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Brief olfactory learning drives perceptive sensitivity in newborn rabbits: new insights in peripheral processing of odor mixtures and induction

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Abstract

Perception of the wide, complex and moving odor world requires that the olfactory system engages processing mechanisms ensuring detection, discrimination and environment adaptation, as early as the peripheral stages. Odor items are mainly elicited by odorant mixtures which give rise to either elemental or configural perceptions. Here, we first explored the contribution of the peripheral olfactory system to configural and elemental perception through odorant interactions at the olfactory receptor (OR) level. This was done in newborn rabbits, which offer the opportunity to pair peripheral electrophysiology and well characterized behavioral responses to two binary mixtures, AB and A'B', which differ in their component ratio (A: ethyl isobutyrate, B: ethyl maltol), and that rabbit pups respectively perceived configurally and elementally. Second, we studied the influence on peripheral reactivity of the brief but powerful learning of one mixture component (odorant B), conditioned by association with the mammary pheromone (MP), which allowed us to assess the possible implication of the phenomenon called induction in neonatal odor learning. Induction is a plasticity mechanism expected to alter both the peripheral electrophysiological responses to, and perceptual detection threshold of, the conditioned stimulus. The results reveal that perceptual modes are partly rooted in differential peripheral processes, the AB configurally perceived mixture mirroring odorant antagonist interactions at OR level to a lesser extent than the A'B' elementally perceived mixture. Further, the results highlight that a single and brief MP-induced odor learning episode is sufficient to alter peripheral responses to the conditioned stimulus and mixtures including it, and shifts the conditioned stimulus detection threshold towards lower concentrations. Thus, MP-induced odor learning relies on induction phenomenon in newborn rabbits.

Keywords

odor mixtures; configural perception; learning; peripheral processing; induction; newborn

1. Introduction

The world of scents is defined by a quasi-limitless number of odor molecules (odorants) and their combinations in mixtures organisms have to deal with. Through behavioral and psychophysical experiments, odor mixtures have been revealed as perceived in a non-linear way since interactions between odorants such as synergy, overshadowing or masking may occur [1, 2] and find their origin, at least in part, at the olfactory system peripheral level [3, 4]. Indeed, in the olfactory mucosa lie olfactory receptor neurons (ORNs) expressing olfactory receptors (ORs), which are mainly weakly selective [5-7]. As a consequence, odorants in mixture combine their simultaneous actions on multiple ORs, resulting in antagonist or agonist interactions through competitive or non-competitive (allosteric) effects [8-12]. At the end of the integration process, certain of or all of the odorants that compose a mixture may be perceived (elemental perception) or a new odor quality may arise, i.e., a configural odor quality specific to the whole mixture and distinct from the element qualities (configural perception) [13, 14]. These two modes may be shown as two poles defining the olfactory perception axis along which there is a perceptive continuum, which goes through a dual perception of both elements and configuration named weak configural perception [15].

Strikingly, elemental and configural perceptions are both functional from early in life. Thus, newborn rabbits perceive weak configurally a binary AB mixture of element A (ethyl isobutyrate) and B (ethyl maltol) [16-18], which is also perceived configurally in human adults [e.g., 19] and adult mice [20]. The ratio of components is a crucial factor in this perception. Indeed, the AB configural perception requires a 30/70 ratio of A/B (AB mixture) while a 68/32 ratio (A'B' mixture) elicits the AB elemental perception in newborn rabbits [21]; see [19] and [20] for convergent results in humans and mice. While some neurophysiological corollaries of AB and A'B' differentiated perceptions have been found in the newborn rabbit brain [22], the question of the processing of these mixtures at the peripheral level and the contribution of this

level to elemental vs. configural perception has remained unexplored so far. The first aim of the present study was to fill that gap in the newborn rabbit model, which offers the advantage of being able to pair peripheral electrophysiology (e.g. [23, 24]) and well characterized behavioral responses.

In olfaction, a major process that implements and modulates perception and contributes to the immediate and long-term adaptation of organisms to their environment is plasticity, which occurs from the peripheral captors. However, much attention has been paid to plasticity mechanisms occurring in higher brain areas (i.e. [25-28]) and much less is known about peripheral mechanisms. Perceptual olfactory plasticity, dealing with peripheral changes and driven by experience, has been first described in human adults. Indeed, Wysocki and colleagues have observed that when patients displaying androstenone specific anosmia were exposed to androstenone for some minutes, three times a day during six weeks, perception of this odorant was restored [29]. These changes have been termed “experienced incidental induction” and because anosmia was suspected to result from a genetic deficit in peripheral captors, they have been assigned to the olfactory system periphery. Repeated exposure to androstenone would result in sensitization of some olfactory receptor neurons (ORNs) equipped with androstenone-binding ORs. Such ORs would be initially at subthreshold density in anosmic patients and the sensitization would increase their expression [29]. The periphery involvement has been supported in experiments reporting that chronic exposures to odorants increased the peripheral olfactory responses (measured through electro-olfactograms, EOG) in anosmic mice and humans [30, 31]. These results concerned with anosmic patients and animals have been then extended to normosmic ones. Subsequently, a body of experimental data, collected throughout the animal kingdom (from insects to various vertebrates, including in humans) confirmed that changes in peripheral reactivity can happen following odorant learning or exposure protocols; such changes could result mainly in an increase [32-42] or sometimes in a decrease in responses

to target stimuli [43] (or even in no change [44,45]). In parallel with these changes, regulation in the expression rate of some ORs [43, 46] or proteins involved in peripheral transduction [47] has been reported.

Thus, the analysis of the literature currently leads to consider induction as a generic phenomenon, with a "positive side" (enhancement of olfactory abilities) that would mainly occur after discontinuous exposure and/or conditioning, whereas passive and continuous exposure to odor cues would rather reveal a "negative side" (loss in olfactory reactivity or habituation). From the mechanistic perspective, induction would quantitatively and/or qualitatively alter the expression of peripheral captors (ORs) leading to an optimized peripheral tuning, without ceasing to favor the detection of new odors [48, 49]. Importantly, in conjunction with the peripheral effect, induction is considered to impact the olfactory perceptual detection threshold [29]. This aspect is unfortunately weakly documented, although it directly links peripheral processing and perceptual/behavioral performances.

In the context of induction, newborn rabbits constitute a relevant model to study to what extent the induction phenomenon is involved in learning-induced plasticity in a newborn mammal, an issue that has never been addressed to date. This constituted the second aim of our study. Rabbit neonates can indeed learn a new stimulus (odorant or odor mixture) after a remarkably powerful (unique, extremely rapid < 5 min) associative conditioning promoted by the mammary pheromone (MP) (e.g., [16, 22, 50]), a signal naturally emitted by all lactating females in their milk and used spontaneously by pups to localize the nipples and suck (e.g., [50, 51]). Twenty-four hours after the conditioning, rabbit pups display in response to the CS the typical orocephalic behavior usually triggered by the MP itself. Thus, in close conjunction with our analysis of the peripheral responses to mixtures vs. mixture components, we therefore tested induction by exploring in naive vs. conditioned pups the two criteria changes observed in humans [29], i.e., the consequences of MP-induced conditioning for odorant B (contained in

the AB and A'B' mixtures) on CS electrophysiological responses (EOG) and detection threshold of this odorant.

2. Methods

2.1. Animals

New-Zealand rabbit pups (Charles River strain, France) originated from the breeding facility of IUT Feyssine (Université Lyon 1, France). Adult males and females were housed individually in cages under constant 12:12 light-dark cycle (light on at 7 am), temperature (21-22°C) and *ad libitum* access to food and water. Nest boxes were fixed to the housing cages of pregnant does two days prior to parturition, to allow nest building. The day of birth was designated as postnatal day 0. To equalize the time of mother-young interaction, females were allowed access to the nest box only once a day for 15 min at 11:30 am. This short daily period of nursing respected the natural rhythm of maternal visits [52]. A total of 239 pups aged 2-4 days from 56 litters were used in this study, 84 for electrophysiological recordings (38 naive, 46 conditioned) and 155 for behavioral procedures. All experiments were performed in accordance with ethic rules enforced by French law and were approved by the ethical committee of Lyon 1 University (CEE-55) and the French Ministry of Higher Education and Research under the no. 9745.

2. Stimuli

The stimuli were the mammary pheromone (MP; 2-methylbut-2-enal; CAS# 497-03-0), ethyl isobutyrate (odorant A; CAS# 97-62-1), ethyl maltol (odorant B; CAS# 4940-11-8), and their mixtures (all the odorants were purchased from Sigma-Aldrich; Saint-Quentin-Fallavier, France). The latter were AB (30/70 ratio), which was reported as generating a configural perception of a pineapple odor in human adults (e.g., [19, 53]), adult mice [20], and weak

configural perception in newborn rabbits (e.g., [16-18, 22]), and A'B' (68/32 ratio), which generates elemental perception in both human adults [19, 53], mice [20] and newborn rabbits (e.g., [16-18, 22]).

For each odorant, stock solutions were prepared at 10^{-2} v/v, first in ethanol and then in successive dilutions in distilled water (ethanol: 0.01% in stock solutions; this solvent has been demonstrated as behaviorally neutral for rabbit pups, see [16]). To induce the associative learning of odorant B, MP was used as unconditioned stimulus at 10^{-5} g/ml (as previously validated (e.g., [16, 17, 22, 50])). The MP-B blend was prepared from 10^{-2} g/ml stock-solutions of each component in distilled water, with a final concentration of 10^{-5} g/ml of MP and B at a 50/50 ratio.

In animals dedicated to the behavioral paired with electrophysiological recordings, two groups were formed, each of which was stimulated with one or the other set of stimuli detailed in Table 2. The two series of stimuli included A, B, AB and MP (AB series) or A', B', A'B' and MP (A'B' series).

In rabbit pups devoted to the behavioral threshold determination, the conditioned odorant B was tested at concentrations ranging from 10^{-5} to 10^{-22} g/ml in 9 distinct groups (successive dilutions in distilled water; Table 1).

3. Odor conditioning

Conditioning sessions were run on postnatal days 1, 2 or 3 in an experimental room close to the breeding room of the animal unit. They occurred 1h before the daily nursing (10:30 a.m.) to equalize the pups' motivational state and limit the impact of satiation on responses [54]. The pups were transferred by groups of 5/litter into a box maintained at room temperature. The MP-induced conditioning was then run following a procedure previously validated (e.g. [16, 17, 22, 50])). Briefly, 6 ml of the B + MP blend were pipetted on a 10 x 15 cm cotton pad, which

was held 2 cm above the pups for 5 min. One minute after the end of conditioning, the pups were individually marked and returned to their nest. Each procedure was run on 3 or 4 pups/litter; to avoid litter effect, only 1 or 2 of these pups were finally used for testing.

4. Behavioral assay

The behavioral assay consisted of an individual oral activation test previously validated (e.g. [16, 17, 21, 22, 50, 51]). Briefly, each pup was gently maintained in one gloved hand of the experimenter, allowing only head movements; with the other hand, the experimenter presented the olfactory stimulus for 10 sec on the tip of a glass rod 0.5 cm in front of the nares. The test was positive when the stimulus induced the on/off typical orocephalic response, i.e., head-searching usually followed by oral grasping movements. Spontaneously, this response is highly selective, meaning that naive animals respond only to the MP. In our three procedures involving behavior, each pup was tested with a maximum of 6 stimuli with an inter-trial interval of 60 sec. The order of stimuli presentation was counterbalanced from one to another pup (except for the MP presentation which always occurred last, as a control). If a pup responded to a stimulus, its nose was softly dried before the next stimulation. The pups were immediately reintroduced to their nest after testing. To minimize litter effects, each group was drawn from several litters, with a maximum of 5 pups tested per litter.

4.1. Procedure 1 - Behavioral test before electrophysiology: pre-recording test

The naive animals were all tested with the 3 odor stimuli from either the AB or A'B' series (Table 2), and systematically to the MP in order to assess that they did not respond spontaneously to unfamiliar stimuli but to the MP. The conditioned animals were tested to the same stimuli as the naive pups, 24h after odor learning, to determine the effectiveness of the conditioning to B. All the animals were tested at 8:00 a.m., i.e., at least 30 min before the

beginning of electrophysiological recordings. Importantly, the same series was then used for the electrophysiological recordings.

4.2. Procedures 2 and 3 - Behavioral test for detection threshold determination

We distinguished two procedures that used the same oral activation test in order to evaluate first the spontaneous detection threshold (ST) of naive animals to odorant B, and second the detection threshold to B after conditioning (CT) to that odorant.

Determining the spontaneous threshold to B was challenging due to the absence of spontaneous behavioral response to that odorant in rabbit pups. Our strategy consisted in a “learning failure” approach meaning that we determined the concentration level at which odorant B could not be learned anymore. Thus, 7 groups of 1- or 2-day-old pups (15 newborns/group; n total = 105) were conditioned with the MP at 7 decreasing concentrations of B from 10^{-10} to 10^{-21} g/ml (one concentration/group). All the pups were then orally tested the day after at B 10^{-5} g/ml.

Regarding the post-conditioning threshold to B, 2 groups of 2-day-old pups (n = 25/group) were conditioned to B at 10^{-5} g/ml by pairing with the MP, then tested 24h later either to B at 10^{-5} , 10^{-10} , 10^{-15} , 10^{-19} and 10^{-20} g/ml or at 10^{-5} , 10^{-17} , 10^{-21} and 10^{-22} g/ml, and systematically to MP 10^{-5} g/ml.

5. Electrophysiology

The electrodes were homemade silver wire ended with a silver ball plated with AgCl (200-500 μ m diameter, impedance \leq 100 k Ω). Each electrode was connected to an amplifier (WPI, DAM80, amplifier, DC, gain 1000), the output signal of which was connected to a computer via an acquisition card (analog to digital interface). The three electrodes were placed on the surface of the mucosa using micromanipulators, each on a turbinate. The electrodes were always placed in the distal part of the turbinates (Figure 1a). However, because the precision of

these locations cannot be based on any coordinate and animals naturally presented variations in morphology (size, inclination and proximity of the turbinates to a greater or lesser extent), this choice was only intended to standardize the measurements without any chemotopic information being derived from them; knowing that the existence of chemotopy has not yet been proven. Recording visualization and acquisition were made on-line using homemade Neurolabscope© software. Data analysis was made off-line through a python script designed by S. Garcia (CRNL, Lyon). Recordings are illustrated in Figure 1, which shows three simultaneously recorded EOGs (raw signals) from the three turbinates and details the amplitude measure.

6. Olfactory stimulation

The olfactory stimuli were delivered in gas phase through an olfactometer derived from a previous version [3]. The gaseous phase of odorants was however sucked off from vial by bubbling 200 ml of odorized solution instead of U-shaped tubes filled with absorbent pellets. This ensured that electrophysiological recordings were done by using exactly the same stimulus concentrations as those used in previously published and the present behavioral experiments. The stimulation consisted of 0.35 sec square pulses at an 80 ml/sec flow rate. The protocol consisted of delivering the same stimulus 3 times successively at 1-min intervals; the 3 measures being averaged.

7. Statistical analyses

7.1. Behavioral data

Proportions of pups responding behaviorally were compared using the Cochran's Q test when the data were dependent (i.e., pups from the same group tested for their response to the three stimuli) or the χ^2 test of Pearson (with Yates correction when necessary) when the data were independent (i.e., distinct groups tested for their response to the same stimulus). When the Cochran's Q test was significant, proportions of responding pups were compared 2x2 by the χ^2

test of McNemar. Degrees of freedom are indicated when > 1 . Data were considered as significant when the two-tailed tests yielded $p < 0.05$. Analyses were made with Statistica software (StatSoft, Tulsa, OK, USA).

7.2. Electrophysiological data

A total of four sets of data were gathered: two sets from naive and two sets from conditioned animals, each of which was stimulated by either the AB or the A'B' series. Mean EOG amplitudes were compared inside each data set using non parametric paired-Friedman test. Then, a Mann-Whitney *post hoc* paired test was used to compare stimuli by pair. For the comparison between naive and conditioned groups, the same tests were used but for unpaired data. The frequency of occurrence of each stimulus as being the least and the most effective within the naive and conditioned animals were tested with the χ^2 test (comparison of k proportions) and Monte Carlo method (distribution of the χ^2 distance based on simulations with the total number of observations for the k groups, followed by the Marascuilo procedure, which carries out to the pairwise comparisons (XLSTAT software, Microsoft, Redmond, USA). The same tests were used to compare, for each stimulus, its occurrence as being the least and the most effective stimulus in naive vs. conditioned pups.

3. Results

3.1. Peripheral processing of odorants and mixtures

3.1.1. Naive animals: spontaneous behavior and peripheral processing

From the 38 pups assigned to the naive group, 31 gave exploitable records, 5 did not and 2 were discarded because they did not respond to MP in the pre-recording behavioral test. Among the 31 recorded pups, 15 were stimulated with the stimulus series AB comprising A, B, AB and MP, while the remaining 16 pups were stimulated with the A'B' series including A', B', A'B' and MP. The behavioral pre-recording test showed that all pups responded positively to the MP,

and none to the other odorants (in each series $Q > 40$, $p < 0.001$ and $\chi^2 > 13$, $p < 0.001$ for global and 2x2 comparisons) (Figure 2; for simplification, results of the two series have been pooled in the Figure).

The mean EOG amplitudes recorded in naive pups in response to the two-stimulus series are shown in Figure 3. For the AB series, regardless of the stimulus, EOGs recorded in turbinate 1 (T1) were statistically smaller than those recorded in T2 and T3 (Friedman test, $p = 0.007$, T1 vs. T2: $p < 0.0001$, T1 vs. T3: $p = 0.002$). For the A'B' series, no statistical differences were observed between the three turbinates.

In the AB series (Figure 3a), the MP induced the largest response in T2 (Wilcoxon: $0.04 \leq p \leq 0.06$) and one of the largest in T1 and T3. Responses to the AB configural mixture were not different from those of its components in T2-T3, lower than responses to A and MP in T1 (Wilcoxon: $p = 0.042$ and 0.037) and lower than response to MP in T2.

In the A'B' series (Figure 3b), the MP elicited one of the largest responses in T1 and the largest one in T2 and T3 (Wilcoxon: $0.0001 \leq p \leq 0.05$). By contrast, the elemental mixture A'B' elicited one of the lowest responses in T1 and T2. In particular, response to A'B' was lower than that to the single A' component (A'B' vs. A' in T1 and T2: $p=0.002$ and <0.001), despite the mixture concentration being very close to that of A'.

To sum up, regardless of the turbinates, the AB and A'B' mixtures spontaneously induced differential responses compared to their components: the AB mixture tended to trigger responses closer to its components than the A'B' mixture, which mostly induced lower responses than A'. Strikingly, the MP appeared in the two series as the most, or one of the most, powerful stimuli (despite a concentration lower than those of A, A', AB and A'B').

3.1.2. Conditioned animals: post-learning behavior and peripheral processing

From the 46 pups assigned to the conditioned group, 36 gave exploitable records, 3 did not and 7 were discarded because they failed to give clear behavioral responses to B (CS). Among these 36 newborn rabbits, 17 pups were stimulated with the AB series and 19 pups with the A'B' series. In terms of behavior, within the AB series, pups strongly responded to B (94.1%) and to the MP (100%) but not to A and AB (whole comparison: $Q = 47$, $df = 3$, $p < 0.001$; 2x2 comparisons between B or MP vs. A or AB: $\chi^2 > 14.06$, $p < 0.001$; B vs. MP: $\chi^2 < 0.5$, $p > 0.05$) (Figure 2; to simplify reading, responses of the two series were pooled in the Figure).

Within the A'B' series, a strong proportion of pups responded behaviorally to B (89.5%) and to the MP (100%) as for the AB series, but also to A'B' (84.2%); however, as for the AB series, the pups did not respond to A' (0%) (whole comparison: $Q = 46$, $df = 3$, $p < 0.001$; 2x2 comparisons between B or MP or A'B' vs. A': $\chi^2 > 14.06$, $p < 0.001$; B vs. MP or AB and MP vs. AB: $\chi^2 < 0.5$, $p > 0.05$) (Figure 2). Thus, as expected, conditioned pups responded behaviorally more to B and to A'B' than naive animals ($\chi^2 > 20.5$, $p < 0.001$ in the 2x2 comparisons between conditioned vs. naive pups) and as much to the MP (100%) (Figure 2).

The mean EOG amplitudes recorded in conditioned pups in response to the two-stimulus series are shown in Figure 4. In the AB series (Figure 4a), EOGs' amplitudes recorded in T1 differed from those recorded in T2 and T3 (Friedman test, $p < 0.0001$, T1 vs. T2 and T1 vs. T3, $p < 0.001$); T1 EOGs being lower regardless of the stimulus. In addition, T2 and T3 responses differed too (Friedman, $p = 0.024$); noticeably in T2, B induced larger EOGs than in T3 (Wilcoxon, $p = 0.041$). In T2, A and B both elicited larger responses than AB (Wilcoxon, $B > AB$, $p = 0.008$; $A > AB$, $p = 0.001$). In T3, odorant A, the odorant with the highest concentration, induced larger responses than AB (Wilcoxon, $p = 0.003$) and MP (Wilcoxon, $p = 0.029$).

In the A'B' series (Figure 4b), EOGs' amplitudes recorded in T1 differed from those recorded in T2 and T3 (Friedman, $p < 0.001$, T1 vs. T2 $p < 0.0001$; T1 vs. T3 $p = 0.0002$); T1 EOGs being lower regardless the stimulus. In T2, B' induced larger responses than A'B' and MP (B'

> A'B'; Wilcoxon, $p = 0.028$, $B' > MP$; Wilcoxon, $p = 0.031$). In T3, B' induced larger responses than A'B' (Wilcoxon $p = 0.037$).

To sum up, regardless of the turbinates, after conditioning to the odorant B, the AB mixture triggered lower responses compared to A, while the A'B' mixture triggered responses that were close to those induced by its components. Strikingly, although the conditioned stimulus (B/B') was the lowest concentration stimulus, it triggered the largest or one of the largest responses.

3.1.3. Comparison of EOG amplitudes in naive vs. conditioned animals

Regarding the AB series (Figure 5a), in conditioned pups the response to B increased in T2 ($p = 0.033$) while the response to MP decreased in the three turbinates ($p < 0.045$). Regarding the A'B' series (Figure 5b), in conditioned pups the response to B' increased in T2 and T3 ($p < 0.05$) as well as the responses to A' and A'B' in T2 ($p < 0.05$ and < 0.01). In T1, the response to MP decreased in conditioned pups ($p = 0.037$).

Thus, the conditioning mainly impacted the responses to MP and B (B'): responses to MP decreased while those to B (B') increased, regardless of the stimulus series. However, the conditioning to B appeared to be more impacting on the responses to A'B' than to AB.

3.1.4. Comparison of stimulus relative effectiveness in naive vs. conditioned animals

Here, we addressed the question of the high variability in EOG amplitude for a same stimulus (from one recording site to another, from one nasal cavity to another and from one animal to another), by analyzing the differences in response between different stimuli in relative terms. To that end, based on their amplitude, the EOG responses were ranked from the lowest to the largest and the corresponding stimulus sequence was used to determine the least or the most effective stimuli; only the sets of recording comprising exploitable responses to the 5 stimuli (i.e., complete stimulus sequence) were taken into account. Doing so, we compared the

occurrence of each stimulus as being the least and the most effective one, first, within the naive and conditioned groups for the AB and A'B' series, respectively, and then between the two groups.

3.1.4.1. Occurrence of stimuli as least effective (Figure 6a)

Within the AB-series naive animals (Figure 6a-left, grey bars), all stimuli occurred as the least effective with the same probability ($p > 0.5$). By contrast, within the A'B'-series naive animals (Figure 6a-right, grey bars) statistical differences appeared between stimuli ($p < 0.0001$, χ^2 and Monte Carlo method): the occurrence of A'B' differed from that of A' and MP, A'B' being most frequently the least effective stimulus ($p < 0.05$, pairwise comparisons by the Marascuilo procedure).

In conditioned animals, statistical differences existed in both stimulus series (χ^2 and Monte Carlo method; $p < 0.0001$). In the AB series (Figure 6a-left, black bars), the stimulus pairwise comparison (Marascuilo procedure) showed that AB and MP occurred more frequently as the least effective stimuli compared to A. In the A'B' series (Figure 6a-right, black bars), A' was most rarely observed as the least effective stimulus in comparison with B', A'B' and MP ($p < 0.05$).

The comparisons between naive and conditioned groups showed that the occurrence of MP as the least effective stimulus increased from 28% to 43% ($p < 0.05$, in the AB series) and from 18% to 34% ($p = 0.02$; in A'B' series) in conditioned neonates. Comparatively, the occurrence of A'B' as the least effective stimulus decreased from 43% in naive pups to 29% in conditioned ones ($p < 0.05$, bilateral test of two proportions, confirmed with Monte-Carlo method).

3.1.4.2. Occurrence of stimuli as most effective (Figure 6b)

Within both naive and conditioned groups, and in both AB and A'B' series, stimuli differed in their occurrence as the most effective stimulus ($p < 0.0001$, χ^2 and Monte Carlo method). In the AB-series naive animals (Figure 6b-left, grey bars), MP and A significantly differed from

B and AB ($p < 0.05$), and were most often the most effective stimuli. In the A'B'-series naive animals (Figure 6b-right, grey bars), MP and A' differed from B and A'B', and B differed from A'B' ($p < 0.05$). MP and A' were most often the most efficient stimuli especially compared to A'B', which never induced the largest EOGs.

In the AB-series conditioned animals (Figure 6b-left, black bars), A and B differed from AB ($p < 0.05$) and were the most effective stimuli. In the A'B'-series conditioned animals (Figure 6b-right, black bars), the odorants B, A' and MP were more frequently the most effective stimulus compared to A'B' ($p < 0.05$).

Comparisons between naive and conditioned pups showed that the occurrence of B as the most effective stimulus increased in conditioned vs. naive pups for both the AB and A'B' series (14 vs. 34 % and 13 vs. 33% respectively; $p \leq 0.002$ for the two groups). Furthermore, the occurrence of A'B' as the most effective stimulus increased from 1% to 15%, ($p = 0.001$). The opposite result was observed for MP after conditioning: its occurrence as the most effective stimulus decreased from 46 to 24% ($p = 0.001$).

To sum up, regardless of the AB and A'B' series, the comparison of relative effectiveness between the stimuli showed that MP, the most effective stimulus in naive animals, lost this status in conditioned pups: since, after conditioning its occurrence increased as the least effective stimulus, while simultaneously decreased as the most effective one. At the same time, the frequency of odorant B (i.e., the conditioned stimulus) as the most effective stimulus, alone (for the AB series) or in the elemental mixture A'B' (for the A'B' series), increased in conditioned animals, whereas the occurrence of B as the lowest effective cue did not change in the two series.

3.2. Detection threshold in naive and conditioned animals

Our original assumption considered that MP-induced conditioning is based on induction. This assumption requires theoretically that some plasticity happens at the periphery of the olfactory system during/after the conditioning (see above), and also that the detection threshold of the conditioned stimulus is influenced by the conditioning. Here, we therefore determined the detection threshold of the odorant B, both before and after it has been learned by pairing with the MP.

3.2.1 Spontaneous detection threshold of the odorant B

Because rabbit pups did not respond spontaneously to B, the determination of B spontaneous threshold (ST) could not be evidenced in really naive animals but in animals which failed to learn. This issue was addressed by using decreasing concentrations of B paired with a constant concentration of MP, expecting that at a given concentration of B this odorant would cease to be perceived and, therefore, would not be learned anymore. The ST was then set between the last concentration of B supporting learning and the first concentration of B failing to induce it. We tested 105 pups divided in 7 groups. Each group was conditioned by pairing B at 10^{-5} , 10^{-10} , 10^{-15} , 10^{-17} , 10^{-20} , 10^{-21} or 10^{-22} g/ml with MP at 10^{-5} g/ml. All the pups were tested 24h later for their orocephalic response to B alone at 10^{-5} g/ml.

The results showed that differences existed in pup responsiveness to B depending on the concentration of the odorant used during the conditioning ($Q = 81.2$, $df = 7$, $p < 0.001$) (Figure 7a). A strong and similar proportion of pups responded to B after conditioning at 10^{-5} , 10^{-10} and 10^{-15} g/ml ($> 80\%$; $\chi^2 < 0.7$, $p > 0.05$ in all 2x2 comparisons) attesting that B was effectively learned, i.e., that it was spontaneously perceived at these concentrations. The level of responsiveness dropped dramatically between 10^{-15} and 10^{-17} g/ml (80 vs. 20%; $\chi^2 = 7.1$, $p < 0.01$) and then remained very low from 10^{-17} to 10^{-22} g/ml ($< 20\%$; $\chi^2 < 0.7$, $p > 0.05$ in all 2x2 comparisons).

Thus, rabbit pups could not learn the odorant B when its concentration fell below 10^{-15} g/ml, setting ST for this odorant between 10^{-15} and 10^{-17} g/ml in our conditions.

3.2.2. *Post-conditioning detection threshold of the odorant B*

To determine the detection threshold of odorant B after MP-induced conditioning (“conditioned threshold”, CT), two groups of 25 pups were conditioned to B 10^{-5} g/ml (paired with MP 10^{-5} g/ml), then each tested 24h later to several concentration steps of B over a range from 10^{-5} and 10^{-22} g/ml, and systematically to MP 10^{-5} g/ml in the end. In the two groups, the responsiveness to B varied according to its concentration ($Q < 73.1$, $df = 3$ and 4 , $p < 0.001$). The proportion of responding pups was strong and similar to B from 10^{-5} to 10^{-19} g/ml ($> 80\%$; $\chi^2 < 3.2$, $p > 0.05$ in all 2x2 comparisons) but it strongly decreased at 10^{-20} g/ml (20%; $\chi^2 > 16.05$, $p < 0.001$ in all 2x2 comparisons between 10^{-20} vs. stronger concentration steps) and then remained low or null until 10^{-22} g/ml ($< 20\%$; $\chi^2 < 3.5$, $p > 0.05$ in all 2x2 comparisons) (Figure 7b).

Between the ST and CT determination procedures, no difference contrasted the pups’ responsiveness to B at 10^{-5} , 10^{-10} , 10^{-15} , 10^{-20} , 10^{-21} and 10^{-22} g/ml ($\chi^2 < 0.5$, $p > 0.05$ in all comparisons). Conversely, in the CT determination procedure, rabbit pups responded clearly more to B at 10^{-17} and 10^{-19} g/ml than in the ST determination procedure ($\chi^2 > 20.6$, $p < 0.001$ in the two comparisons).

Thus, after a single and brief conditioning to B at 10^{-5} g/ml, the conditioned threshold appeared to be set between 10^{-19} and 10^{-20} g/ml, i.e., 100 times lower than the spontaneous threshold.

4. Discussion

The present study provides original insights into the peripheral contribution to odor mixtures’ perception, by focusing on the AB and A’B’ mixtures initially characterized as

perceived configurally and elementally, respectively, in both human adults and newborn rabbits, and also more recently in adult rodents [20]; this by using here the same concentrations and ratios of components for acquisition of electrophysiological and behavioral data. At the same time, the use of MP as a reference stimulus led us to observe the peripheral processing of this pheromone. In addition, by assessing in parallel potential alterations of peripheral electrophysiological activity and behavioral detection threshold, we tested whether the induction phenomenon contributes to MP-induced odor learning in the newborn rabbit.

4.1. Spontaneous peripheral processing of odor mixtures

In this work, EOG measures have been done simultaneously from 3 turbinates and successively from the two nasal cavities of pups. This strategy primarily intended to optimize the number of measures and, as expected, no clear-cut chemotopic reactivity between turbinates and stimuli was evidenced; all stimuli induced EOG in the three turbinates. However, this does not mean that there is no differential sensitivity over the turbinates for the used stimuli, but establishing such a difference would require the use of ranges of concentrations for each. This was not the purpose of our work.

Regarding the AB mixture, the mean EOG response to AB was lower than to A in turbinate T1, but not different than those to A and B in T2 and T3, and close to MP, the most effective stimulus in T3. According to the relative comparison of effectiveness, AB was the least effective stimulus in 30% of cases (which is similar to its components, A and B), whereas it was the most powerful in 12% of cases (which is similar to B, only).

Compared to what happened with AB, the interactions between components seem to operate differently regarding the A'B' mixture. In naive animals, A'B' responses were smaller than those to A' in T1 and to the components in T2. When considering the relative effectiveness of

stimuli, A'B' was the least effective stimulus in 44% of cases and the most effective stimulus in only 1%; such frequencies ranked it as the least effective of all the stimuli.

Mixture processing at the peripheral level is reported to as involving mainly suppressive interactions between odorants, whether in binary [3, 4, 11, 55-57] or more complex mixtures [58-60]. Moreover, such a predominance of suppressive interactions seems true whatever the overlapping, or absence of overlapping ORs' assemblies activated by the odorants [60]. In line with this, the responses to AB and A'B' both appeared as mainly resulting from suppressive interactions between the components. However, such interactions seem to be consistently weaker in AB, which would involve either hypoadditive or at least less marked suppressive interactions than A'B'. This result fits with some others gathered in adult rats, suggesting that AB involves hypoaddition, synergy and only then suppression, in descending order of importance [61, 62 and personal observations]. Comparatively, the elemental A'B' mixture would engage stronger suppressive interactions, as shown in rats for the elemental isoamyl acetate (ISO)/whiskey lactone (WL) mixture [4, 61]. Strikingly, both A'B' and ISO/WL combine a high vapor pressure component (A' and ISO, respectively) with a low vapor pressure one (B' and WL). Regarding the AB and A'B' concentrations, the high vapor pressure of A means that in both AB (A/B ratio: 30/70) and A'B' (68/32), the odorants A and A' are largely dominant in concentration: 1.23×10^{-6} and 16.16×10^{-6} mol/l, respectively, vs. 2.38×10^{-12} mol/l for B (and B'). Therefore, the theoretical concentrations of the mixtures are close to those of A and A', while A'B' is about 5 times more concentrated than AB; thus, the higher the concentration of A in mixture (A'B'), the stronger the suppressive interaction with B (and B').

Taken together, the results obtained in rabbit neonates here, and previous ones obtained in adult rats, argue that the peripheral responsiveness to a mixture mainly results from suppressive or antagonistic interactions, whatever the elemental or configural perceptual attributes of the mixture. Additionally, our observations lead us to go further by suggesting that if A and B in

mixture mainly show negative interactions, these interactions are less marked in AB than A'B' depending on the concentration of A/A' (which impacts *de facto* the ratio with B). The observation that AB is perceived configurally, (even weakly) in rabbit pups, may lie in the fact that A and B at the 30/70 ratio and current concentrations set in motion suppressive interactions to a lesser extent than A' and B' do in the A'B' mixture. The reason why A'B' peripheral responses would induce stronger inhibitory interaction than AB remains unknown. It could rely on the higher concentration of A' and negative action of B/B' on OR responding to A' [57].

4.2. Conditioning impacts the peripheral processing of odorant B

In naive animals, as expected because it was the weakest concentrated stimulus in the AB and A'B' series, odorant B induced the smallest or one of the smallest EOG responses. However, B gained in effectiveness after conditioning, in T2 in the AB series' recordings and in T2-T3 in the A'B' series. This gain was confirmed when considering the relative effectiveness of B over the recorded sites: its occurrence as the most effective stimulus increased from 13 to 34%. Noteworthy here is that B learning contemporarily impacted the relative effectiveness of MP in conditioned animals: indeed, the gain in B effectiveness led to a loss of MP peripheral stimulating hegemony in favor of B.

The increase of peripheral responses to a conditioned stimulus, observed here in newborn rabbits, is in agreement with previous studies in adult rodents including peripheral recordings [30, 31, 35-37, 42] and optical imaging of olfactory bulb inputs [38, 63]. However, in rabbit neonates, it is remarkable that this gain observed at the periphery appeared after only a single and extremely brief conditioning episode (5 min), a result that highlights the rapidity of the physiological (i.e., cellular, molecular) mechanisms engaged in peripheral plasticity.

4.3. Influence of odorant B learning on peripheral processing of mixtures including it

The conditioning to B enhanced the contrast between the AB and A'B' mixtures' peripheral processing. Indeed, the mean response amplitude to A'B' was clearly increased in conditioned vs. naive pups, which may mirror weaker suppressive interactions occurring between A' and B' after the conditioning. In parallel, in terms of relative efficacy, A'B' elicited less frequently the lowest response (decreasing from 44 to 29%) and more the highest one (rising from 1 to 15%) in conditioned vs. naive animals. By contrast, responses to AB did not appear to be influenced by B conditioning, neither through the comparison of the absolute mean amplitudes nor the relative effectiveness. Such a differential impact of B learning on peripheral responsiveness to A'B' and AB further supports the different processing of these mixtures and the particular (configural) perception of AB. It can be proposed that after learning, AB and A'B' mixtures faced a differently tuned population of ORs, at least in part. Indeed, odor learning could have increased the expression of ORs presenting high affinity for B [29, 46]; this might contribute to improving the peripheral processing of the elemental A'B' mixture by increasing the perceptual weight of B' (i.e., B) component.

These results from EOGs are fully consistent with those obtained in behavioral tests during which the pups conditioned to B responded to A'B' but not to AB. Interestingly, if a single conditioning to B does not alter the peripheral processing and configural perception of AB, it may already modulate the processing of A'B'. Along the same line, the observation that three or nine repeated conditionings to A or B alters the AB perception and leads rabbit pups to perceive the AB mixture more elementally [64] might also involve OR expression changes.

Altogether, the results highlighting that AB and A'B' are spontaneously processed differently at the periphery and differently influenced by the learning of B are fully in line with previous ones, which indicated the distinct processing of these mixtures both before and after learning at the olfactory bulb and piriform cortex brain levels in newborn rabbits [22].

4.4. Molecular and cellular mechanisms underlying peripheral plasticity and EOG alterations

The current observations of EOG alterations in response to B and to mixtures including B, after the brief B-learning could be supported by OR expression regulation. ORs are expressed in a monogenic, monoallelic and seemingly stochastic location [65, 66]. Furthermore, it has been reported that OR transcription (and then expression) can be induced by odorant stimulation, especially during ORN differentiation, but also in mature neurons, thanks to *de novo* DNA methyltransferase [67]; this enzyme would be responsible for both repressive and active transcriptional states within neurons, which generate the full set of gene responses associated with neuronal plasticity. Regulation of specific ORs' expression have been observed in honeybees in correlation with change in EAG (i.e., EOG in vertebrates; [43]) and with more central plasticity like long term memory [68, 69]. A role of down and up-regulations of ORs would be to ensure that new floral scents are detected and would allow the honeybees to adapt to their ever-changing scent environment [48, 49]. From a broader view, OR plasticity linked to olfactory environment influence appears a widespread phenomenon [46, 48, 49, 70, 71]. Here, the increase of peripheral response to B supports a parallel increase of either the density of ORs dedicated to B processing or of the number of ORNs expressing such ORs (mainly through an activity dependent orientation in OR selection in newly mature ORNs, or both). The reported results [63] that odorant exposure did not alter spatial pattern, peak magnitude, or odorant-selectivity of aldehyde-evoked OSN input to olfactory bulb glomeruli, but did alter the temporal dynamics of that input, plead in favor of an increase of density of B sensitive ORs expressed by ORNs spontaneously responsive to B.

4.5. MP peripheral processing is spontaneously prioritized in rabbit neonates

In a previous *ex vivo* study in rabbit pups, high concentrations of MP (1.17×10^{-5} and 10^{-6} mol/l) induced large EOGs (up to 8 mV; [24]). Strikingly, here, despite the MP delivery at a lower

concentration (7.18×10^{-7} mol/l), which was ranked in the optimal concentration range known to elicit behavioral responses [72], MP induced one of the largest, even the largest EOG in naive pups. This observation supports the conclusion that spontaneous peripheral processing of MP is favored in rabbit neonates' olfactory mucosa, a result consistent with the crucial function of this pheromonal signal in newborn rabbits [e.g., 24, 50, 51, 73]. This raises the question of the origin of MP prioritization at the olfactory system periphery. Since MP is absent in the uterine environment (no trace of the molecule has been found in the amniotic fluid or blood of pregnant rabbit females; [51]), the hypothesis of prenatal MP learning can be rejected. Moreover, the releasing activity of MP is immediately displayed at birth, even before any postnatal exposure to the natural MP contained in the rabbit milk [50, 51]. Therefore, even if in our conditions the peripheral sensitivity to MP could be putatively enhanced thanks to the sucking experience of the first postnatal days, it would only complement a predisposed MP-OR tuning effective immediately at birth. Our results give the first demonstration of the strong and direct link that may exist between peripheral and behavioral processing for a pheromone in mammals as soon as early in life.

4.6. Impact of learning on odorant B detection threshold

MP-induced learning of B resulted in an increase of the B sensitivity of newborn rabbits, in our conditions by a factor of at least 100. This validated the second criteria of induction [29]. Strikingly, in both naive and conditioned pups, the B threshold concentrations were extremely low, i.e., around 3.40×10^{-22} mol/l (1×10^{-15} g/ml) for the spontaneous threshold and 3.40×10^{-26} mol/l (1×10^{-19} g/ml) for the conditioned threshold. Thus, the spontaneous and conditioned thresholds were respectively 5.3×10^{11} and 5.3×10^{15} times lower than the detection threshold observed for MP in a previous study (1.79×10^{-10} mol/l gas estimated concentration; [72]). It may seem surprising that the MP threshold was higher than that of B, a spontaneously non-relevant

odorant. However, in ecological conditions, the MP threshold in newborn rabbits must fit with an optimal detection, discrimination and recognition of that signal in the mother milk. Furthermore, B concentration (at saturated vapor pressure value) is about 7.35×10^6 lower than the MP one. Thus, it can be proposed that the olfactory system adapted to the reality of odor molecule concentrations in order to detect low volatile and (or) concentrated odorants; this may explain, at least partly, why the B threshold value is very low even spontaneously. In line with this hypothesis, the sexual Bombykol pheromone in *Bombyx Mori* (Lepidoptera) is perceived at around 2.82×10^{-22} mol/l (gas estimated concentration from [74]), thus far lower than the MP one but close to the ST observed for odorant B in our conditions (3.40×10^{-22} mol/l). However, although sharing pheromonal status, MP is contained in the milk and detected by rabbit pups in the near proximity of the nipples, whereas Bombykol must be detected in the air during the flying of moths, thus at very low concentrations, requiring highly sensitive and specialized OR(s). The olfactory system of this invertebrate is adapted for such an extreme sensitivity since "one pheromone molecule is sufficient to elicit a nerve impulse" [75]. In rabbit neonates, according to our results, B presents a spontaneous threshold quite close to that of Bombykol.

Thus, it can be summed up first that the olfactory system of rabbit neonate is amazingly potent to spontaneously detect low vapor pressure compounds at very low concentration, and second, that by promoting the rapid learning of the odorant B, MP would further increase such exceptional detection abilities to the odorant that became relevant through conditioning. This may be adaptive in the sense that, in nature, some odorants present in the nest or carried by the maternal body may be learned by MP co-perception, and then become key information for the neonates in their immediate environment, or later on when the pups leave the nest and are autonomous [76].

4.7. Brain plasticity mechanisms underlying post-learning perception of odorant B

In rodents, the anatomical connection between the first two stages of the olfactory system, namely ORNs and olfactory bulb glomeruli takes place very early in life and for instance ORNs respond to odorants immediately at birth ([77] and personal observations). It has been proposed that ORN/OR activity is a prerequisite to the basal expression of ORs [78, 79] and to the correct projection of the axons defining the initial glomerular map [80]. Indeed, the selective suppression of activity in a set of ORNs prevents the formation of the targeted glomeruli [81]. Conversely, chronic exposition to heptaldehyde and acetophenone in adult mice induced the formation of supernumerary glomeruli receiving I7 and M72 axons (these two OR types interacting with the two odor ligands [81]), while exposition to acetophenone and isopropyl tiglate around birth enlarged the size of glomeruli receiving M72 and M71 inputs [82]. Not only simple exposure but also and mainly odor associative learning can induce alteration of the glomerular map [36, 83, 84]. Thus, the literature converges towards the idea that olfactory experience not only influences OR expression and OR signaling (i.e., ORN activity) but governs the development and plasticity of the glomerular map with sensory experience, resulting in the permanent formation or reorganization of glomeruli through a “constructivist” process [85].

Our results suggest that B paired to MP during the conditioning episode triggers or (and) up-regulates the expression of ORs having high affinity to B. The weight of B inputs at the olfactory bulb would be thus increased leading the pups to have a lower perceptual threshold. Because we are testing neonates here, and are using a powerful and crucial pheromone for them as unconditioned stimulus during the associative conditioning procedure, olfactory bulb and targeted central neuroanatomic structures may quickly adapt in less than 24h (see also [22]). In addition, the co-input of B and MP confers a high level of biological pertinence to B, likely involving the amygdala, which mediates emotionally arousing experiences by reinforcing memorization (e.g., [22]).

5. Conclusions

First, our results highlight that, as soon as the periphery, the olfactory system differentially and spontaneously processes two mixtures containing the same elements, making more or less salient antagonistic interactions between odorants, and leading to their distinct perception in the same animals (i.e., AB and A'B' mixtures in newborn rabbits here). Such a differential process probably results from the concentration (dependent on the volatility) and ratio of the components; the periphery being adapted to deal with low saturated vapor pressure components, such as odorant B, allowing them to largely influence the mixture processing, with respect to one component with high saturated vapor pressure (as for example odorant A). Thus, peripheral processes may contribute, at least in part, to the configural vs. elemental perception of odor mixtures. Moreover, MP-induced odor learning of B altered the peripheral processing of the A'B' mixture by decreasing suppressive interaction between the components, which may facilitate the detection of the learned component in that elemental mixture and explain the behavioral responsiveness of the pups to A'B' (although the mixture is initially inactive on the behavior). Conversely, the configural perception of AB would be favored by less suppressive interactions between its odorants.

Second, our results demonstrate that MP-induced single trial odor learning involves (at least partly) an induction phenomenon in newborn rabbits. Indeed, the conditioning promoted by MP increased extremely rapidly (24h) and in parallel the peripheral reactivity to the learned stimulus and its detection abilities by the animal. These changes totally agree with the seminal results pinpointing induction in anosmic human patients [29] and extend the notion of induction to the neonatal period in normosmic animals. After learning, MP loses its spontaneous and unique privileged peripheral processing to the shared benefit of the conditioned odorant. As a consequence, some new elements (odorants) from the external chemical environment appears to become relevant for the young organism, the olfactory system favoring their detection from

the OR level, while remaining reactive to spontaneously valuable stimuli (as the MP). In ecological conditions of mother-young interactions, MP-induced odor learning is functional when the mother comes into the nest to nurse her litter and newborns are exposed to odor cues carried by her body during nursing, allowing them to acquire odors contemporarily present in the living environment of the mother and social group [18, 76].

Induction is consistent with the idea that the olfactory system structure and functioning operates according to constructivist theory, perfectly adapted to a sensory system facing a quasi-infinite number of stimuli. Thus, even if over phylogenetic evolution, neocortex, auditory and visual information apparently take the place of the chemosensory systems primarily contributing to decision-making in most mammals, olfaction supports detection and perception performances even more remarkable than visual and acoustic ones [86]. This is highlighted here very early in life, in organisms still partly immature.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed were approved by the ethical committee of Lyon 1 University (CEEA-55) and covered by the authorization no. 9745 from the French Ministry of Higher Education and Research.

Informed consent

This article does not contain any studies with human participants performed by any of the authors.

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Table 1. Concentrations of odorant B used in behavioral tests

| Stimulus reference | Concentration in water (g/ml) in vials | Estimated gaseous concentration (mol/l)* | Estimated gaseous concentration (ppm v/v) |
|--------------------|--|--|---|
| B ⁻⁵ | 10 ⁻⁵ | 3.40 x 10 ⁻¹² | 7.61 x 10 ⁻⁵ |
| B ⁻¹⁰ | 10 ⁻¹⁰ | 3.40 x 10 ⁻¹⁷ | 7.61 x 10 ⁻¹⁰ |
| B ⁻¹⁵ | 10 ⁻¹⁵ | 3.40 x 10 ⁻²² | 7.61 x 10 ⁻¹⁵ |
| B ⁻¹⁷ | 10 ⁻¹⁷ | 3.40 x 10 ⁻²⁴ | 7.61 x 10 ⁻¹⁷ |
| B ⁻¹⁹ | 10 ⁻¹⁹ | 3.40 x 10 ⁻²⁶ | 7.61 x 10 ⁻¹⁹ |
| B ⁻²⁰ | 10 ⁻²⁰ | 3.40 x 10 ⁻²⁷ | 7.61 x 10 ⁻²⁰ |
| B ⁻²¹ | 10 ⁻²¹ | 3.40 x 10 ⁻²⁸ | 7.61 x 10 ⁻²¹ |
| B ⁻²² | 10 ⁻²² | 3.40 x 10 ⁻²⁹ | 7.61 x 10 ⁻²² |

*: calculated by using Henry law constant [(Pi/Ci) Pa.m3/mol]

Table 2. Stimuli used in electrophysiological recordings

| Stimuli | 2-methylbut-2-enal | Ethyl Maltol | Ethyl Isobutyrate | | Configural mixture | Elemental mixture |
|---|-------------------------|--------------------------|-------------------------|-------------------------|-------------------------|-------------------------|
| Abbreviation | MP | B and B' | A | A' | AB | A'B' |
| Concentration in liquid phase (g/ml) | 1 x 10 ⁻⁵ | 7 x 10 ⁻⁶ | 3 x 10 ⁻⁶ | 1.5 x 10 ⁻⁵ | 1 x 10 ⁻⁵ | 2.2 x 10 ⁻⁵ |
| Estimated gaseous concentration*: (mol/l) | 7.18 x 10 ⁻⁷ | 2.38 x 10 ⁻¹² | 1.23 x 10 ⁻⁶ | 6.16 x 10 ⁻⁶ | 1.23 x 10 ⁻⁶ | 6.16 x 10 ⁻⁶ |
| Estimated gaseous concentration (ppm) | 1.61 x 10 ¹ | 5.33 x 10 ⁻⁵ | 2.76 x 10 ¹ | 1.38 x 10 ² | 2.76 x 10 ¹ | 1.38 x 10 ² |

*: calculated by using Henry law constant [(Pi/Ci) Pa.m3/mol]

Figure Legends

Figure 1: a. Positioning of the three electrodes on the three turbinates (T1, T2, T3) on the hemi-head of a rabbit neonate (the large arrow indicating an enlargement of the area). The olfactometer nozzle is positioned so that the odorized puff (materialized in grey shadow) sweeps the 3 turbinates, as if it was naturally entering through the animal's nostril.

b. Illustration of raw recordings simultaneously obtained from the three turbinates. The EOG amplitude (here in response to the odorant A) is measured between the baseline level sampled within 100 ms prior to the start of olfactory stimulation (STIM) and the peak amplitude.

Figure 2: Proportions of newborn rabbits (in %) responding in the oral activation test either spontaneously (naive pups in grey, $n = 31$) or 24h after MP-induced acquisition of the odorant B (conditioned pups in black, $n = 36$) to the odorant B (B') and the stimulus series comprising single odorants and mixtures (see Table 1 for stimuli concentrations). Stimuli are ordered from the left to the right in ascending level of concentration except for MP, which was always tested at the end as a control. Distinct geometrical symbols above the bars indicate significant differences within naive or conditioned pups, respectively, at $p < 0.05$, and ** differences between naive and conditioned pups at $p < 0.01$ (Cochran, McNemar and χ^2 tests).

Figure 3: Mean EOG amplitudes (\pm SD) recorded in the three nasal turbinates of naive rabbit neonates in response to **a.** AB series ($n = 15$ pups); **b.** A'B' series ($n = 16$ pups). Stimuli are ordered in ascending level of concentration from the left to the right except for MP, which was always tested in the end as a control. Within each graph, distinct geometrical symbols indicate statistical differences at $p \leq 0.05$ (Wilcoxon test for matched samples).

Figure 4: Mean EOG amplitudes (\pm SD) recorded in the three nasal turbinates of conditioned rabbit neonates in response to **a.** the AB series (n = 19 pups); **b.** the A'B' series (n = 17 pups). Stimuli are ordered in ascending level of concentration from the left to the right except for MP, which was always tested in the end as a control. Within each graph, distinct geometrical symbols indicate statistical differences at $p \leq 0.05$ (Wilcoxon test for matched samples).

Figure 5: Comparisons of mean EOG amplitudes (\pm SD) in response to **a.** the AB series, and **b.** the A'B' series, in nasal turbinates 1, 2 and 3 of naive and conditioned rabbit neonates. Stimuli are ordered in ascending level of concentration from left to right except for MP, which was always tested at the end as a control. *, **, *** indicate differences at $p < 0.05$, 0.01 and 0.001 (Wilcoxon test for non-matched samples).

Figure 6: Occurrences of each odor stimulus as being **a.** the least effective (i.e., inducing the smallest EOGs) or **b.** the most effective (i.e., inducing the largest EOGs) in naive (grey bars) and conditioned (black bars) rabbit neonates for the stimulus series AB (left) and A'B' (right). Within the naive and conditioned conditions, χ^2 test and Monte Carlo method were applied on the distributions. Then the Marascuilo procedure gives the statistical classification of stimuli. Distinct geometrical symbols above each bar (grey symbols for: naive pups; black symbols: conditioned pups) indicate statistical differences at $p < 0.05$, and *, **, *** differences between naive and conditioned pups at $p < 0.05$, 0.01 and 0.001, respectively; bilateral test of two proportions, confirmed with Monte Carlo method.

Figure 7: **a.** Proportions of newborn rabbits responding to the odorant B at 10^{-5} g/ml, as a function of the concentration of B used during the conditioning (ran 24h before). The conditioning concentrations were ranged from 10^{-5} to 10^{-22} g/ml (grey bars). This experiment

1000 allowed to assess the spontaneous threshold (ST) of B in the animals (n = 50); **b.** Proportions
1001 of newborn rabbits responding to the odorant B at concentrations ranged from 10^{-5} to 10^{-22} g/ml
1002 24h after conditioning to B at 10^{-5} g/ml. This experiment pinpointed the conditioning threshold
1003 (CT) of B in the animals (n = 105).