

# Effects of *in utero* odorant exposure on neuroanatomical development of the olfactory bulb and odour preferences

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Human babies and other young mammals prefer food odours and flavours of their mother's diet during pregnancy as well as their mother's individually distinctive odour. Newborn mice also prefer the individual odours of more closely related—even unfamiliar—lactating females. If exposure to *in utero* odorants—which include metabolites from the mother's diet and the foetus's genetically determined individual odour—helps shape the neuroanatomical development of the olfactory bulb, this could influence the perception of such biologically important odours that are preferred after birth. We exposed gene-targeted mice during gestation and nursing to odorants that activate GFP-tagged olfactory receptors (ORs) and then measured the effects on the size of tagged glomeruli in the olfactory bulb where axons from olfactory sensory neurons (OSNs) coalesce by OR type. We found significantly larger tagged glomeruli in mice exposed to these activating odorants in amniotic fluid, and later in mother's milk, as well as significant preferences for the activating odour. Larger glomeruli comprising OSNs that respond to consistently encountered odorants should enhance detection and discrimination of these subsequently preferred odours, which in nature would facilitate selection of palatable foods and kin recognition, through similarities in individual odours of relatives.

**Keywords:** odour exposure; olfactory bulb; odour preferences; neuroanatomical development; mice

## 1. INTRODUCTION

Babies' preferences for odours of foods their mothers ate during pregnancy and nursing [1–4] and for their own mother's odour [5–7] demonstrated, along with similar preferences of rabbits [8], lambs [9], dogs [10], rats [11,12] and mice [13], that infant mammals respond preferentially to familiar odours they encountered *in utero* and prior to weaning. At the same time, newborn mouse pups' preferences for the nipple odour of unfamiliar lactating females that are more genetically similar to themselves (whether paternal aunts to unrelated females or distant conspecific to heterospecific females) [14] demonstrated that newborns can assess degrees of genetic relatedness across a broad relatedness continuum [15,16]. Individual odours of more genetically similar conspecifics are perceptually similar compared with odours of less genetically similar conspecifics [15,17]. Rodents from different species and of all ages use this similarity to respond to unfamiliar individuals of differing degrees of genetic relatedness as though they were assessing the extent of the common qualities in their individual odours and then preferring individuals whose odours are more similar to their own [14–16]. Such preferences (for familiar odours and for genetically similar conspecifics' odours) depend on perceptual discriminations among alternative stimuli and then a response based on the relative attractiveness of the distinctive differences. We sought to investigate processes occurring during initial

neuroanatomical development of the olfactory system that could prepare young mammals to make the perceptual discriminations underlying the described adaptive responses to odours of unfamiliar conspecifics and also to familiar food and maternal odours.

Odorants entering the nasal cavity through the nose during sniffing or through the mouth (as flavours) during eating are detected by olfactory receptor (OR) proteins in olfactory sensory neurons (OSNs) spread across the olfactory epithelium (OE) [18,19]. In the mouse genome, there are about 1000 different OR genes, each coding for a different type of OR protein [20]. Each OSN expresses only one type of OR protein, which determines the OR identity of the particular OSN [18,19] and which odorants will activate it. ORs are activated by multiple odorants, and odorants activate multiple ORs in different combinations [21]. The chemical composition and concentration of the odorants entering the nasal cavity determines which subsets of OSNs will be activated and how strongly the various types respond. During its development, each OSN extends a single axon that grows, over a period of days, from the OE to the olfactory bulb (OB) where it coalesces with other OSN axons of the same OR type in a neuropil mass, called a glomerulus, and synapses on dendrites of mitral/tufted cells and periglomerular cells [19,22,23]. Although the relative proportions of OSNs of the various OR types (among the millions of OSNs in the OE) are not known, these proportions must (because of differences in the accessibility of particular OR types and the extent to which the entering odorants activate them) influence

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both the sensation and the ultimate perception of the odour. If the proportions of particular OSN types that are expressed or survive in a mammal's developing olfactory system are affected by odour exposure during foetal and early postnatal life, each individual—with its unique odour environment—would have its own unique proportion of each type of OSN. This would determine a unique pattern of activation across OSNs when an odour enters the nose. Likewise, other odour-exposure-dependent changes in the glomeruli would lead to an individualized, distinctive perception of the odour. Exposure to the individual's own genetically determined and diet-dependent odorous metabolites excreted into the amniotic fluid [5,6,12,24], which bathes the developing nasal cavity and OE, could modulate the proportions of mature OSNs and the neuroanatomical structure of the glomeruli, which would shape the odour perception during subsequent odour exposure. In short, odour exposure would configure the developing olfactory system to optimize perception based on individual experience. Moreover, although the processes may be somewhat different when OSNs are regenerated later in life [19,25], consistent exposure to odourants—such as dietary odours while eating and the individual's own odour while grooming or in the nest—could maintain or modify the proportion of OSN types and the structure of the glomeruli across the lifespan. It has long been suggested that prenatal and early postnatal experience could influence the development of the neural structures underlying observed behavioural effects (e.g. [26,27]), but the hypotheses about what and how this occurs have not previously been formulated or tested as they are here.

Glomeruli are visually distinct in histological sections, and most have acquired the characteristics of the mature olfactory system by the third week after birth [22,23,28]. With the advent of genetic fluorescence tagging [29,30], it became possible to identify a particular glomerulus by the type of OR expressed in the axons projecting to it by co-expression of  $\tau$ GFP with the OR protein, making the fluorescence evident in the glomeruli when they are formed.

In this work, we studied the effects of odour exposure during gestation and prior to weaning on the neuroanatomy of developing glomeruli—the site of the first sensory synapse—in the olfactory bulbs of mice. The findings reported here indicate that glomeruli formed from OSNs that respond to particular activating odourants are larger in mice exposed to these odourants during the initial development of their olfactory system. Our findings using these same odourants also confirm previous findings [1–13] that odour exposure *in utero* and/or during nursing elicits a preference for that odour at the time of weaning.

## 2. METHODS

### (a) *Animals*

Breeding pairs of gene-targeted mice with either the M71 (M71-IRES- $\tau$ GFP) or the M72 (M72-IRES- $\tau$ GFP) olfactory receptor gene tagged with GFP were purchased from Jackson Laboratories and housed in the animal facility at the University of Colorado Denver in a reversed light cycle (11.00 h off; 23.00 h on). The offspring from the initial pairs were used as parents for the pups (M71:  $n = 46$ ; M72:

$n = 26$ ) in the experimental litters. For the M71s, there were three litters for each group; for the M72s, owing to breeding difficulties, there was one litter for each group.

### (b) *Procedure*

#### (i) *Odour exposure and experimental design*

Genetically determined individual odour constituents in the uterine environment cannot be altered in a natural way, but the composition of dietary metabolites changes with the mother's diet. Thus, for this experiment, we used mice in which OSNs expressing a specific OR express GFP and selected OSN types with receptors that respond strongly to an odourant that can safely be added as a supplemental flavour to the mother's diet: M71 receptors [29,31] are among the receptors activated by acetophenone (Sigma), described as 'cherry'; M72 receptors [32] are among the receptors activated by isopropyl tiglate (Sigma), described as 'mint'. Standard flavour laboratory mouse chow (Harlan) was scented (1 ml per 100 g crushed chow) with acetophenone or isopropyl tiglate suspended in filtered water. The flavour-supplemented pellets (reformed in ice cube trays) were dried for 3 days under a fume hood to reduce the odour to a suitable level—as judged by a human nose—before presenting them to the mothers.

For this initial study, we chose a parsimonious design (figure 1): giving mothers of pups in the four experimental groups a flavour-supplemented diet instead of the standard flavour diet throughout 'gestation' (M71:  $n = 13$ ; M72:  $n = 7$ ), throughout 'nursing' (M71:  $n = 11$ ; M72:  $n = 7$ ), throughout 'both' pregnancy and nursing (M71:  $n = 11$ ) or during the last 10 days of 'late nursing' (M72:  $n = 6$ ) and then comparing the sizes of the tagged glomeruli of their pups with those of control group pups (M71:  $n = 11$ ; M72:  $n = 6$ ) from mothers that ate the standard diet. (We chose to feed the mothers of the control pups the standard flavour chow rather than chow supplemented with a different flavour that did not activate the tagged glomerulus. This avoids confounding effects from additional odour exposure that cannot be quantified in the untagged glomeruli. This design also enables comparisons of each experimental group with a single control group.)

#### (ii) *Preference tests*

Pups at postnatal day (P) 20 were tested in clean cages under red light in the first 2 h of their dark phase. One 6 g flavour-supplemented pellet of each type was placed at each end (varied randomly). The time spent sniffing within 1 cm of each pellet during the 3 min test was recorded with stopwatches by experimenters who were blind to the pellet flavours. Pups from the control litters were tested to confirm that there was no intrinsic preference for either flavour-supplemented diet when both flavours were novel. Pups from the experimental litters were tested to determine whether they preferred the flavour their mothers ate to the novel flavour.

#### (iii) *Perfusion*

To enable comparison of the effects of odour exposure during gestation and nursing, all pups were sacrificed at three weeks old. Mice (P21) were anaesthetized and perfused transcardially with 4 per cent paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Olfactory bulbs were harvested and post-fixed for 2 h and then transferred to 25 per cent sucrose in phosphate-buffered saline (PBS) for

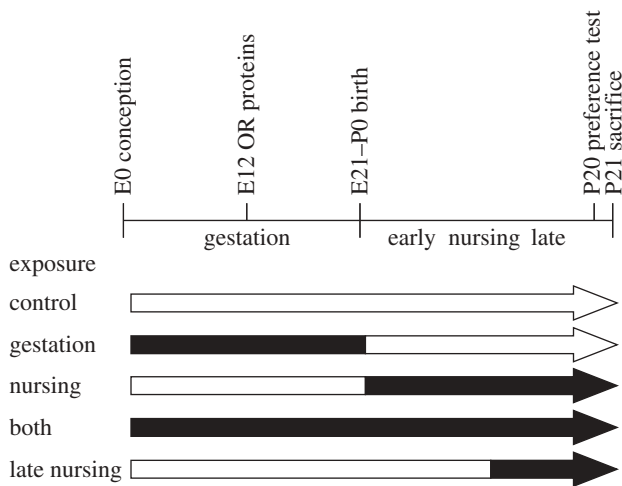


Figure 1. Filled portions of the arrows under the developmental timeline indicate when the mother of the tested pups ate the flavour-supplemented diet. OR proteins become evident from E12.

cryoprotection overnight. Brains were embedded in Neg50 (Richard-Allan Scientific) cutting medium on dry ice and sliced at 20 µm on a Leica cryostat at -13°C. Slides were washed (2 × 5 min) with 0.1 M PBS before coverslipping with Fluoromount-G (SouthernBiotech).

(c) Data analysis

Fluorescence of axons and their coalescence into glomeruli are readily distinguishable in coronal slices when viewed under 4× magnification. Images of single glomeruli (four per mouse) were captured at 40× on a Nikon Eclipse 600 microscope/camera using Nikon Imaging Software. In IMAGEJ software, the fluorescent glomerulus in each image was delineated using ‘adjust > threshold’ from the image menu (then delineating the boundary between the glomerulus and the incoming axons using ‘freehand selection’ from the draw menu when necessary), and areas were calculated using ‘analyse particles’ from the analyse menu. The volume (µm<sup>3</sup>) of each glomerulus was determined by summing the areas of serial coronal sections and multiplying by the depth of the slice (20 µm).

Main effects were assessed with ANOVA, and post hoc comparisons were assessed with Tukey HSD tests using STATISTICA software. The glomerulus types were analysed separately because the M72s were larger. The initial analysis showed a main effect of litter on weight and glomerular volume. This was because the average weight of pups in the control groups was significantly higher than that of pups in the gestation groups. We did not consider this an issue because if higher weight had been associated with larger glomeruli, this would have worked against our finding significantly larger glomeruli in the gestation group. Although the findings are comparable either way at the reported level of precision, the weight range was broad enough (6–12.6 g) that we normalized volumes by multiplying the individual glomerulus volume by the average animal weight (8.9 g) and dividing by the individual animal’s weight. There were no differences in the volumes of medial versus lateral glomeruli or those in the left or right OB. There were no differences in the findings when using the average volume per group or average volume per mouse in the group as the statistical unit. (Because the M71 glomerulus matures later than the M72 [28], there was a possibility of

finding doublets in M71 mice sacrificed at weaning. We excluded the 7.2 per cent (n = 13) doublets found in the M71 pups from the analysis because we could not be sure that the combined volume of the doublets was comparable to that of the single glomeruli. There was one doublet in the control group and the other 12 doublets were distributed across the three treatment groups, but the sample was too small to find statistically significant differences in occurrences of doublets across groups (F<sub>3,172</sub> = 0.835, p = 0.4765.) We used non-parametric Wilcoxon matched pairs tests to analyse the preference data because the data were not normally distributed.

3. RESULTS AND DISCUSSION

By exposing foetal and pre-weaning mouse pups with GFP-tagged OSNs to odorants that activate the tagged receptors and measuring the effect of this exposure on the sizes of developing glomeruli where these OSN axons coalesce, we have shown that odour exposure *in utero* and/or during the first few weeks after birth shapes neuroanatomical development in the olfactory system. The fluorescent glomeruli of three-week-old mice whose mothers had eaten lightly flavour-supplemented chow during gestation or nursing or both (figure 1) were significantly larger than those whose mothers had eaten the standard flavour chow (figure 2a,b; ANOVA: M71: F<sub>3,159</sub> = 22.57, p = 0.000001; M72: F<sub>3,98</sub> = 55.20, p = 0.000001), demonstrating the efficacy of odour exposure in modulating the neuroanatomical development of both types of tagged glomeruli. For M72s, the effects were significantly larger in pups exposed to mint during gestation than during nursing (figure 2b), suggesting that, although OR proteins are expressed during the last 10 embryonic days [33], exposure to odorants in the womb could be decisive in establishing the dimensions of the developing glomeruli. Fluorescent glomeruli of M71 pups exposed to cherry during gestation plus nursing were not significantly larger than those exposed only during gestation (figure 2a), further emphasizing the importance of *in utero* exposure. Odour exposure for just the last 10 days of nursing did not significantly affect the size of fluorescent glomeruli compared with controls (although the average weight of pups in the late nursing group was significantly higher than that of the control pups), and these glomeruli were significantly smaller than those of pups exposed throughout nursing (figure 2b), either pointing to a perinatal critical period in OSN/glomerular development or indicating that 10 days of odour exposure just prior to performing histology is not sufficient to establish increases in the volume of glomeruli.

It is reasonable to infer, although it could not be measured, that the odour-exposure-induced increases in glomerular size occurred in all the glomeruli that were activated by the supplemental odorant and not just the tagged glomeruli. It is a logical inference, noting again that all odorants activate multiple glomeruli and that all glomeruli are activated by multiple odorants, and that this odour-exposure-dependent neuroanatomical tuning occurs broadly across the OE and OB irrespective of the odour source. Because glomeruli formed by OSNs that are activated by consistently encountered odorants become larger than glomeruli that do not receive such

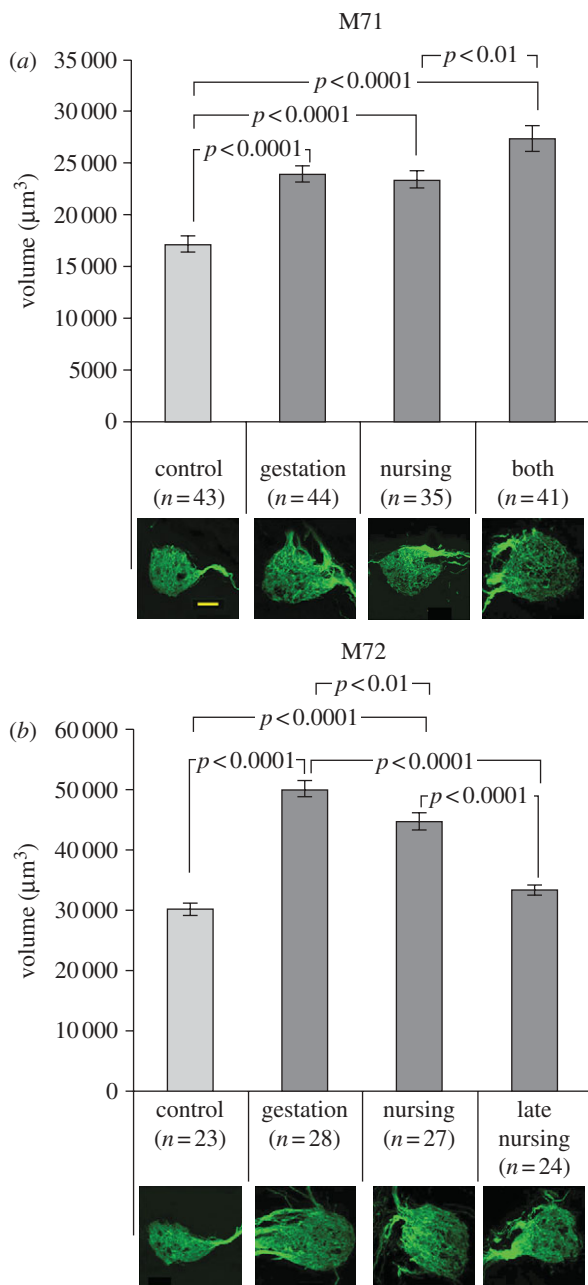


Figure 2. Mean ( $\pm$  s.e.m.) glomerulus volume ( $\mu\text{m}^3$ ), normalized to average weight pup. (a,b) Mothers of ‘control’ pups ate standard flavour chow (light grey bars); mothers of treatment pups ate flavour-supplemented chow (dark grey bars) during gestation, nursing or both. Late nursing mothers ate flavour-supplemented chow for the last 10 days of nursing. Cross-sections of exemplary GFP-tagged glomeruli from each group appear below each bar. Note that there are more OSN axons entering the glomeruli in the treated than the control groups. The scale bar (20  $\mu\text{m}$ ) applies to all images.

activation and because each individual foetus or infant encounters a unique combination of odorants—including metabolites from the mother’s diet and its own genetically determined metabolites—the relative sizes of the 1000 types of glomeruli in each individual’s developing OB depend to some extent on the relative presence of odorants that activate their constituent OSNs. Individually distinctive combinations of odorants during development help configure the olfactory system by establishing individually distinctive patterns of differentially sized glomeruli.

There are a number of possible explanations for the odour-exposure-induced increase in glomerular volume. A glomerulus could become larger because there are more OSNs in the OE with axons projecting to it. During foetal OSN development, growing dendritic knobs reach the OE surface in the nasal cavity before OR proteins are evident in the cell, but they are detectable in the dendritic knobs before the cilia start growing the following day [33]. This developmental sequence, which also must occur later in life during OSN regeneration and replacement [25], could allow exposure to environmental odorants to influence which OR gene is expressed and the functional identity of the maturing OSN, as has been suggested in adult mice [34]. It is also possible that OR gene expression is determined by intrinsic genomic factors [35,36] and that differential odour exposure during their continued development affects their survival as the OSN axons grow from the epithelium to the glomerulus [37]. Another possibility is that activation through odour exposure modulates branching of the axon terminals once they reach the glomerulus [38]. Activation could also enhance dendritic arborizations of mitral cells in the glomerulus [39]. Future studies will be necessary to distinguish among these and other possibilities and thus to shed additional light on the exact processes by which odour exposure determines the proportions of OSN types in the individual’s OE and/or the sizes of developing glomeruli. Nonetheless, it is clear from the results of the gestation groups in comparison with the controls (figure 2a,b) that exposure to activating odorants makes a substantial contribution to the increase in the size of the glomerulus even though it reaches maturity two or more weeks after birth [22,23,28]. Ongoing studies in our laboratory are also addressing the longevity of the effects of odour exposure during gestation in older mice that either have or have not experienced the activating odour after birth and the effects of constant versus intermittent odour exposure during other stages of the life cycle.

Pups at P20 whose mothers had eaten the standard flavour chow showed no preference between the two flavour-supplemented chows (figure 3). In contrast, P20 pups from both mouse lines whose mothers had eaten flavour-supplemented chow during gestation, nursing or both preferred the odour of their mother’s diet to a differently flavoured diet (figure 3). Note that this preference was robust even if their only exposure to the odour had been during gestation and thus they had not had any opportunity to smell the odour after birth (figure 3). Interestingly, the pups in the late nursing group showed a clear preference for the familiar odour even though their tagged glomeruli were not significantly bigger than those of the control group, indicating that preferences for familiar food odours over readily discriminable novel food odours can be established in the absence of enhanced glomerular volume. The preferences described here are consistent with those reported in previous studies of prenatal and early postnatal learning [1–3,8–11]. The association between odour or flavour exposure and preferences for those odours or flavours appears robust, but the neurocircuitry connecting the stimulus to the response has never been clear. In the gestation, nursing and both groups, the preferences parallel increased volume of tagged glomeruli that are activated by the exposed

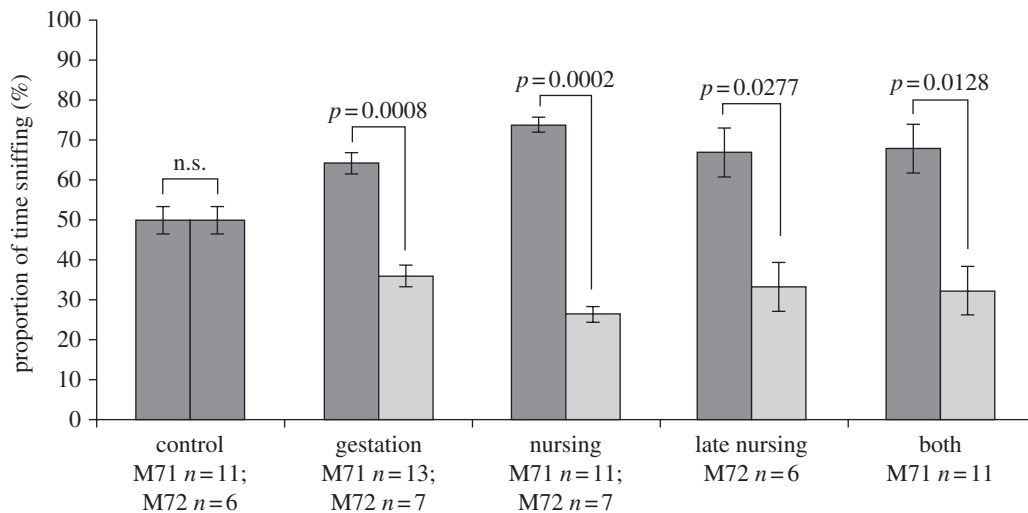


Figure 3. Odour exposure elicits odour preferences. Mean ( $\pm$  s.e.m.) proportion of time during a 3 min preference test that pups spent investigating the cherry- and mint-flavoured pellets. Pups that had not been exposed to either flavour showed no preference (grey bars). Pups whose mothers had eaten a flavoured diet (cherry for M71s; mint for M72s) during gestation, nursing or both significantly preferred the flavour their mother ate (dark grey bars) to the novel flavour—mint for M71s; cherry for M72s (light grey bars).

flavour, but the late nursing group showed a significant preference without a significant increase in the volume of the tagged glomeruli (figures 2 and 3). This suggests that odour or flavour preferences can be established before the odour exposure has enhanced the volume of the glomeruli, uncoupling the preferential response and the perception of the odour stimulus and raising new questions about the role of a process that configures the neuroanatomy through odour exposure during initial development.

We had hypothesized that exposure to odorants in the womb (and later prior to weaning) may regulate the maturation of the sensory apparatus in each individual's developing olfactory system so that it becomes especially sensitive to consistently encountered *in utero* odorants. These odorants would be a unique combination of the growing foetus's excreted genetically determined 'individual odour' metabolites and the mother's dietary metabolites [5,6,12,24]. Such enhanced sensitivity could help young mammals make more finely tuned discriminations [40–43] among odours of conspecifics and familiar foods, enabling them to demonstrate more subtle preferential responses. Although the results of the current study do not address direct connections between the reported odour-exposure-modulated neuroanatomical changes during olfactory system development and enhanced sensitivity to the activating odours, it is possible to draw some inferences by putting our results in the context of findings from other studies. A previous study in rabbit pups linked postnatal preferences for the odour of juniper berries with increased electro-olfactogram (EOG) activity in the OE to juniper berry odour after *in utero* exposure to the odour through the mother's juniper berry-supplemented diet [8]. This suggests not only that *in utero* odour exposure enhances sensitivity but also that the effects are evident in the OE as well as in the OB. There is more substantial evidence of such connections in adult mammals. Adenovirus-driven OR gene expression in adult rats led to greater EOG responsiveness in the OE to the exposed odorants [44]. In humans, the

enhanced EOG response following repetitive odour exposure was also significantly correlated with lower detection thresholds [45]. Previous studies with adult M71 GFP-tagged mice showed an increase in the number of M71 OSNs in the OE and larger M71 glomeruli after exposure to the odour of acetophenone [31]. In addition, in mice and humans, consistent odour exposure has enhanced both the sensitivity to those odorants [34,40,45–48] and the discriminability of similar odours [41–42]. This leads us to speculate (recognizing the complexity of the underlying neurocircuitry) that having larger glomeruli following consistent odorant exposure (figure 2) facilitates both detection and discrimination of odorants that activate them.

The enhanced sensitivity and discriminability afforded by larger glomeruli would not be necessary (or obvious) in distinctions between readily discriminable odours, particularly if one of the odours is familiar and the other novel, as was the case in previous studies [1–3,8–11] and in the preference tests reported here, for example in the late nursing group, between the familiar mint-scented chow and the unfamiliar cherry-scented chow. The enhanced sensitivity and discriminability would be advantageous, however, in subtle distinctions between similar, unfamiliar odours such as those made by newborn mouse pups that were differentially attracted to the nipple odour of their lactating paternal aunt over that of an unrelated lactating female [14]. Additional studies will be necessary to reveal whether these speculations are correct.

Whatever the precise circuitry from odour detection through discrimination to preferential response may be, it would usually be adaptive to shape the neuroanatomy of the developing olfactory system to enhance the perceptual sensitivity to *in utero* odorants if this sensitivity contributes in any way to facilitating subtle odour preferences. The mother's dietary metabolites typically indicate the safety as well as the availability of foods in the environment. Enhanced sensitivity to their odours would be advantageous in locating these foods and distinguishing them from similar but less suitable items, and inborn

preferences should promote acceptance of these available palatable foods after weaning. In humans, enhanced sensitivity and inborn preferences may not always be advantageous, however, because even when 'available' and 'palatable' do not equate with 'healthy' foods, the mother's food choices may influence her unborn child's preferences nonetheless. This could explain how the process can go awry, leading to maladaptive preferences, such as the attraction to ethanol odour and flavour in humans and other mammals [49] whose mothers consumed alcohol during pregnancy. It is sobering to recognize from the findings presented here that odour exposure during gestation and nursing affects not only early flavour learning [50] but the neuroanatomical development of the olfactory system as well.

The described tuning of the developing olfactory system is certainly advantageous for the genetic relatedness assessment mechanism underlying newborn mouse pups' attraction to the odour of more genetically similar females [14]: the constant presence of the foetus's genetically determined individual odour metabolites in the amniotic fluid bathing the nasal cavity should enhance the size of glomeruli of OSNs that are activated by these metabolites. These larger glomeruli should enhance the newborn or infant's ability to detect and discriminate between subtle differences in conspecifics' individual odours, which are composed of genetically determined odorous metabolites that are similar to their own but in differing proportions (see discussion in [15]). The greater the overlap between the newborn's odour and the odour of the encountered individual, the stronger the sensory activation would be when the newborn smells the other's odour. Thus, stronger sensation from receptors in the network of glomeruli that respond to individual odours would indicate closer genetic relatedness of the encountered individual, and subsequent perceptual distinctions could facilitate the process of using individual odour similarities to assess degrees of genetic relatedness demonstrated in newborn [14] and adult mice [16] and other rodents [15]. If this enhanced sensitivity could be linked—in some yet to be determined way—to differential attraction, it could also facilitate an adaptive preferential choice. Perhaps human babies [5,6] and rodent pups [12] prefer their mother's odour not simply because it is familiar but because her proportion of genetically determined odorous compounds, being more similar to their own, provides a better activating match in their olfactory system. In any case, the reported findings help clarify genetic relatedness assessment (kin recognition) mechanisms [15] by suggesting a likely neuroanatomical basis of differential individual odour perception that occurs at the sensory periphery.

Two of the most important decisions that animals make are what to eat and with whom to affiliate. Previous research has confirmed the importance of odour memory in differential responses to familiar as opposed to novel foods and individuals. It is now clear that the neuroanatomical development of the olfactory system enables distinctions, even early in life, at the sensory level—apart from odour learning—that could affect both these types of choices. The mechanisms that fine-tune the neural circuitry in the olfactory bulb and the higher cortical processes through which these sensory discriminations are manifest in adaptive behaviours await further elucidation. Nonetheless, the new conceptualization of the interaction between environmental odours

and neuroanatomical development and the behavioural implications of the individualized tuning tested and reported here represent a substantial advance in understanding the importance of experience in the concurrent shaping of neuroanatomy and behaviour.

Animals were maintained in accordance with NIH and institutional guidelines.

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