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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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1 **Brief olfactory learning influences perceptive sensitivity in newborn rabbits: new**  
2 **insights in peripheral processing of odor mixtures and induction**

3

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17

18 **Abstract**

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

41 **Keywords**

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

## 43 **1. Introduction**

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

59 Strikingly, elemental and configural perceptions are both functional from early in life.  
60 Thus, newborn rabbits perceive weak configurally a binary AB mixture of element A (ethyl  
61 isobutyrate) and B (ethyl maltol) [16-18], which is also perceived configurally in human  
62 adults [e.g., 19] and adult mice [20]. The ratio of components is a crucial factor in this  
63 perception. Indeed, the AB configural perception requires a 30/70 ratio of A/B (AB mixture)  
64 while a 68/32 ratio (A'B' mixture) elicits the AB elemental perception in newborn rabbits  
65 [21]; see [19] and [20] for convergent results in humans and mice. While some  
66 neurophysiological corollaries of AB and A'B' differentiated perceptions have been found in  
67 the newborn rabbit brain [22], the question of the processing of these mixtures at the

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

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

93 in an increase [32-42] or sometimes in a decrease in responses to target stimuli [43] (or even  
94 in no change [44,45]). In parallel with these changes, regulation in the expression rate of some  
95 ORs [43, 46] or proteins involved in peripheral transduction [47] has been reported.

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

106 In the context of induction, newborn rabbits constitute a relevant model to study to what  
107 extent the induction phenomenon is involved in learning-induced plasticity in a newborn  
108 mammal, an issue that has never been addressed to date. This constituted the second aim of  
109 our study. Rabbit neonates can indeed learn a new stimulus (odorant or odor mixture) after a  
110 remarkably powerful (unique, extremely rapid < 5 min) associative conditioning promoted by  
111 the mammary pheromone (MP) (e.g., [16, 22, 50]), a signal naturally emitted by all lactating  
112 females in their milk and used spontaneously by pups to localize the nipples and suck (e.g.,  
113 [50, 51]). Twenty-four hours after the conditioning, rabbit pups display in response to the CS  
114 the typical orocephalic behavior usually triggered by the MP itself. Thus, in close conjunction  
115 with our analysis of the peripheral responses to mixtures vs. mixture components, we  
116 therefore tested induction by exploring in naive vs. conditioned pups the two criteria changes  
117 observed in humans [29], i.e., the consequences of MP-induced conditioning for odorant B

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

120

## 121 **2. Methods**

### 122 *2.1. Animals*

123 New-Zealand rabbit pups (Charles River strain, France) originated from the breeding  
124 facility of IUT Feyssine (Université Lyon 1, France). Adult males and females were housed  
125 individually in cages under constant 12:12 light-dark cycle (light on at 7 am), temperature  
126 (21-22°C) and *ad libitum* access to food and water. Nest boxes were fixed to the housing  
127 cages of pregnant does two days prior to parturition, to allow nest building. The day of birth  
128 was designated as postnatal day 0. To equalize the time of mother-young interaction, females  
129 were allowed access to the nest box only once a day for 15 min at 11:30 am. This short daily  
130 period of nursing respected the natural rhythm of maternal visits [52]. A total of 239 pups  
131 aged 2-4 days from 56 litters were used in this study, 84 for electrophysiological recordings  
132 (38 naive, 46 conditioned) and 155 for behavioral procedures. All experiments were  
133 performed in accordance with ethic rules enforced by French law and were approved by the  
134 ethical committee of Lyon 1 University (CEEA-55) and the French Ministry of Higher  
135 Education and Research under the no. 9745.

136

### 137 *2. Stimuli*

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

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

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

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

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

160

### 161 *3. Odor conditioning*

162 Conditioning sessions were run on postnatal days 1, 2 or 3 in an experimental room  
163 close to the breeding room of the animal unit. They occurred 1h before the daily nursing  
164 (10:30 a.m.) to equalize the pups' motivational state and limit the impact of satiation on  
165 responses [54]. The pups were transferred by groups of 5/litter into a box maintained at room  
166 temperature. The MP-induced conditioning was then run following a procedure previously  
167 validated (e.g. [16, 17, 22, 50])). Briefly, 6 ml of the B + MP blend were pipetted on a 10 x 15



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

172

#### 173 *4. Behavioral assay*

174 The behavioral assay consisted of an individual oral activation test previously  
175 validated (e.g. [16, 17, 21, 22, 50, 51]). Briefly, each pup was gently maintained in one gloved  
176 hand of the experimenter, allowing only head movements; with the other hand, the  
177 experimenter presented the olfactory stimulus for 10 sec on the tip of a glass rod 0.5 cm in  
178 front of the nares. The test was positive when the stimulus induced the on/off typical  
179 orocephalic response, i.e., head-searching usually followed by oral grasping movements.  
180 Spontaneously, this response is highly selective, meaning that naive animals respond only to  
181 the MP. In our three procedures involving behavior, each pup was tested with a maximum of  
182 6 stimuli with an inter-trial interval of 60 sec. The order of stimuli presentation was  
183 counterbalanced from one to another pup (except for the MP presentation which always  
184 occurred last, as a control). If a pup responded to a stimulus, its nose was softly dried before  
185 the next stimulation. The pups were immediately reintroduced to their nest after testing. To  
186 minimize litter effects, each group was drawn from several litters, with a maximum of 5 pups  
187 tested per litter.

##### 188 *4.1. Procedure 1 - Behavioral test before electrophysiology: pre-recording test*

189 The naive animals were all tested with the 3 odor stimuli from either the AB or A'B'  
190 series (Table 2), and systematically to the MP in order to assess that they did not respond  
191 spontaneously to unfamiliar stimuli but to the MP. The conditioned animals were tested to the  
192 same stimuli as the naive pups, 24h after odor learning, to determine the effectiveness of the

193 conditioning to B. All the animals were tested at 8:00 a.m., i.e., at least 30 min before the  
194 beginning of electrophysiological recordings. Importantly, the same series was then used for  
195 the electrophysiological recordings.

#### 196 *4.2. Procedures 2 and 3 - Behavioral test for detection threshold determination*

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

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

207 Regarding the post-conditioning threshold to B, 2 groups of 2-day-old pups (n = 25/group)  
208 were conditioned to B at  $10^{-5}$ g/ml by pairing with the MP, then tested 24h later either to B at  
209  $10^{-5}$ ,  $10^{-10}$ ,  $10^{-15}$ ,  $10^{-19}$  and  $10^{-20}$  g/ml or at  $10^{-5}$ ,  $10^{-17}$ ,  $10^{-21}$  and  $10^{-22}$  g/ml, and systematically  
210 to MP  $10^{-5}$  g/ml.

211

#### 212 *5. Electrophysiology*

213 The electrodes were homemade silver wire ended with a silver ball plated with AgCl  
214 (200-500  $\mu$ m diameter, impedance  $\leq$  100 k $\Omega$ ). Each electrode was connected to an amplifier  
215 (WPI, DAM80, amplifier, DC, gain 1000), the output signal of which was connected to a  
216 computer via an acquisition card (analog to digital interface). The three electrodes were  
217 placed on the surface of the mucosa using micromanipulators, each on a turbinate. The

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

228

## 229 *6. Olfactory stimulation*

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

238

## 239 *7. Statistical analyses*

### 240 *7.1. Behavioral data*

241 Proportions of pups responding behaviorally were compared using the Cochran's Q  
242 test when the data were dependent (i.e., pups from the same group tested for their response to  
243 the three stimuli) or the  $\chi^2$  test of Pearson (with Yates correction when necessary) when the

244 data were independent (i.e., distinct groups tested for their response to the same stimulus).  
245 When the Cochran's Q test was significant, proportions of responding pups were compared  
246 2x2 by the  $\chi^2$  test of McNemar. Degrees of freedom are indicated when  $> 1$ . Data were  
247 considered as significant when the two-tailed tests yielded  $p < 0.05$ . Analyses were made with  
248 Statistica software (StatSoft, Tulsa, OK, USA).

## 249 *7.2. Electrophysiological data*

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

262

## 263 **3. Results**

### 264 *3.1. Peripheral processing of odorants and mixtures*

#### 265 *3.1.1. Naive animals: spontaneous behavior and peripheral processing*

266 From the 38 pups assigned to the naive group, 31 gave exploitable records, 5 did not and 2  
267 were discarded because they did not respond to MP in the pre-recording behavioral test.  
268 Among the 31 recorded pups, 15 were stimulated with the stimulus series AB comprising A,

269 B, AB and MP, while the remaining 16 pups were stimulated with the A'B' series including  
270 A', B', A'B' and MP. The behavioral pre-recording test showed that all pups responded  
271 positively to the MP, and none to the other odorants (in each series  $Q > 40$ ,  $p < 0.001$  and  $\chi^2 >$   
272  $13$ ,  $p < 0.001$  for global and 2x2 comparisons) (Figure 2; for simplification, results of the two  
273 series have been pooled in the Figure).

274 The mean EOG amplitudes recorded in naive pups in response to the two-stimulus series  
275 are shown in Figure 3. For the AB series, regardless of the stimulus, EOGs recorded in  
276 turbinate 1 (T1) were statistically smaller than those recorded in T2 and T3 (Friedman test,  $p$   
277  $= 0.007$ , T1 vs. T2:  $p < 0.0001$ , T1 vs. T3:  $p = 0.002$ ). For the A'B' series, no statistical  
278 differences were observed between the three turbinates.

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

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

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

293

294 *3.1.2. Conditioned animals: post-learning behavior and peripheral processing*

295 From the 46 pups assigned to the conditioned group, 36 gave exploitable records, 3 did not  
296 and 7 were discarded because they failed to give clear behavioral responses to B (CS). Among  
297 these 36 newborn rabbits, 17 pups were stimulated with the AB series and 19 pups with the  
298 A'B' series. In terms of behavior, within the AB series, pups strongly responded to B (94.1%)  
299 and to the MP (100%) but not to A and AB (whole comparison:  $Q = 47$ ,  $df = 3$ ,  $p < 0.001$ ; 2x2  
300 comparisons between B or MP vs. A or AB:  $\chi^2 > 14.06$ ,  $p < 0.001$ ; B vs. MP:  $\chi^2 < 0.5$ ,  $p >$   
301  $0.05$ ) (Figure 2; to simplify reading, responses of the two series were pooled in the Figure).

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

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

318 In the A'B' series (Figure 4b), EOGs' amplitudes recorded in T1 differed from those recorded  
319 in T2 and T3 (Friedman,  $p < 0.001$ , T1 vs. T2  $p < 0.0001$ ; T1 vs. T3  $p = 0.0002$ ); T1 EOGs  
320 being lower regardless the stimulus. In T2, B' induced larger responses than A'B' and MP ( $B' > A'B'$ ;  
321 Wilcoxon,  $p = 0.028$ ,  $B' > MP$ ; Wilcoxon,  $p = 0.031$ ). In T3, B' induced larger  
322 responses than A'B' (Wilcoxon  $p = 0.037$ ).

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

328

### 329 *3.1.3. Comparison of EOG amplitudes in naive vs. conditioned animals*

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

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

338

### 339 *3.1.4. Comparison of stimulus relative effectiveness in naive vs. conditioned animals*

340 Here, we addressed the question of the high variability in EOG amplitude for a same  
341 stimulus (from one recording site to another, from one nasal cavity to another and from one  
342 animal to another), by analyzing the differences in response between different stimuli in

343 relative terms. To that end, based on their amplitude, the EOG responses were ranked from  
344 the lowest to the largest and the corresponding stimulus sequence was used to determine the  
345 least or the most effective stimuli; only the sets of recording comprising exploitable responses  
346 to the 5 stimuli (i.e., complete stimulus sequence) were taken into account. Doing so, we  
347 compared the occurrence of each stimulus as being the least and the most effective one, first,  
348 within the naive and conditioned groups for the AB and A'B' series, respectively, and then  
349 between the two groups.

#### 350 *3.1.4.1. Occurrence of stimuli as least effective (Figure 6a)*

351 Within the AB-series naive animals (Figure 6a-left, grey bars), all stimuli occurred as the  
352 least effective with the same probability ( $p > 0.5$ ). By contrast, within the A'B'-series naive  
353 animals (Figure 6a-right, grey bars) statistical differences appeared between stimuli ( $p <$   
354  $0.0001$ ,  $\chi^2$  and Monte Carlo method): the occurrence of A'B' differed from that of A' and MP,  
355 A'B' being most frequently the least effective stimulus ( $p < 0.05$ , pairwise comparisons by  
356 the Marascuilo procedure).

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

363 The comparisons between naive and conditioned groups showed that the occurrence of MP  
364 as the least effective stimulus increased from 28% to 43% ( $p < 0.05$ , in the AB series) and  
365 from 18% to 34% ( $p = 0.02$ ; in A'B' series) in conditioned neonates. Comparatively, the  
366 occurrence of A'B' as the least effective stimulus decreased from 43% in naive pups to 29%



367 in conditioned ones ( $p < 0.05$ , bilateral test of two proportions, confirmed with Monte-Carlo  
368 method).

#### 369 *3.1.4.2. Occurrence of stimuli as most effective (Figure 6b)*

370 Within both naive and conditioned groups, and in both AB and A'B' series, stimuli  
371 differed in their occurrence as the most effective stimulus ( $p < 0.0001$ ,  $\chi^2$  and Monte Carlo  
372 method). In the AB-series naive animals (Figure 6b-left, grey bars), MP and A significantly  
373 differed from B and AB ( $p < 0.05$ ), and were most often the most effective stimuli. In the  
374 A'B'-series naive animals (Figure 6b-right, grey bars), MP and A' differed from B and A'B',  
375 and B differed from A'B' ( $p < 0.05$ ). MP and A' were most often the most efficient stimuli  
376 especially compared to A'B', which never induced the largest EOGs.

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

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

387

388 To sum up, regardless of the AB and A'B' series, the comparison of relative effectiveness  
389 between the stimuli showed that MP, the most effective stimulus in naive animals, lost this  
390 status in conditioned pups: since, after conditioning its occurrence increased as the least  
391 effective stimulus, while simultaneously decreased as the most effective one. At the same

392 time, the frequency of odorant B (i.e., the conditioned stimulus) as the most effective  
393 stimulus, alone (for the AB series) or in the elemental mixture A'B' (for the A'B' series),  
394 increased in conditioned animals, whereas the occurrence of B as the lowest effective cue did  
395 not change in the two series.

396

### 397 *3.2. Detection threshold in naive and conditioned animals*

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

#### 404 *3.2.1 Spontaneous detection threshold of the odorant B*

405 Because rabbit pups did not respond spontaneously to B, the determination of B  
406 spontaneous threshold (ST) could not be evidenced in really naive animals but in animals  
407 which failed to learn. This issue was addressed by using decreasing concentrations of B paired  
408 with a constant concentration of MP, expecting that at a given concentration of B this odorant  
409 would cease to be perceived and, therefore, would not be learned anymore. The ST was then  
410 set between the last concentration of B supporting learning and the first concentration of B  
411 failing to induce it. We tested 105 pups divided in 7 groups. Each group was conditioned by  
412 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  
413 were tested 24h later for their orocephalic response to B alone at  $10^{-5}$  g/ml.

414 The results showed that differences existed in pup responsiveness to B depending on the  
415 concentration of the odorant used during the conditioning ( $Q = 81.2$ ,  $df = 7$ ,  $p < 0.001$ )  
416 (Figure 7a). A strong and similar proportion of pups responded to B after conditioning at  $10^{-5}$ ,

417  $10^{-10}$  and  $10^{-15}$  g/ml ( $> 80\%$ ;  $\chi^2 < 0.7$ ,  $p > 0.05$  in all 2x2 comparisons) attesting that B was  
418 effectively learned, i.e., that it was spontaneously perceived at these concentrations. The level  
419 of responsiveness dropped dramatically between  $10^{-15}$  and  $10^{-17}$  g/ml (80 vs. 20%;  $\chi^2 = 7.1$ ,  $p$   
420  $< 0.01$ ) and then remained very low from  $10^{-17}$  to  $10^{-22}$  g/ml ( $< 20\%$ ;  $\chi^2 < 0.7$ ,  $p > 0.05$  in all  
421 2x2 comparisons).

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

### 424 3.2.2. *Post-conditioning detection threshold of the odorant B*

425 To determine the detection threshold of odorant B after MP-induced conditioning  
426 (“conditioned threshold”, CT), two groups of 25 pups were conditioned to B  $10^{-5}$  g/ml (paired  
427 with MP  $10^{-5}$  g/ml), then each tested 24h later to several concentration steps of B over a range  
428 from  $10^{-5}$  and  $10^{-22}$  g/ml, and systematically to MP  $10^{-5}$  g/ml in the end. In the two groups, the  
429 responsiveness to B varied according to its concentration ( $Q < 73.1$ ,  $df = 3$  and  $4$ ,  $p < 0.001$ ).  
430 The proportion of responding pups was strong and similar to B from  $10^{-5}$  to  $10^{-19}$  g/ml  
431 ( $> 80\%$ ;  $\chi^2 < 3.2$ ,  $p > 0.05$  in all 2x2 comparisons) but it strongly decreased at  $10^{-20}$  g/ml  
432 (20%;  $\chi^2 > 16.05$ ,  $p < 0.001$  in all 2x2 comparisons between  $10^{-20}$  vs. stronger concentration  
433 steps) and then remained low or null until  $10^{-22}$  g/ml ( $< 20\%$ ;  $\chi^2 < 3.5$ ,  $p > 0.05$  in all 2x2  
434 comparisons) (Figure 7b).

435 Between the ST and CT determination procedures, no difference contrasted the pups’  
436 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  
437 comparisons). Conversely, in the CT determination procedure, rabbit pups responded clearly  
438 more to B at  $10^{-17}$  and  $10^{-19}$  g/ml than in the ST determination procedure ( $\chi^2 > 20.6$ ,  $p < 0.001$   
439 in the two comparisons).

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

443

#### 444 **4. Discussion**

445 The present study provides original insights into the peripheral contribution to odor  
446 mixtures' perception, by focusing on the AB and A'B' mixtures initially characterized as  
447 perceived configurally and elementally, respectively, in both human adults and newborn  
448 rabbits, and also more recently in adult rodents [20]; this by using here the same  
449 concentrations and ratios of components for acquisition of electrophysiological and  
450 behavioral data. At the same time, the use of MP as a reference stimulus led us to observe the  
451 peripheral processing of this pheromone. In addition, by assessing in parallel potential  
452 alterations of peripheral electrophysiological activity and behavioral detection threshold, we  
453 tested whether the induction phenomenon contributes to MP-induced odor learning in the  
454 newborn rabbit.

455

##### 456 *4.1. Spontaneous peripheral processing of odor mixtures*

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

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

469 Compared to what happened with AB, the interactions between components seem to  
470 operate differently regarding the A'B' mixture. In naive animals, A'B' responses were  
471 smaller than those to A' in T1 and to the components in T2. When considering the relative  
472 effectiveness of stimuli, A'B' was the least effective stimulus in 44% of cases and the most  
473 effective stimulus in only 1%; such frequencies ranked it as the least effective of all the  
474 stimuli.

475 Mixture processing at the peripheral level is reported to as involving mainly suppressive  
476 interactions between odorants, whether in binary [3, 4, 11, 55-57] or more complex mixtures  
477 [58-60]. Moreover, such a predominance of suppressive interactions seems true whatever the  
478 overlapping, or absence of overlapping ORs' assemblies activated by the odorants [60]. In  
479 line with this, the responses to AB and A'B' both appeared as mainly resulting from  
480 suppressive interactions between the components. However, such interactions seem to be  
481 consistently weaker in AB, which would involve either hypoadditive or at least less marked  
482 suppressive interactions than A'B'. This result fits with some others gathered in adult rats,  
483 suggesting that AB involves hypoaddition, synergy and only then suppression, in descending  
484 order of importance [61, 62 and personal observations]. Comparatively, the elemental A'B'  
485 mixture would engage stronger suppressive interactions, as shown in rats for the elemental  
486 isoamyl acetate (ISO)/whiskey lactone (WL) mixture [4, 61]. Strikingly, both A'B' and  
487 ISO/WL combine a high vapor pressure component (A' and ISO, respectively) with a low  
488 vapor pressure one (B' and WL). Regarding the AB and A'B' concentrations, the high vapor

489 pressure of A means that in both AB (A/B ratio: 30/70) and A'B' (68/32), the odorants A and  
490 A' are largely dominant in concentration:  $1.23 \times 10^{-6}$  and  $16.16 \times 10^{-6}$  mol/l, respectively, vs.  
491  $2.38 \times 10^{-12}$  mol/l for B (and B'). Therefore, the theoretical concentrations of the mixtures are  
492 close to those of A and A', while A'B' is about 5 times more concentrated than AB; thus, the  
493 higher the concentration of A in mixture (A'B'), the stronger the suppressive interaction with  
494 B (and B').

495 Taken together, the results obtained in rabbit neonates here, and previous ones obtained in  
496 adult rats, argue that the peripheral responsiveness to a mixture mainly results from  
497 suppressive or antagonistic interactions, whatever the elemental or configural perceptual  
498 attributes of the mixture. Additionally, our observations lead us to go further by suggesting  
499 that if A and B in mixture mainly show negative interactions, these interactions are less  
500 marked in AB than A'B' depending on the concentration of A/A' (which impacts *de facto* the  
501 ratio with B). The observation that AB is perceived configurally, (even weakly) in rabbit  
502 pups, may lie in the fact that A and B at the 30/70 ratio and current concentrations set in  
503 motion suppressive interactions to a lesser extent than A' and B' do in the A'B' mixture. The  
504 reason why A'B' peripheral responses would induce stronger inhibitory interaction than AB  
505 remains unknown. It could rely on the higher concentration of A' and negative action of B/B'  
506 on OR responding to A' [57].

507

#### 508 4.2. Conditioning impacts the peripheral processing of odorant B

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

515 relative effectiveness of MP in conditioned animals: indeed, the gain in B effectiveness led to  
516 a loss of MP peripheral stimulating hegemony in favor of B.

517 The increase of peripheral responses to a conditioned stimulus, observed here in newborn  
518 rabbits, is in agreement with previous studies in adult rodents including peripheral recordings  
519 [30, 31, 35-37, 42] and optical imaging of olfactory bulb inputs [38, 63]. However, in rabbit  
520 neonates, it is remarkable that this gain observed at the periphery appeared after only a single  
521 and extremely brief conditioning episode (5 min), a result that highlights the rapidity of the  
522 physiological (i.e., cellular, molecular) mechanisms engaged in peripheral plasticity.

523

#### 524 *4.3. Influence of odorant B learning on peripheral processing of mixtures including it*

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

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

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

549

#### 550 *4.4. Molecular and cellular mechanisms underlying peripheral plasticity and EOG alterations*

551 The current observations of EOG alterations in response to B and to mixtures including B,  
552 after the brief B-learning could be supported by OR expression regulation. ORs are expressed  
553 in a monogenic, monoallelic and seemingly stochastic location [65, 66]. Furthermore, it has  
554 been reported that OR transcription (and then expression) can be induced by odorant  
555 stimulation, especially during ORN differentiation, but also in mature neurons, thanks to  
556 *de novo* DNA methyltransferase [67]; this enzyme would be responsible for both repressive  
557 and active transcriptional states within neurons, which generate the full set of gene responses  
558 associated with neuronal plasticity. Regulation of specific ORs' expression have been  
559 observed in honeybees in correlation with change in EAG (i.e., EOG in vertebrates; [43]) and  
560 with more central plasticity like long term memory [68, 69]. A role of down and up-  
561 regulations of ORs would be to ensure that new floral scents are detected and would allow the  
562 honeybees to adapt to their ever-changing scent environment [48, 49]. From a broader view,  
563 OR plasticity linked to olfactory environment influence appears a widespread phenomenon



564 [46, 48, 49, 70, 71]. Here, the increase of peripheral response to B supports a parallel increase  
565 of either the density of ORs dedicated to B processing or of the number of ORNs expressing  
566 such ORs (mainly through an activity dependent orientation in OR selection in newly mature  
567 ORNs, or both). The reported results [63] that odorant exposure did not alter spatial pattern,  
568 peak magnitude, or odorant-selectivity of aldehyde-evoked OSN input to olfactory bulb  
569 glomeruli, but did alter the temporal dynamics of that input, plead in favor of an increase of  
570 density of B sensitive ORs expressed by ORNs spontaneously responsive to B.

571

#### 572 *4.5. MP peripheral processing is spontaneously prioritized in rabbit neonates*

573 In a previous *ex vivo* study in rabbit pups, high concentrations of MP ( $1.17 \times 10^{-5}$  and  $10^{-6}$   
574 mol/l) induced large EOGs (up to 8 mV; [24]). Strikingly, here, despite the MP delivery at a  
575 lower concentration ( $7.18 \times 10^{-7}$  mol/l), which was ranked in the optimal concentration range  
576 known to elicit behavioral responses [72], MP induced one of the largest, even the largest  
577 EOG in naive pups. This observation supports the conclusion that spontaneous peripheral  
578 processing of MP is favored in rabbit neonates' olfactory mucosa, a result consistent with the  
579 crucial function of this pheromonal signal in newborn rabbits [e.g., 24, 50, 51, 73]. This raises  
580 the question of the origin of MP prioritization at the olfactory system periphery. Since MP is  
581 absent in the uterine environment (no trace of the molecule has been found in the amniotic  
582 fluid or blood of pregnant rabbit females; [51]), the hypothesis of prenatal MP learning can be  
583 rejected. Moreover, the releasing activity of MP is immediately displayed at birth, even before  
584 any postnatal exposure to the natural MP contained in the rabbit milk [50, 51]. Therefore,  
585 even if in our conditions the peripheral sensitivity to MP could be putatively enhanced thanks  
586 to the sucking experience of the first postnatal days, it would only complement a predisposed  
587 MP-OR tuning effective immediately at birth. Our results give the first demonstration of the

588 strong and direct link that may exist between peripheral and behavioral processing for a  
589 pheromone in mammals as soon as early in life.

590

#### 591 *4.6. Impact of learning on odorant B detection threshold*

592 MP-induced learning of B resulted in an increase of the B sensitivity of newborn rabbits, in  
593 our conditions by a factor of at least 100. This validated the second criteria of induction [29].

594 Strikingly, in both naive and conditioned pups, the B threshold concentrations were extremely

595 low, i.e., around  $3.40 \times 10^{-22}$  mol/l ( $1 \times 10^{-15}$  g/ml) for the spontaneous threshold and  $3.40 \times$   
596  $10^{-26}$  mol/l ( $1 \times 10^{-19}$  g/ml) for the conditioned threshold. Thus, the spontaneous and

597 conditioned thresholds were respectively  $5.3 \times 10^{11}$  and  $5.3 \times 10^{15}$  times lower than the  
598 detection threshold observed for MP in a previous study ( $1.79 \times 10^{-10}$  mol/l gas estimated

599 concentration; [72]). It may seem surprising that the MP threshold was higher than that of B, a  
600 spontaneously non-relevant odorant. However, in ecological conditions, the MP threshold in

601 newborn rabbits must fit with an optimal detection, discrimination and recognition of that

602 signal in the mother milk. Furthermore, B concentration (at saturated vapor pressure value) is

603 about  $7.35 \times 10^6$  lower than the MP one. Thus, it can be proposed that the olfactory system

604 adapted to the reality of odor molecule concentrations in order to detect low volatile and (or)

605 concentrated odorants; this may explain, at least partly, why the B threshold value is very low

606 even spontaneously. In line with this hypothesis, the sexual Bombykol pheromone in *Bombyx*

607 *Mori* (Lepidoptera) is perceived at around  $2.82 \times 10^{-22}$  mol/l (gas estimated concentration from  
608 [74]), thus far lower than the MP one but close to the ST observed for odorant B in our

609 conditions ( $3.40 \times 10^{-22}$  mol/l). However, although sharing pheromonal status, MP is

610 contained in the milk and detected by rabbit pups in the near proximity of the nipples,

611 whereas Bombykol must be detected in the air during the flying of moths, thus at very low

612 concentrations, requiring highly sensitive and specialized OR(s). The olfactory system of this

613 invertebrate is adapted for such an extreme sensitivity since "one pheromone molecule is  
614 sufficient to elicit a nerve impulse" [75]. In rabbit neonates, according to our results, B  
615 presents a spontaneous threshold quite close to that of Bombykol.

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

624

#### 625 *4.7. Brain plasticity mechanisms underlying post-learning perception of odorant B*

626 In rodents, the anatomical connection between the first two stages of the olfactory system,  
627 namely ORNs and olfactory bulb glomeruli takes place very early in life and for instance  
628 ORNs respond to odorants immediately at birth ([77] and personal observations). It has been  
629 proposed that ORN/OR activity is a prerequisite to the basal expression of ORs [78, 79] and  
630 to the correct projection of the axons defining the initial glomerular map [80]. Indeed, the  
631 selective suppression of activity in a set of ORNs prevents the formation of the targeted  
632 glomeruli [81]. Conversely, chronic exposition to heptaldehyde and acetophenone in adult  
633 mice induced the formation of supernumerary glomeruli receiving I7 and M72 axons (these  
634 two OR types interacting with the two odor ligands [81]), while exposition to acetophenone  
635 and isopropyl tiglate around birth enlarged the size of glomeruli receiving M72 and M71  
636 inputs [82]. Not only simple exposure but also and mainly odor associative learning can  
637 induce alteration of the glomerular map [36, 83, 84]. Thus, the literature converges towards

638 the idea that olfactory experience not only influences OR expression and OR signaling (i.e.,  
639 ORN activity) but governs the development and plasticity of the glomerular map with sensory  
640 experience, resulting in the permanent formation or reorganization of glomeruli through a  
641 “constructivist” process [85].

642 Our results suggest that B paired to MP during the conditioning episode triggers or (and)  
643 up-regulates the expression of ORs having high affinity to B. The weight of B inputs at the  
644 olfactory bulb would be thus increased leading the pups to have a lower perceptual threshold.  
645 Because we are testing neonates here, and are using a powerful and crucial pheromone for  
646 them as unconditioned stimulus during the associative conditioning procedure, olfactory bulb  
647 and targeted central neuroanatomic structures may quickly adapt in less than 24h (see also  
648 [22]). In addition, the co-input of B and MP confers a high level of biological pertinence to B,  
649 likely involving the amygdala, which mediates emotionally arousing experiences by  
650 reinforcing memorization (e.g., [22]).

651

## 652 **5. Conclusions**

653 First, our results highlight that, as soon as the periphery, the olfactory system differentially  
654 and spontaneously processes two mixtures containing the same elements, making more or less  
655 salient antagonistic interactions between odorants, and leading to their distinct perception in  
656 the same animals (i.e., AB and A'B' mixtures in newborn rabbits here). Such a differential  
657 process probably results from the concentration (dependent on the volatility) and ratio of the  
658 components; the periphery being adapted to deal with low saturated vapor pressure  
659 components, such as odorant B, allowing them to largely influence the mixture processing,  
660 with respect to one component with high saturated vapor pressure (as for example odorant A).  
661 Thus, peripheral processes may contribute, at least in part, to the configural vs. elemental  
662 perception of odor mixtures. Moreover, MP-induced odor learning of B altered the peripheral

663 processing of the A'B' mixture by decreasing suppressive interaction between the  
664 components, which may facilitate the detection of the learned component in that elemental  
665 mixture and explain the behavioral responsiveness of the pups to A'B' (although the mixture  
666 is initially inactive on the behavior). Conversely, the configural perception of AB would be  
667 favored by less suppressive interactions between its odorants.

668 Second, our results demonstrate that MP-induced single trial odor learning involves (at  
669 least partly) an induction phenomenon in newborn rabbits. Indeed, the conditioning promoted  
670 by MP increased extremely rapidly (24h) and in parallel the peripheral reactivity to the  
671 learned stimulus and its detection abilities by the animal. These changes totally agree with the  
672 seminal results pinpointing induction in anosmic human patients [29] and extend the notion of  
673 induction to the neonatal period in normosmic animals. After learning, MP loses its  
674 spontaneous and unique privileged peripheral processing to the shared benefit of the  
675 conditioned odorant. As a consequence, some new elements (odorants) from the external  
676 chemical environment appears to become relevant for the young organism, the olfactory  
677 system favoring their detection from the OR level, while remaining reactive to spontaneously  
678 valuable stimuli (as the MP). In ecological conditions of mother-young interactions, MP-  
679 induced odor learning is functional when the mother comes into the nest to nurse her litter and  
680 newborns are exposed to odor cues carried by her body during nursing, allowing them to  
681 acquire odors contemporarily present in the living environment of the mother and social group  
682 [18, 76].

683 Induction is consistent with the idea that the olfactory system structure and functioning  
684 operates according to constructivist theory, perfectly adapted to a sensory system facing a  
685 quasi-infinite number of stimuli. Thus, even if over phylogenetic evolution, neocortex,  
686 auditory and visual information apparently take the place of the chemosensory systems  
687 primarily contributing to decision-making in most mammals, olfaction supports detection and

688 perception performances even more remarkable than visual and acoustic ones [86]. This is  
689 highlighted here very early in life, in organisms still partly immature.

690

691

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703

## 704 **Conflict of Interest**

705 The authors declare that they have no conflict of interest.

706

## 707 **Ethical approval**

708 All applicable international, national, and/or institutional guidelines for the care and use of  
709 animals were followed. All procedures performed were approved by the ethical committee of  
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712

713 **Informed consent**

714 This article does not contain any studies with human participants performed by any of the  
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716

717 **7. References**

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959



960 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 <sup>-5</sup>	10 <sup>-5</sup>	3.40 x 10 <sup>-12</sup>	7.61 x 10 <sup>-5</sup>
B <sup>-10</sup>	10 <sup>-10</sup>	3.40 x 10 <sup>-17</sup>	7.61 x 10 <sup>-10</sup>
B <sup>-15</sup>	10 <sup>-15</sup>	3.40 x 10 <sup>-22</sup>	7.61 x 10 <sup>-15</sup>
B <sup>-17</sup>	10 <sup>-17</sup>	3.40 x 10 <sup>-24</sup>	7.61 x 10 <sup>-17</sup>
B <sup>-19</sup>	10 <sup>-19</sup>	3.40 x 10 <sup>-26</sup>	7.61 x 10 <sup>-19</sup>
B <sup>-20</sup>	10 <sup>-20</sup>	3.40 x 10 <sup>-27</sup>	7.61 x 10 <sup>-20</sup>
B <sup>-21</sup>	10 <sup>-21</sup>	3.40 x 10 <sup>-28</sup>	7.61 x 10 <sup>-21</sup>
B <sup>-22</sup>	10 <sup>-22</sup>	3.40 x 10 <sup>-29</sup>	7.61 x 10 <sup>-22</sup>

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

962

963

964 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 <sup>-5</sup>	7 x 10 <sup>-6</sup>	3 x 10 <sup>-6</sup>	1.5 x 10 <sup>-5</sup>	1 x 10 <sup>-5</sup>	2.2 x 10 <sup>-5</sup>
Estimated gaseous concentration*: (mol/l)	7.18 x 10 <sup>-7</sup>	2.38 x 10 <sup>-12</sup>	1.23 x 10 <sup>-6</sup>	6.16 x 10 <sup>-6</sup>	1.23 x 10 <sup>-6</sup>	6.16 x 10 <sup>-6</sup>
Estimated gaseous concentration (ppm)	1.61 x 10 <sup>1</sup>	5.33 x 10 <sup>-5</sup>	2.76 x 10 <sup>1</sup>	1.38 x 10 <sup>2</sup>	2.76 x 10 <sup>1</sup>	1.38 x 10 <sup>2</sup>

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

966

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968

## Figure Legends

969

970

971 **Figure 1: a.** Positioning of the three electrodes on the three turbinates (T1, T2, T3) on the  
972 hemi-head of a rabbit neonate (the large arrow indicating an enlargement of the area). The  
973 olfactomer nozzle is positioned so that the odorized puff (materialized in grey shadow)  
974 sweeps the 3 turbinates, as if it was naturally entering through the animal's nostril.  
975 **b.** Illustration of raw recordings simultaneously obtained from the three turbinates. The EOG  
976 amplitude (here in response to the odorant A) is measured between the baseline level sampled  
977 within 100 ms prior to the start of olfactory stimulation (STIM) and the peak amplitude.

978

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

987

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

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

998

999 **Figure 5:** Comparisons of mean EOG amplitudes ( $\pm$  SD) in response to **a.** the AB series, and  
1000 **b.** the A'B' series, in nasal turbinates 1, 2 and 3 of naive and conditioned rabbit neonates.  
1001 Stimuli are ordered in ascending level of concentration from left to right except for MP,  
1002 which was always tested at the end as a control. \*, \*\*, \*\*\* indicate differences at  $p < 0.05$ ,  
1003 0.01 and 0.001 (Wilcoxon test for non-matched samples).

1004

1005 **Figure 6:** Occurrences of each odor stimulus as being **a.** the least effective (i.e., inducing the  
1006 smallest EOGs) or **b.** the most effective (i.e., inducing the largest EOGs) in naive (grey bars)  
1007 and conditioned (black bars) rabbit neonates for the stimulus series AB (left) and A'B' (right).  
1008 Within the naive and conditioned conditions,  $\chi^2$  test and Monte Carlo method were applied on  
1009 the distributions. Then the Marascuilo procedure gives the statistical classification of stimuli.  
1010 Distinct I geometrical symbols above each bar (grey symbols for: naive pups; black symbols:  
1011 conditioned pups) indicate statistical differences at  $p < 0.05$ , and \*, \*\*, \*\*\* differences  
1012 between naive and conditioned pups at  $p < 0.05$ , 0.01 and 0.001, respectively; bilateral test of  
1013 two proportions, confirmed with Monte Carlo method.

1014

1015 **Figure 7:** **a.** Proportions of newborn rabbits responding to the odorant B at  $10^{-5}$  g/ml, as a  
1016 function of the concentration of B used during the conditioning (ran 24h before). The  
1017 conditioning concentrations were ranged from  $10^{-5}$  to  $10^{-22}$  g/ml (grey bars). This experiment

1018 allowed to assess the spontaneous threshold (ST) of B in the animals (n = 50); **b.** Proportions  
1019 of newborn rabbits responding to the odorant B at concentrations ranged from  $10^{-5}$  to  $10^{-22}$   
1020 g/ml 24h after conditioning to B at  $10^{-5}$  g/ml. This experiment pinpointed the conditioning  
1021 threshold (CT) of B in the animals (n = 105).