamp-Viret *et al*., 1989). It was achieved using the statistical Mann–Whitney test. The quantification was performed on the spike train statistically determined as being included in the initial part of the response using the previous test and was given in spikes/s.

Odour stimuli

Basic stimuli were pure chemical compounds purchased from Merck-France. The whole odour set comprised 10 and 4 molecules reported as inducing an increase in cAMP and IP_3, respectively (Sklar *et al*., 1986; Breer & Boekhoff, 1991; Noe & Breer, 1998). They are termed cAMP and IP_3 molecules, respectively, in the text. cAMP odour molecules were camphor (CAM), cineole (CIN), citralva (CIT), menthone (MENT), isoamyl acetate (ISO), methyl-amyl-ketone (MAK), anisole (ANI), acetophenone (ACE) and L- and D- carvone (L-, D-CAR). IP_3 putative odour molecules were limonene (LIM), ethylvanillin (EVAN), lilial (LIL) and lyral (LYR).

Stimuli were delivered using a dynamic flow multistage olfactometer which ensured a precise control of the stimulation parameters and allowed delivery of up to 12 different concentrations (Vigouroux *et al*., 1988). Using this olfactometer, OSN responses have been previously shown to be reproducible for the same odour at the same concentration and as not displaying a run-down or decrement over time of recording (Duchamp-Viret *et al*., 2000). Odours were stored in U-shaped tubes filled with absorbent pellets. Twelve different concentrations were available from discrete dilutions ranging from 1 : 1124 to 1 : 2 of the saturated vapour (SV) of compounds at atmospheric pressure. Depending on their SV values (Table 1), the lowest and highest concentrations were between 2.2×10^{-7} and 5.9×10^{-6} M and between 1.2×10^{-4} and 3.2×10^{-3} M, respectively. Mixtures were achieved from saturated vapour phases of the two compounds. Between stimulation procedures, pure air entered in parallel in the dilution stage of the olfactometer through two U-shaped tubes filled only with absorbent pellets (blank tubes). For single odour molecule delivery, one of the blank tubes was replaced by the tube containing the selected odour. Then, to deliver binary mixtures containing this odour, the second blank tube was replaced by the tube containing the second selected molecule so that, for each odour, the same quantity of molecules was delivered during single and mixture presentation.

TABLE 1. Saturated vapour pressures (SVP) and concentrations of the staturated vapour of the different compounds

Compound	SVP at 25 °C		Concentration (M)
	(Pa)	(mm Hg)	
CAM	27†	0202	2.44×10^{-04}
CIN	260	1950	2.35×10^{-03}
CIT	NA		
MENT	43†	0322	3.89×10^{-04}
ISO	728	5460	6.58×10^{-03}
MAK	490	3675	4.43×10^{-03}
ANI	472	3540	4.27×10^{-03}
ACE	49	0367	4.43×10^{-04}
L-, D-CAR	NA		
LIM	259	1942	2.34×10^{-03}
EVAN	NA		
LIL	NA		
LYR	NA		

SVP values were obtained from the CRC Handbook of Chemistry and Physics (76th ed.) and (†) from Parfums Cosmetiques Savons de France (Vol. 1, n°5, 1971); NA, values not available. Concentrations (in M) were calculated using the formula PV = nRT, where P is the saturated vapour pressure in mm of Hg, V is the volume of one mole (22.4 L), R is the universal gas constant (62.381 L/mmHg/°K/M), and T is the absolute temperature.

Stimulus presentation consisted of 2-s square odour pulses delivered on the mucosa at a constant temperature (22 °C) and flow rate (200 mL/min.). During stimulation procedure, stimuli were delivered from the lowest to the highest concentrations and a delay of at least 2 min was left between successive presentations.

Experimental paradigm

Single-cell activities and EOGs were recorded simultaneously. As the EOG reflects the global response of the OSN population, its amplitude directly reflected stimulus efficiency. This allowed us to verify that the mucosa remained in good health during the whole experimental procedure. Each single-cell response profile was determined by testing each available stimulus at a concentration close to SV. This led us then to choose two effective stimuli whose concentration–response curves were established when each stimulus was delivered singly and mixed. Stimuli were applied directly near the surface of the olfactory mucosa. For each recorded OSN, a minimum of five concentrations of each odour molecule and mixture were tested, so that potential interactions between odours were quantified dynamically from threshold to the maximal concentration. This constitutes a major point of the present paradigm because results from biochemical assays (Breer & Boekhoff, 1991; Noe & Breer, 1998) and patch-clamp studies (Olson & Derby, 1995) have shown that binding interactions between mixture components depend on their concentration.

Analysis and comparison of concentration–response curves

Currently, recording a single ORN long enough to repeat several times the presentation of the same stimulus at the same concentration and to

establish the concentration–response curves for two single molecules and their mixture is not possible. This is why only threshold concentration tests were repeated at least twice. Because the present study planned to describe mixture interactions occurring at single OSN level by comparing the concentration–response curves, the question of the reliability of the comparison was crucial. The comparison between curves was based on criteria which combined statistical tests used for response identifications and our knowledge of intensity coding. First, we previously observed that response firing frequencies of single OSNs displayed intertrial variabilities, for the same odour and concentration, which were $<15\%$. Thus higher differences were judged as potentially significant. Then, the general aspect of curves was analysed: two curves were judged as being different only if several successive points (at least two) differed by $>15\%$. Moreover, to confirm our decision, the minimum interspike interval in the response firing burst (defined with the statistical Mann–Whitney test; see above) and the latency of the response were taken into account. A higher response had to combine a firing increase $>15\%$, a lower minimum interspike interval and a shorter latency.

Results

General odour responsiveness

We recorded 132 OSNs in 51 rats. Seventy-two percent of the OSNs were responsive to both cAMP and IP_3 odour compounds. When all odour tests were considered ($n = 1157$), 58.5% induced excitatory responses, 2.5% induced suppressive responses and 39% did not evoke any response. These values are similar to those reported in a previous study (Duchamp-Viret *et al*., 1999, 2000). According to the odour molecule, the percentage of cells showing an excitatory response ranged from 43 to 71% and those showing an inhibitory response ranged from 1 to 9% of the tested cells (Table 2). The 'best' inhibitory stimuli were CIN and CAM, two odour molecules qualitatively classified as camphoraceous compounds (Duchamp-Viret & Duchamp, 1997) and biochemically shown as cAMP molecules (Sklar *et al*., 1986; Firestein & Shepherd, 1991).

Interactions of odour molecules in mixture: general observations over the concentration range

Fifty-two OSNs were tested with two single molecules and the corresponding binary mixture both delivered at five concentrations at least through a total of 735 trials. OSN responses to binary mixtures cannot be predicted by simply summing the effects of their components and reveal that complex interactions occurred between stimuli. Several types of mixture interactions were observed. They are described according the terminology used in psychophysiology to compare the perceived intensities of single odours and mixtures (Laing *et al*., 1989; Laska & Hudson, 1993; Cometto-Muniz *et al*., 1999). To do so, the perceived intensity was replaced by the discharge spike frequency of single OSN responses. Responses to mixtures were found to reflect the occurrence of 'hypoadditivity' mechanisms, i.e. the response frequency to the mixture was equivalent to, but not higher than, the response frequency induced by the most effective component at the same concentration. The two other types of interactions observed were termed 'suppression' and 'synergy'. 'Suppression' referred to the case where the response frequency to the mixture was lower than the frequency induced by the most effective component at the same concentration. Conversely, 'synergy' (also termed 'complete additivity'; see Cometto-Muniz *et al*., 1999) was used when the response frequency to the binary mixture was higher than that induced by the most effective component at the same concentration.

Forty-eight percent of recorded OSNs expressed the same type of mixture interaction from the threshold to the highest concentration; 40% showed hypoadditivity and 8% suppression. By contrast, for the remaining 52%, mixture interactions changed as follows with increasing concentrations: synergy toward hypoadditivity (17.5%) or vice-versa (6%), and hypoadditivity toward suppression (17.5%) or vice-versa (11%). No change from synergy toward suppression was seen. The distribution of the interaction types as a function of concentrations for the three types of odour couples (IP_3/IP_3, cAMP/cAMP, cAMP/IP_3) is given in Fig. 1. Independently from all criteria, the most common type of mixture interaction is hypoadditivity. Hypoadditivity varied from 58 to 68% from low to high concentrations against 21–29% for suppression and 20–3% for synergy. On the whole, suppression and hypoadditivity increased with increasing concentration while synergy decreased.

Interactions of odour molecules in mixture: how spike discharge responses of single OSNs express hypoadditivity, synergy and suppression

Hypoadditivity interactions of two excitatory compounds acting in a mixture on a single OSN are illustrated in Fig. 2. In this case, hypoadditivity occurs over the whole concentration range and the mixture curve closely reflects that obtained with the best stimulating component (in term of maximum response frequency), the less effective one in no way antagonizing the action of the former.

TABLE 2. Numbers and percentages of activation, inhibition and no response for the different odour molecules

Odour	Tests (*n*)	Activation (*n*)	Inhibition (*n*)	No response (*n*)	Activation (%)	Inhibition (%)	No response (%)
CAM	69	37	5	27	54	7	39
CIN	35	15	3	17	43	9	49
CIT	119	82	1	36	69	1	30
MENT	109	77	1	31	71	1	28
ISO	63	45	3	15	71	5	24
MAK	58	38	1	19	66	2	33
ANI	66	41	3	22	62	5	33
ACE	60	39	3	18	65	5	30
L-CAR	74	50	0	24	68	0	32
D-CAR	78	50	2	26	64	3	33
LIM	86	51	0	35	59	0	41
EVAN	108	53	0	55	49	0	51
LIL	116	46	0	70	40	0	60
LYR	116	46	2	68	40	2	59
Total	1157	670	24	463			
Mean					58.5	2.7	38.8
SEM					9.7	2.4	9.4

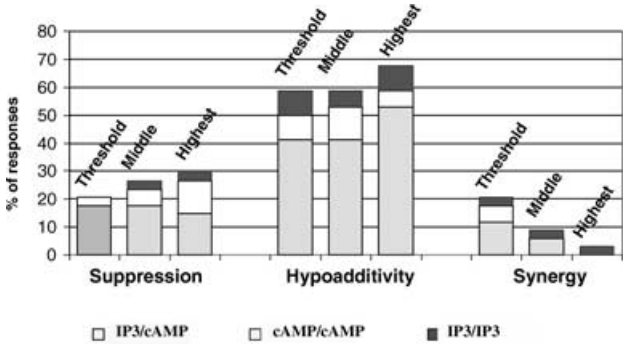

FIG. 1. Distribution of binary interactions. They were determined at three levels of the concentration–response curves (the threshold, middle and maximal levels of the curve kinetic) for the three different odour couples (IP_3/IP_3, cAMP/cAMP, and IP_3/cAMP).

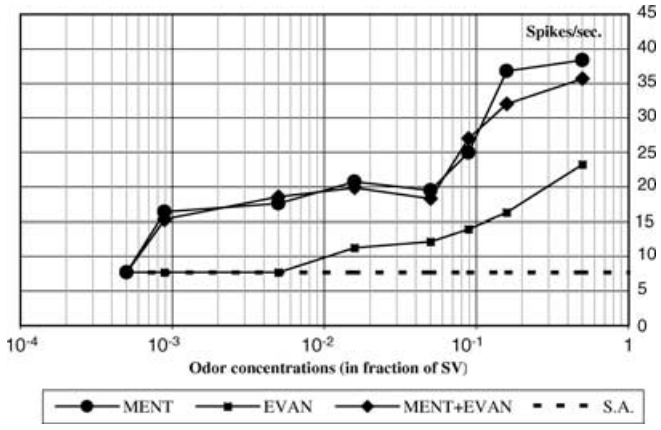

FIG. 2. Example of mixture hypoadditivity: concentration-response curves of the same OSN established with MENT, EVAN and MENT + EVAN binary mixture. Dotted lines indicate the discharge rate of spontaneous activity (SA). Details are in the text.

For mixtures formed of two excitatory molecules, 'synergy' may be expected to occur especially at threshold or medium concentrations at which neurons do not reach their maximal firing. In Fig. 3, CIT and LIL act clearly in synergy at threshold and medium concentrations because the CIT + LIL concentration–response curve shows a steeper slope

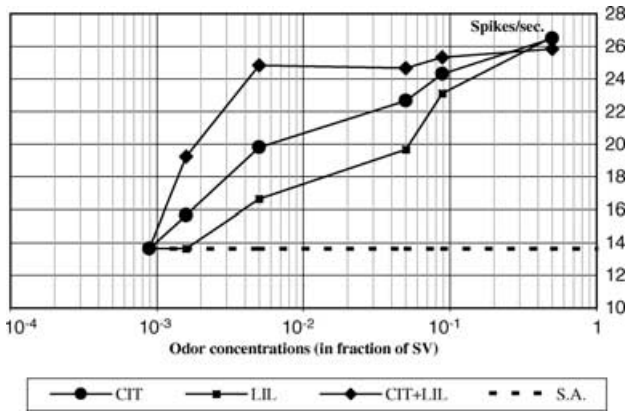

FIG. 3. Example of mixture synergy: concentration–response curves of the same OSN established with CIT, LIL and CIT + LIL binary mixture. Same conventions as Fig. 2.

than curves obtained with single molecules so that higher firing frequencies were reached for lower concentrations. A similar synergy is illustrated in Fig. 4 through temporal response pattern series of the same OSN stimulated with increasing concentration of LIM, MENT and LIM + MENT. Synergistic actions of LIM and MENT in the mixture are visible by eye through the frequency of response bursts obtained at 0.9×10^{-1} and 1.6×10^{-1} M of the SV pressure. For higher concentrations, response frequencies reflected mixture interactions rather close to hypoadditivity.

By contrast, in 'suppression' interactions, the two stimulating odour molecules seemed to show a 'negative cooperation'; the less effective component seemed to counteract the action of the more effective one, at least over part of the concentration range. For example, in Fig. 5, LYR antagonizes the action of CIT at threshold and medium concentrations. Then, for higher concentrations, CIT recovers its efficiency, in spite of the presence of LYR.

A few experiments related to binary mixtures involving two molecules with single opposite actions, i.e. excitation and inhibition, were also performed. This provides the possibility to analyse, as a function of concentration, how an inhibitory molecule counteracts the action of an excitatory one. Such an interaction is illustrated in Fig. 6 where the two mixed molecules, namely MENT and CIN, counteract each other at high concentrations. As a consequence, the inhibitory action of CIN was shifted toward the highest concentrations due to the presence of MENT in the stimulus. Following our definition, such an interaction may also be categorized as 'suppression'.

Discussion

The present study is the first of its kind in mammalian olfaction and might be of interest for further studying transduction and molecular organization of OSNs. It opens the door for studying the mechanisms of mixture effects in mammalian OSNs.

The results demonstrate that interactions between odour molecules in mixtures originate from integration mechanisms taking place at single OSN level. With the design of our study, especially in terms of the stimulus concentration tested, we are able to clearly describe a wealth and variety of mixture effects. These effects were categorized into three types: synergy, suppression and hypoadditivity. A given type may be observed over the whole OSN dynamic range or may shift to another as a function of the concentration. Interaction mechanisms already appear complex even in the simplest case where only two odour molecules are mixed together. This leads to the supposition that, in all probability, the complexity of interactions would increase with the number of odour molecules forming a mixture, especially when the concentrations of the odour molecules differ one from the other. This was approached *de facto* in this study because mixtures were prepared from molecules with various SV pressures. Thus, even delivered at the same fraction of SV, the two components of the mixture were at different concentrations, as is the case in natural odours. This has allowed us to demonstrate that low vapour pressure compounds are able to counteract the action of high vapour pressure molecules (see Fig. 5). This should mean that if the odour molecule concentration appears often as a determinant parameter in mixture interactions, it is not the sole parameter which defines the predominance for an odour molecule in a mixture. This hypothesis is reinforced by our results showing that suppression or synergy mechanisms for mixtures may occur for infraliminar concentrations of one of the components (see Figs 3 and 4).

With regard to the duality of transduction pathways, the analysis of spike activities induced in OSNs could not lead us to objective differences between cAMP and IP_3 odours. The only features which

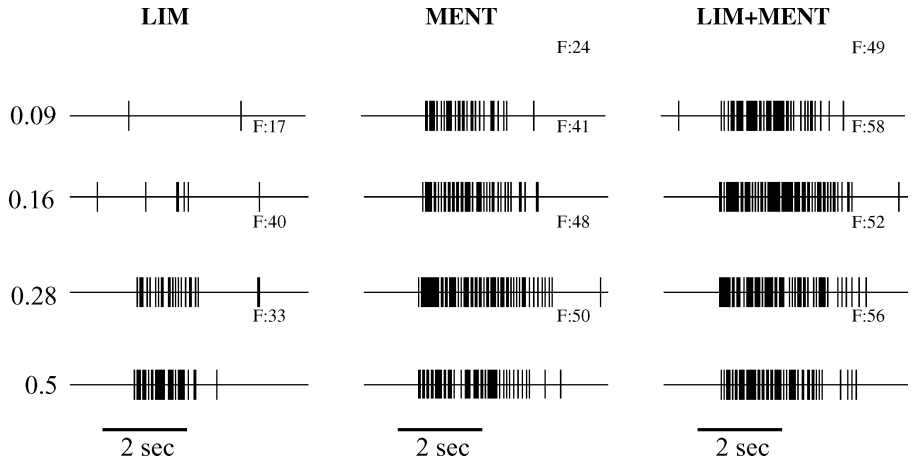

FIG. 4. Series of temporal response patterns of the same OSN stimulated with increasing concentrations of LIM, MENT and LIM + MENT binary mixture. On the left, stimulus concentrations (×SV) are indicated. On the right of each line, the quantified response frequency (F) is given in spikes/s. At the bottom is marked the 2-s stimulus pulse. Here is an example of an additive action of two molecules even when LIM was delivered at infraliminar concentrations.

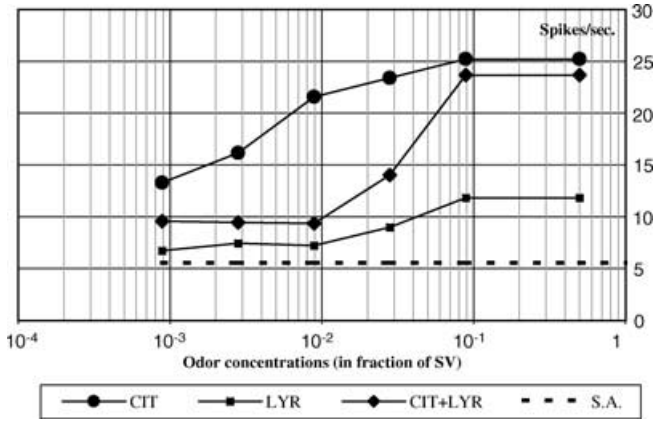

FIG. 5. Example of mixture suppression obtained with two excitatory odour molecules: Concentration-response curves of the same OSN established with CIT, LYR and CIT + LYR binary mixture. Same conventions as Fig. 2.

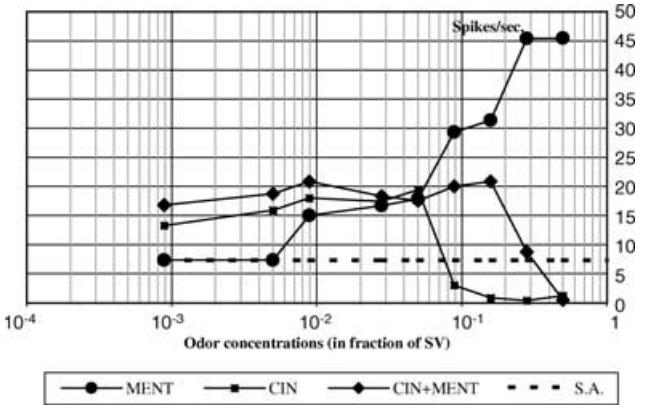

FIG. 6. Example of mixture suppression obtained with one excitatory molecule and one inhibitory molecule: concentration–response curves of the same OSN established with MENT, CIN and MENT + CIN binary mixture. For threshold and medium concentrations, CIN was excitatory, then at high concentrations it became inhibitory. This shift between excitation and inhibition may be ascribed to 'cross-talk' between two transduction pathways which may initiate ionic currents with opposite effects. Same conventions as Fig. 2.

seem to individualize IP_3-termed compounds may be ascribed to their low SV pressure, which probably explains the fact that they induce weaker depolarizing actions than the other compounds. Thus, lack of obvious differences between the action of cAMP and IP_3 odours on OSN electrophysiological responses corroborate recent studies in mammals where the cAMP pathway was advocated as the main olfactory transduction pathway (Gold, 1999; Chen *et al.*, 2000; Wong *et al.*, 2000). At the present time, the IP_3 pathway appears to be a secondary pathway which may modulate the cAMP pathway (Spehr *et al.*, 2002).

Synergy in binary mixtures may result from compounds binding either to the same OR (Buck & Axel, 1991; Chess *et al.*, 1992; Kishimoto *et al.*, 1994) or to different ORs (Cromarty & Derby, 1997). Synergy implies, at least, that the two odour molecules do not counteract each other in binding processes and, at best, that they act in 'positive cooperation'. This may require that each molecule involves additive transduction mechanisms. Nevertheless, synergy would be limited by the fact that the recorded neuron reached its maximum depolarization or by OR saturation. This would explain why synergy may turn into hypoadditivity at high concentrations.

In contrast to synergy, suppression in binary mixtures comprising two excitatory compounds may suppose that the two odour molecules counteract each other either through inhibitory binding mechanisms on the same OR or by setting in motion inhibitory intracellular cross-talk between two excitatory transduction cascades. It has been suggested that, in lobsters and fishes, the ability of an OR to bind one odour molecule composing a binary mixture can be inhibited by the other molecule in a way that seems largely noncompetitive (Ngai *et al.*, 1993; Burgess *et al.*, 1994; Olson & Derby, 1995; Cromarty & Derby, 1998). Such a binding inhibition should depend on the concentration of odours forming the binary mixture and reduces the OSN response to its best stimulus when that stimulus is presented in mixture. These assumptions are in agreement with our observations performed in mammals. Indeed, for a mixture comprising two high vapour pressure components, suppression mechanisms increase with the concentration, which suggests that an allosteric blocking action may be involved. However, low vapour pressure components were also shown to suppress or reduce the action of high vapour pressure components, this suppression being antagonized by an increasing concentration (see Fig. 5). This suggests that low vapour pressure components such as LYR, LIL and EVAN (putative IP_3 molecules) might 'qualitatively'

dominate binding processes due to their high affinity to their binding sites at low concentration whereas their action might be 'quantitatively' supplanted by the high vapour pressure component at high concentrations. Such a shift of the dominating molecule with concentration may have direct consequences on the mechanisms of odour perception.

Suppression in binary mixtures where CIN or CAM showed a dominating inhibiting action when mixed with an excitatory compound (Fig. 6) has to be ascribed to mechanisms different from binding inhibition. As assumed in lobsters (Ache, 1994), such a negative cooperation may be attributed to opposite effects of currents initiated by the two mixture components. Thus, as shown in lobsters and in newts, one odour could counteract the depolarizing current initiated by another excitatory chemical by hyperpolarizing the OSNs (McClintock & Ache, 1989; Michel *et al*., 1991; Michel & Ache, 1992, 1994; Kurahashi *et al*., 1994; Daniel *et al*., 1996; Cromarty & Derby, 1997, 1998). However, given the few inhibitory responses observed in mammalian OSNs in this study, this type of mixture suppression cannot be considered to be preponderant.

Hypoadditivity interactions, which appear as the most common interaction type, may be interpreted as a form of partial suppression regarding synergy (also termed complete additivity; see Laing *et al*., 1989; Laska & Hudson, 1993; Cometto-Muniz *et al*., 1999). In this type of mixture interaction, the best stimulus clearly dominates. This dominance may be ascribed to its higher molecular concentration or to its higher affinity to binding sites and pleads in favour of an action of the two odour molecules on common ORs (Kajiya *et al*., 2001; Touhara *et al*., 2002). By revealing a dominating action of one of the mixture components, mixture interactions such as hypoadditivity and suppression support the hypothesis that odour masking mechanisms are already initiated at the peripheral level of the olfactory system.

Among the three types of binary interactions, synergy suggests that odour molecules may act on different ORs whereas hypoadditivity and suppression would rather imply an action on the same OR. Thus, although we are well aware that numerous unknown parameters remain to be deciphered, we propose that the one OSN–one OR hypothesis might be valid for most OSNs, it being understood that a single OR would have a wide molecular receptive range (Malnic *et al*., 1999; Kajiya *et al*., 2001; Touhara *et al*., 2002).

The fact that the hypoadditivity interaction is the most common interaction fully corroborates psychophysical observations of Laing and coworkers (Laing *et al*., 1984). These authors have reported that the total perceived intensity of a mixture is generally less than the sum of the perceived intensities of its two components, but is never less than the intensity of the weaker component.

Conclusion

In order to be less speculative as to the mechanisms responsible for mixture effects, further electrophysiological data and knowing the molecular structure and sequence of ORs, which receptor types are expressed by each OSN, and the molecular binding range of each OSN would certainly help to explain and refine the mixture odour coding model and to predict mixture effects. This first approach strongly suggests that changes in perception of odours in a mixture, as reported in psychophysical studies, may originate from interactions between odour molecules during their OSN/OR binding phase. Thus, we demonstrate the hypothesis previously proposed by several authors (Bell *et al*., 1987; Derby, 2000; Jinks *et al*., 2001) that decisive integrative neural mechanisms leading to the odour mixture perception would already take place at the peripheral olfactory stage. Even though this may be considered as ensuing directly from a definitive recognition of the OSNs' abilities to interact with various molecules, it has never been experimentally demonstrated by exploring the whole concentration range over which OSNs can work. Thanks to this possibility, our results allow us to draw links between peripheral odour reception and central odour coding and can serve as a future basis for exploring rules which may govern the dominating, even masking, action of odour molecules in mixtures and how a minor component of a mixture may be determinant in the final perception of odour quality. Giving that natural odours comprise several tens of compounds, each of them exhibiting different vapour pressures, molecular structures and binding affinities, our observations with binary mixtures anticipate the complexity of processes which may rise at the level of a single OSN in physiological conditions. OSNs are probably able to integrate the action of several molecules among those forming a complex natural odour. These properties may refine odour coding and play a crucial role in the final performances of the olfactory system.

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Abbreviations

ACE, acetophenone; ANI, anisole; CAM, camphor; cAMP, cyclic adenosine monophosphate; CIN, cineole; CIT, citralva; EOG, electro-olfactogram; EVAN, ethylvanillin; IP_3, inositol triphosphate; ISO, isoamyl acetate; L-, D-CAR, L- and D-carvone; LIL, lilial; LIM, limonene; LYR, lyral; MAK, methyl-amylketone; MENT, menthone; OR, olfactory receptor; OSN, olfactory sensory neuron; SV, saturated vapour.

References

Ache, B. (1994) Towards a common strategy for transducing olfactory information. *Semin. Cell. Biol.*, **5**, 55–63.

Bell, G.A., Laing, D.G. & Panhuber, H. (1987) Odour mixture suppression: evidence for a peripheral mechanism in human and rat. *Brain Res.*, **426**, 8–18.

Breer, H. & Boekhoff, I. (1991) Odorants of the same odor class activate different 2nd messenger pathways. *Chem. Senses*, **16**, 19–29.

Buck, L. & Axel, R. (1991) A novel multigene family may encode odorant receptors – a molecular basis for odor recognition. *Cell*, **65**, 175–187.

Burgess, M., Olson, K., Derby, C. & Daniel, P. (1994) Relationship between inhibition of odorant–receptor binding and physiological binary mixture interactions for glutamate, taurine and 5′AMP receptors in the olfactory organ of the spiny lobster. *Chem. Senses*, **19**, 448.

Chen, S., Lane, A.P., Bock, R., Leinders-Zufall, T. & Zufall, F. (2000) Blocking adenylyl cyclase inhibits olfactory generator currents induced by 'IP (3)-odors'. *J. Neurophysiol.*, **84**, 575–580.

Chess, A., Buck, L., Dowling, M.M., Axel, R. & Ngai, J. (1992) Molecular biology of smell – expression of the multigene family encoding putative odorant receptors. *Cold Spring Harbor Symposia Quantitative Biol.*, **57**, 505–516.

Cometto-Muniz, J.E., Cain, W.S., Abraham, M.H. & Gola, J.M. (1999) Chemosensory detectability of 1-butanol and 2-heptanone singly and in binary mixtures. *Physiol. Behav.*, **67**, 269–276.

Cromarty, S.I. & Derby, C.D. (1997) Multiple excitatory receptor types on individual olfactory neurons: implications for coding of mixtures in the spiny lobster. *J. Comp. Physiol. [a]*, **180**, 481–491.

Cromarty, S.I. & Derby, C.D. (1998) Inhibitory receptor binding events among the components of complex mixtures contribute to mixture suppression in responses of olfactory receptor neurons of spiny lobsters. *J. Comp. Physiol. [a] Sensory Neural Behav. Physiol.*, **183**, 699–707.

Daniel, P.C., Burgess, M.F. & Derby, C.D. (1996) Responses of olfactory receptor neurons in the spiny lobster to binary mixtures are predictable using a noncompetitive model that incorporates excitatory and inhibitory transduction pathways. *J. Comp Physiol. [a]*, **178**, 523–536.

Derby, C.D. (2000) Learning from spiny lobsters about chemosensory coding of mixtures. *Physiol. Behav.*, **69**, 203–209.

Duchamp-Viret, P. (1988) Le codage intensitif du stimulus olfactif: rôle des neurorécepteurs et de deutoneurones, étude électrophysiologique chez la grenouille. PhD Thesis. Université Claude Bernard, Lyon, France.

Duchamp-Viret, P., Chaput, M.A. & Duchamp, A. (1999) Odor response properties of rat olfactory receptor neurons. *Science*, **284**, 2171–2174.

Duchamp-Viret, P. & Duchamp, A. (1997) Odor processing in the frog olfactory system. *Prog. Neurobiol.*, **53**, 561–602.

Duchamp-Viret, P., Duchamp, A. & Chaput, M.A. (2000) Peripheral odor coding in the rat and frog: Quality and intensity specification. *J. Neurosci.*, **20**, 2383–2390.

Duchamp-Viret, P., Duchamp, A. & Vigouroux, M. (1989) Amplifying role of convergence in olfactory system. A comparative study of receptor cell and second order neuron sensitivities. *J. Neurophysiol.*, **61**, 1085–1094.

Fadool, D.A. & Ache, B.W. (1992) Plasma membrane inositol 1,4,5-trisphosphate-activated channels mediate signal transduction in lobster olfactory receptor neurons. *Neuron*, **9**, 907–918.

Firestein, S. & Shepherd, G.M. (1991) A kinetic model of the odor response in single olfactory receptor neurons. *J. Steroid Biochem. Mol. Biol.*, **39**, 615–620.

Gold, G.H. (1999) Controversial issues in vertebrate olfactory transduction. *Annu. Rev. Physiol.*, **61**, 857–871.

Hatt, H. & Ache, B.W. (1994) Cyclic nucleotide- and inositol phosphate-gated ion channels in lobster olfactory receptor neurons. *Proc. Natl Acad. Sci. USA*, **91**, 6264–6268.

Jinks, A., Laing, D.G. & Hutchinson, I. (2001) A psychophysical study of the relationship between temporal processing in odor mixtures and transduction pathways. *Brain Res. Cogn. Brain Res.*, **12**, 273–288.

Kajiya, K., Inaki, K., Tanaka, M., Haga, T., Kataoka, H. & Touhara, K. (2001) Molecular bases of odor discrimination: Reconstitution of olfactory receptors that recognize overlapping sets of odorants. *J. Neurosci.*, **21**, 6018–6025.

Kang, J. & Caprio, J. (1997) In vivo responses of single olfactory receptor neurons of channel catfish to binary mixtures of amino acids. *J. Neurophysiol.*, **77**, 1–8.

Kishimoto, J., Cox, H., Keverne, E.B. & Emson, P.C. (1994) Cellular localization of putative odorant receptor mRNAs in olfactory and chemosensory neurons: a non radioactive in situ hybridization study. *Mol. Brain Res.*, **23**, 33–39.

Kurahashi, T., Lowe, G. & Gold, G.H. (1994) Suppression of odorant responses by odorants in olfactory receptor cells. *Science*, **265**, 118–120.

Laing, D.G., Panhuber, H. & Slotnick, B.M. (1989) Odor masking in the rat. *Physiol. Behav.*, **45**, 689–694.

Laing, D.G., Panhuber, H., Willcox, M.E. & Pittman, E.A. (1984) Quality and intensity of binary odor mixtures. *Physiol. Behav.*, **33**, 309–319.

Laska, M. & Hudson, R. (1993) Discriminating parts from the whole – determinants of odor mixture perception in squirrel monkeys, Saimiri-sciureus. *J. Comp. Physiol. [a] – Sensory Neural Behav. Physiol.*, **173**, 249–256.

Malnic, B., Hirono, J., Sato, T. & Buck, L.B. (1999) Combinatorial receptor codes for odors. *Cell*, **96**, 713–723.

McClintock, T.S. & Ache, B.W. (1989) Hyperpolarizing receptor potentials in lobster olfactory receptor cells – implications for transduction and mixture suppression. *Chem. Senses*, **14**, 637–647.

Michel, W.C. & Ache, B.W. (1992) Cyclic nucleotides mediate an odor-evoked potassium conductance in lobster olfactory receptor cells. *J. Neurosci.*, **12**, 3979–3984.

Michel, W.C. & Ache, B.W. (1994) Odor-evoked inhibition in primary olfactory receptor neurons. *Chem. Senses*, **19**, 11–24.

Michel, W.C., McClintock, T.S. & Ache, B.W. (1991) Inhibition of lobster olfactory receptor cells by an odor-activated potassium conductance. *J. Neurophysiol.*, **65**, 446–453.

Ngai, J., Chess, A., Dowling, M.M. & Axel, R. (1993) Expression of odorant receptor genes in the catfish olfactory epithelium. *Chem. Senses*, **18**, 209–216.

Noe, J. & Breer, H. (1998) Functional and molecular characterization of individual olfactory neurons. *J. Neurochem.*, **71**, 2286–2293.

Olson, K.S. & Derby, C.D. (1995) Inhibition of taurine and 5′AMP olfactory receptor sites of the spiny lobster Panulirus argus by odorant compounds and mixtures. *J. Comp. Physiol. [a]*, **176**, 527–540.

Sklar, P.B., Anholt, R.R.H. & Snyder, S.H. (1986) The odorant-sensitive adenylate cyclase of olfactory receptor cells. *J. Biol. Chem.*, **261**, 15538–15543.

Spehr, M., Wetzel, C.H., Hatt, H. & Ache, B.W. (2002) 3-phosphoinositides modulate cyclic nucleotide signaling in olfactory receptor neurons. *Neuron*, **33**, 731–739.

Touhara, K. (2002) Odor discrimination by G protein-coupled olfactory receptors. *Microscopy Res. Technique*, **58**, 135–141.

Touhara, K., Sengoku, S., Inaki, K., Tsuboi, A., Hirono, J., Sato, T., Sakano, H. & Haga, T. (1999) Functional identification and reconstitution of an odorant receptor in single olfactory neurons. *Proc. Natl Acad. Sci. USA*, **96**, 4040–4045.

Vigouroux, M., Viret, P. & Duchamp, A. (1988) A wide concentration range olfactometer for delivery of short reproducible odor pulses. *J. Neurosci. Meth*, **24**, 57–63.

Wong, S.T., Trinh, K., Hacker, B., Chan, G.C., Lowe, G., Gaggar, A., Xia, Z., Gold, G.H. & Storm, D.R. (2000) Disruption of the type III adenylyl cyclase gene leads to peripheral and behavioral anosmia in transgenic mice. *Neuron*, **27**, 487–497.