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In mice, discrete odors can selectively promote the neurogenesis of sensory neuron subtypes that they stimulate

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eLife Assessment

This study provides **valuable** insights into the role of sensory stimulation in neurogenesis in the mammalian olfactory epithelium, where new olfactory sensory neurons are continually born throughout an animal's lifespan. The authors show that exposure to two different musk-related odors specifically increases the birth rates of those neurons that respond to these odors. This potentially results in adaptive changes in the subtype composition of the olfactory sensory neuron population. **Solid** evidence, well supported by control experiments, is presented to support these findings, though further work is needed to confirm that this phenomenon generalizes to olfactory sensory neurons expressing other types of odorant receptor and to explore the mechanisms underlying the stimulus specificity of neurogenesis.

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Abstract

In mammals, olfactory sensory neurons (OSNs) are born throughout life, ostensibly solely to replace neurons lost *via* turnover or injury. This assumption follows from the hypothesis that olfactory neurogenesis is stochastic with respect to neuron subtype, as defined by the single odorant receptor that each neural precursor stochastically chooses out of hundreds of possibilities. This assumption is challenged, however, by recent findings that the birthrates of a fraction of OSN subtypes are selectively reduced by olfactory deprivation. These findings raise questions about how, and why, olfactory stimuli are required to accelerate the neurogenesis rates of some subtypes, including whether the stimuli are specific (e.g., discrete odorants) or generic (e.g., broadly activating odors or mechanical stimuli). Based on previous findings that the exposure of mice to sex-specific odors can increase the representations of subtypes responsive to those odors, we hypothesized that the neurogenic stimuli comprise discrete odorants that selectively stimulate OSNs of the same subtypes whose birthrates are accelerated. In support of this, we have found, using scRNA-seq and subtype-specific OSN birthdating, that exposure to male and exogenous musk odors can accelerate the birthrates of subtypes responsive to those odors. These findings reveal that certain odor experiences can

selectively ‘amplify’ specific OSN subtypes and suggest that persistent OSN neurogenesis serves, in part, an adaptive function.

Introduction

Mammalian olfactory epithelia (OE) contain hundreds of distinct olfactory sensory neuron (OSN) subtypes, each of which expresses a single odorant receptor (OR) and can thereby be stimulated and/or inhibited by a distinct set of odorant molecules [1,2]. The olfactory epithelium is one of a few regions of the mammalian nervous system where neurogenesis occurs throughout life [3–5]. In the hippocampus and olfactory bulb, persistent neurogenesis plays vital roles in learning and memory [6–8]. By contrast, life-long neurogenesis within the mammalian OE is generally assumed to function solely to replace OSNs that are lost due to normal turnover or environmentally induced damage. This assumption is consistent with the prevailing hypothesis that OSN neurogenesis is strictly stochastic with respect to subtype since it is based on the evidently stochastic process of OR choice [9–11].

Several studies have observed that the relative quantities of distinct OSN subtypes can be altered by manipulating olfactory experience in mice [12–24]. Olfactory deprivation on one side of the OE *via* unilateral naris occlusion (UNO), for example, has been found to cause both increases and decreases in the representations of different OSN subtypes on the closed side relative to the open [21,23]. Similar bidirectional changes in subtype representations have been observed following olfactory enrichment *via* exposure to discrete odors in mice [17,19,24]. Experience-induced changes in the representations of specific OSN subtypes have generally been attributed entirely to altered OSN survival [12,13,18–26], which could theoretically account for both increases and decreases in subtype representations. By contrast, the stochastic nature of OR choice would seem to preclude the contribution of a mechanism involving subtype-selective changes in OSN birthrates.

Unexplained observations that olfactory deprivation *via* UNO reduces both the overall rate of OSN neurogenesis [27–30] and the representations of a fraction of OSN subtypes [14,16,19,21,23,30] motivated a recent study to test the possibility that the birthrates of these subtypes depend on olfactory stimulation. To do so, newborn OSNs of subtypes that exhibit altered representations following UNO were quantified on the open side relative to the closed side of the OEs from UNO-treated mice [30]. Remarkably, subtypes previously found to have reduced representations on the closed side of the OE also exhibited significantly lower birthrates on the closed side, while subtypes with elevated representations on the closed side showed no differences in birthrates between the two sides. These findings indicated that olfactory stimulation affects the representations of specific OSN subtypes *via* two distinct mechanisms. One mechanism reduces the representations of specific subtypes on the closed side of the OE *via* selective reductions in their birthrates as a result of olfactory deprivation. By contrast, a second mechanism elevates on the closed side the representations of subtypes with extremely high levels of baseline activity, presumably *via* lengthened OSN lifespans due to protection from overstimulation [30].

Findings that the birthrates of a fraction of subtypes depend on olfactory stimulation raise several fascinating questions related to the mechanism and function of this phenomenon. One key question concerns the nature of the stimuli that promote neurogenesis. Because naris occlusion reduces exposure to potentially thousands of odors, as well as mechanical stimuli, and likely causes additional physiological changes [31], we envisioned that the neurogenic stimuli could be either non-specific with respect to the subtypes whose birthrates are reduced by olfactory deprivation (e.g., generic odors, mechanical stimuli, or other physiological effects of UNO) or, alternatively, discrete odorants that selectively stimulate the same OSN subtypes whose birthrates are affected. If the neurogenic stimuli are non-specific, this would imply a generic mechanism and, perhaps, an unknown homeostatic function. By contrast, if the stimuli are discrete odorants

that selectively activate the same subtypes whose birthrates are affected, this would imply a highly specific mechanism in which exposure to certain odors can "amplify" subtypes responsive to those odors.

In this study, we tested the possibility that the neurogenic stimuli comprise discrete odorants. One prediction of this hypothesis is that the extent to which naris occlusion reduces the birthrates of specific subtypes should vary depending on the odor environment to which animals are exposed. Consistent with this, open-side biases in the birthrates of specific subtypes have been found to depend on whether a mouse was in the nursing or post-weaning stage at the time of birthrate assessment [30]. A second prediction is that exposure of intact (non-occluded) mice to specific odors should selectively increase the representations of subtypes responsive to those odors. Support for this comes from two separate studies that identified a set of subtypes that were differentially represented within the OEs of male and female mice that were housed in a sex-separated manner until six months [19] or up to 43 weeks of age [24], and that these differences were attenuated in mice that were housed sex-combined. Notably, several of these subtypes were found to selectively respond to sex-specific odors [19,24], indicating that the observed differences were at least partly odor-dependent. As with UNO, the differentially represented subtypes fell into two categories: those for which stimulation increased their representations and those for which stimulation had the opposite effect. OSNs in the former category were exemplified by subtype Olfr235, which exhibited a higher representation in mice exposed to males [19,24] and selective responsivity to male-emitted odors [19], and was previously shown to respond to musk odors [32–34], whose emission by mice has not been documented. In analogy to findings from UNO-based studies [30], we speculated that these subtypes undergo sex-specific odor-dependent selective birthrate acceleration. Here we present evidence that the neurogenesis rates of Olfr235 and other musk-responsive OSN subtypes are increased in mice exposed to male-specific and musk odors. These findings support the hypothesis that discrete odors can selectively accelerate the birthrates of OSN subtypes that they stimulate and suggest that the function of persistent OSN neurogenesis is not limited to the replacement of neurons lost through damage or normal turnover, but may also enable adaptive changes to the subtype composition of the OSN population.

Results

Long-term exposure of mice to male odors is associated with increased representations of musk-responsive OSN subtypes

To test the possibility that discrete odorants can selectively accelerate the birthrates of the OSN subtypes that they stimulate, we sought to identify subtypes that exhibit odor exposure-dependent increases in representations and for which stimulating odors have been identified. A challenge of this approach is that odorant ligands remain unidentified for most OSN subtypes [35]. To overcome this, we speculated that candidate subtypes might be identified among those previously found to be more highly represented in mice exposed to odors emitted specifically by male or female mice [19,24]. OSN subtypes in this category, which include Olfr235, were observed to be more highly represented in mice exposed to male conspecifics (sex-separated males; sex-combined males and females) compared to mice isolated from males (sex-separated females) [19,24] (**Appendix 1–figure 1**). These findings, combined with observations that Olfr235 OSNs show selective responsivity to male odors [19], suggest that the exposure of mice to one or more odor components specific to males causes an increase in the representation of subtype Olfr235 within the OSN population. Interestingly, Olfr235, which expresses the OR-encoding gene *Or5an11*, belongs to a group of subtypes that express homologous ORs and respond to different musk odorants with varying levels of sensitivity and selectivity [32,33]. Notably, like Olfr235, other subtypes within this group, which includes Olfr1440 *(Or5an6)*, Olfr1437 *(Or5an1b)*, Olfr1431 *(Or5an9)*, and Olfr1434 *(Or5an1)*, exhibited higher transcript levels in the OEs of mice exposed to

male odors compared to their unexposed counterparts (except subtype Olfr1434, whose transcript levels were too low to be accurately assessed) [19] (**Appendix 1–figure 1**). Accordingly, RNA-fluorescent *in situ* hybridization (RNA-FISH) analyses of a subset of these ORs, Olfr235 and Olfr1437, confirmed that the elevated transcript levels observed in mice exposed to male odors reflected greater representations of these subtypes within the OSN population [19]. By contrast, subtypes Olfr912 *(Or8b48)* and Olfr1295 (*Or4k45)*, which detect the male-specific non-musk odorants 2-(sec-Butyl)-4,5-dihydrothiazole (SBT) and (methylthio)methanethiol (MTMT), respectively [24], exhibited lower representations and/or transcript levels in mice exposed to male odors [19,24] (**Appendix 1–figure 1**), possibly reflecting reduced survival due to overstimulation. Taken together, these findings indicate that OSN subtypes that are responsive to musk odors are selectively increased in their representations upon long-term exposure to male mice, consistent with the hypothesis that components of male odors selectively accelerate the birthrates of these subtypes.

Olfactory deprivation via UNO reduces quantities of newborn OSNs of musk-responsive subtypes in male mice

If the birthrates of musk-responsive OSN subtypes are accelerated by exposure to male odors, quantities of newborn OSNs of these subtypes would be expected to be reduced following olfactory deprivation in male mice. To test this, we analyzed two single-cell RNA sequencing (scRNA-seq) datasets (one generated previously [OE 1] [30] and the other as part of this study [OE 2]) corresponding to the open and closed sides of the OEs from male mice that had been UNO- treated at postnatal day (PD) 14, weaned sex-separated at PD 21, and dissected at PD 28 (**Figure 1A, B**; **Figure 1–figure supplement 1A**). Within each dataset, newborn OSNs of specific subtypes can be identified based on the co-expression of *Gap43+* (an established marker of immature OSNs [36–38]) and specific OR genes [30] (**Figure 1C, D**; **Figure 1–figure supplement 1B, C**). Using this approach, we mapped newborn OSNs of each of the five known or putative musk-responsive subtypes within the feature plots corresponding to the open and closed sides of each OE (**Figure 1D**; **Figure 1–figure supplement 1C**) and quantified them as a proportion to total OSNs (**Figure 1E**-*left*; **Figure 1–figure supplement 1D**) and total cells (**Figure 1–figure supplement 1E**) within the open and closed data subsets. Remarkably, musk-responsive OSN subtypes exhibited, on average, 3.1-fold greater quantities of newborn OSNs within the open-sides of the OEs compared to the closed, with all subtypes except Olfr1437 showing open-side biases (**Figure 1D, E**-*left*; **Figure 1–figure supplement 1C-E**). By contrast, newborn OSNs of 15 randomly chosen subtypes located within approximately the same region of the OE where musk-responsive subtypes reside (canonical zones 2 and 3) [39] comprise, on average, nearly equal quantities within the open and closed sides (1.07-fold difference) (**Figure 1E**-*right*). To verify that the differences in newborn OSN quantities are not due to aberrant OR expression regulation (e.g, co-expression of multiple ORs), we analyzed cell-specific OR expression at three stages of OSN differentiation: immediate neuronal precursor 3 (INP3), immature OSN (iOSN), and mature OSN (mOSN) (**Figure 1–figure supplement 2**). As expected, individual iOSNs and mOSNs of specific subtypes exhibit robust and singular OR expression on both the open and closed sides of OEs from UNO-treated mice, while INP3 cells co-express low levels of multiple OR transcripts, as observed previously [38,40–45].

To further test whether olfactory deprivation reduces quantities of newborn OSNs of musk-responsive subtypes in mice exposed to male odors, we employed an established histological assay in which EdU-birthdated OSNs of specific subtypes are identified *via* EdU staining and OR-specific RNA-FISH [30,46]. Using this approach, we quantified the number of newborn OSNs of four musk-responsive subtypes per half-section, on the open and closed sides of the OEs of male mice that had been UNO-treated at PD 14, weaned sex-separated at PD 21, EdU-injected at PD 28, and dissected at PD 35 (**Figure 2A, B**). The EdU injection timepoint was based on the timepoint used for identification of immature (*Gap43*-expressing) OSNs in the scRNA-seq datasets, while the dissection timepoint was chosen to provide a chase period of 7 days, which is sufficient to enable

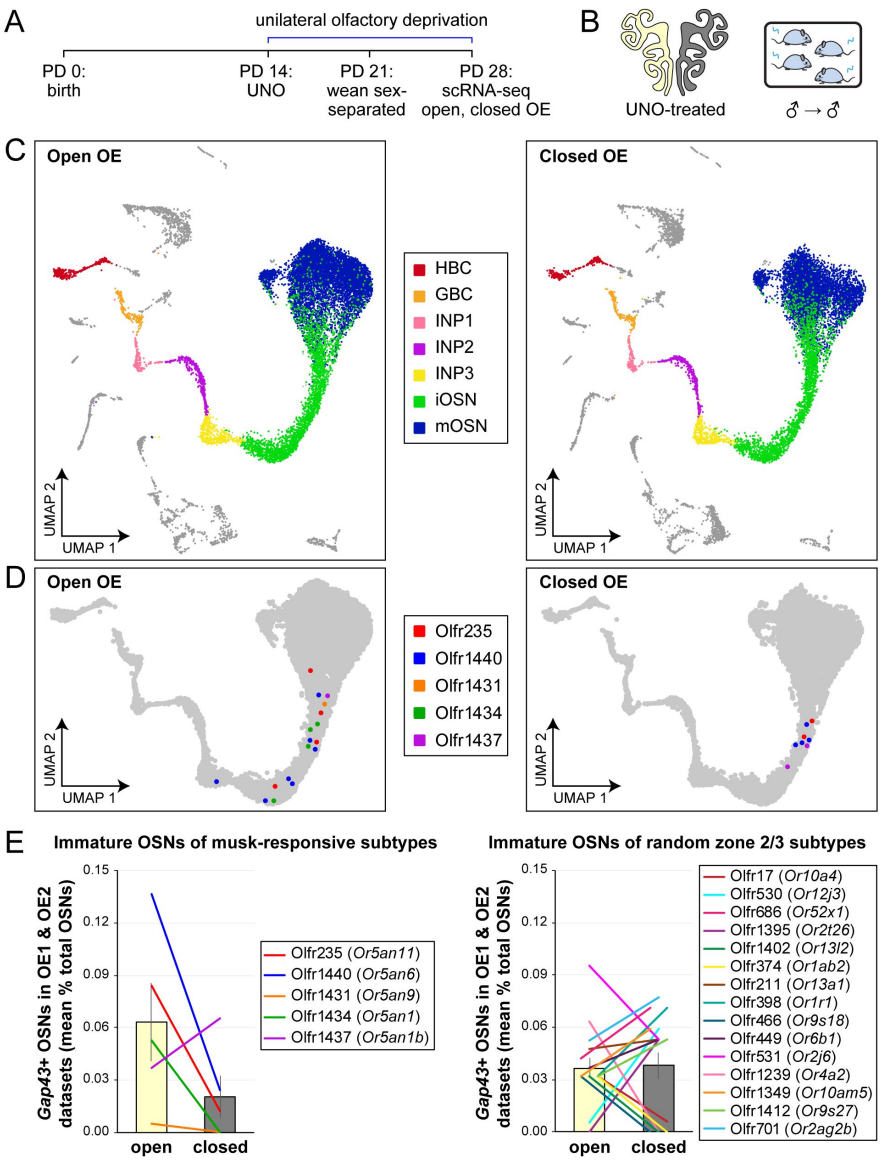


Figure 1.

scRNA-seq analyses of the open and closed sides of whole OEs from UNO-treated adolescent male mice reveal greater quantities of newborn OSNs of musk-responsive subtypes on the open side of the OE relative to the closed.

A, B. Experimental timeline (A) and conditions (B) used to generate scRNA-seq datasets for assessing the effects of UNO on quantities of newborn OSNs of specific subtypes in the OEs of adolescent male mice. Datasets were generated from the open and closed side of the OEs of mice that were UNO-treated at PD 14, weaned sex-separated at PD 21, and sacrificed at PD 28 [30]. C. A UMAP representation of all cells within the open (*left*) and closed (*right*) side datasets corresponding to OE sample 2 (OE 2). Cells within the OSN lineage are represented by colored dots, as defined in the legend: horizontal basal cells (HBC; *red*), globose basal cells (GBC; *orange*), immediate neuronal precursor 1 cells (INP1; *pink*), immediate neuronal precursor 2 cells (INP2; *purple*) immediate neuronal precursor 3 cells (INP3; *yellow*), immature OSNs (iOSN; *green*), and mature OSNs (mOSNs; *blue*). D. UMAP representation of cells within the OSN lineage of the open (*left*) and closed (*right*) side datasets of OE 2. Immature (*Gap43+*) OSNs of the 5 known musk-responsive subtypes are represented by colored dots, as indicated in the legend. E. Quantification of individual (*lines*) and mean (*bars*) percentages of the OSN population represented by immature OSNs of musk-responsive subtypes (*left*) or randomly chosen zone 2/3 subtypes (*right*) within the open and closed side datasets. Quantifications represent the averages of subtype-specific iOSN quantities obtained from scRNA-seq datasets corresponding to the OEs from 2 different mice (OE 1 and OE 2). Error bars: SEM. See also **Figure 1–figure supplements 1** and **2**.

robust and stable expression of an OR [30]. In agreement with findings from scRNA-seq, EdU-labeled OSNs of subtypes Olfr235, Olfr1440, and Olfr1431 exhibited significantly greater quantities on the open side of the OE compared to the closed side in adolescent male mice ($P < 0.05$; 2.3-fold, 1.8-fold, and 2.5-fold, respectively) (**Figure 2C-F**). By contrast, subtype Olfr1437 did not show a significant open-side bias ($P > 0.05$; 1.01-fold) (**Figure 2G**), nor did two non-musk-responsive subtypes, Olfr912 and Olfr1463 (*Or5b109*) (1.05- fold for both subtypes) (**Figure 2H**; **Figure 2–figure supplement 1**). To verify that observed open-side biases in quantities of newborn OSNs are not an artifact of the normalization method used, which was based on the number of OE half-sections quantified, we compared this method to two alternatives: based on the number of EdU+ cells or on the total DAPI+ area. Notably, all three methods yielded open-side biases of similar magnitude, with no significant differences in UNO effect sizes (defined as the $\log_2$ [open/closed] ratio) observed between the three methods (**Figure 2–figure supplement 2**). Taken together, these findings indicate that olfactory deprivation *via* UNO selectively reduces quantities of newborn OSNs of some musk-responsive subtypes in adolescent male mice, consistent with the possibility that the birthrates of these subtypes are selectively accelerated by exposure to male odors.

The exposure of mice to male-specific odors elevates quantities of newborn Olfr235 OSNs

Observations that naris occlusion reduces quantities of newborn OSNs of subtypes Olfr235, Olfr1440, and Olfr1431 in adolescent male mice, together with previous findings that mice exposed to males from weaning until adulthood exhibit elevated representations of all three subtypes, are consistent with the hypothesis that exposure to male odors accelerates the birthrates of these subtypes. If so, we predicted that UNO-induced open-side biases in newborn OSNs of these subtypes may be attenuated in mice that are not exposed to male odors. To test this, we quantified newborn OSNs of subtype Olfr235 in UNO-treated female mice that were housed either sex-separated or sex-combined starting from the age of weaning (PD 21) and thus either unexposed or exposed, respectively, to male odors at the time of EdU labeling (PD 28) (**Figure 3A, B**; **Figure 3–figure supplement 1A**). Unlike sex-separated males, sex-separated females showed no significant difference in quantities of newborn Olfr235 OSNs on the open side of the OE compared to the closed (1.1-fold) (**Figure 3C, E**-*left*) and, correspondingly, a significantly lower UNO effect size ($P < 0.05$; 14-fold) (**Figure 3E**-*right*). By contrast, sex-combined females showed a significantly greater quantity of newborn Olfr235 OSNs on the open side of the OE compared to the closed ($P < 0.01$; 2.2-fold) and, consequently, a UNO effect size significantly greater than that observed in sex-separated females ($P < 0.05$; 11-fold), but not significantly different from sex-separated males (0.8-fold) (**Figure 3D, E**). Analogous open-side biases and differences in UNO effect sizes were observed for total Olfr235 OSN quantities (**Figure 3–figure supplement 1B**; **Figure 3F**).

Findings that exposure to male odors is required for occlusion-induced reductions in quantities of newborn Olfr235 OSNs are consistent with the hypothesis that a component of male odors can accelerate the birthrate of this subtype. If so, the exposure of non-occluded mice to male odors should increase observed quantities of newborn Olfr235 OSNs. To test this, we compared quantities of newborn Olfr235 OSNs in non-occluded sex-separated females, sex-separated males, and sex-combined females (**Figure 3A, B**). Remarkably, compared to sex-separated females, sex-separated males and sex-combined females exhibited significantly greater quantities of newborn Olfr235 OSNs ($P < 0.01$; 2.2-fold and 3.1-fold, respectively) (**Figure 3G**). By contrast, quantities of newborn OSNs of the SBT-responsive subtype Olfr912 showed no significant differences between the three experimental groups (**Figure 3H**). These findings strongly support the conclusion that the exposure of mice to male odors increases quantities of newborn Olfr235 OSNs within the OE.

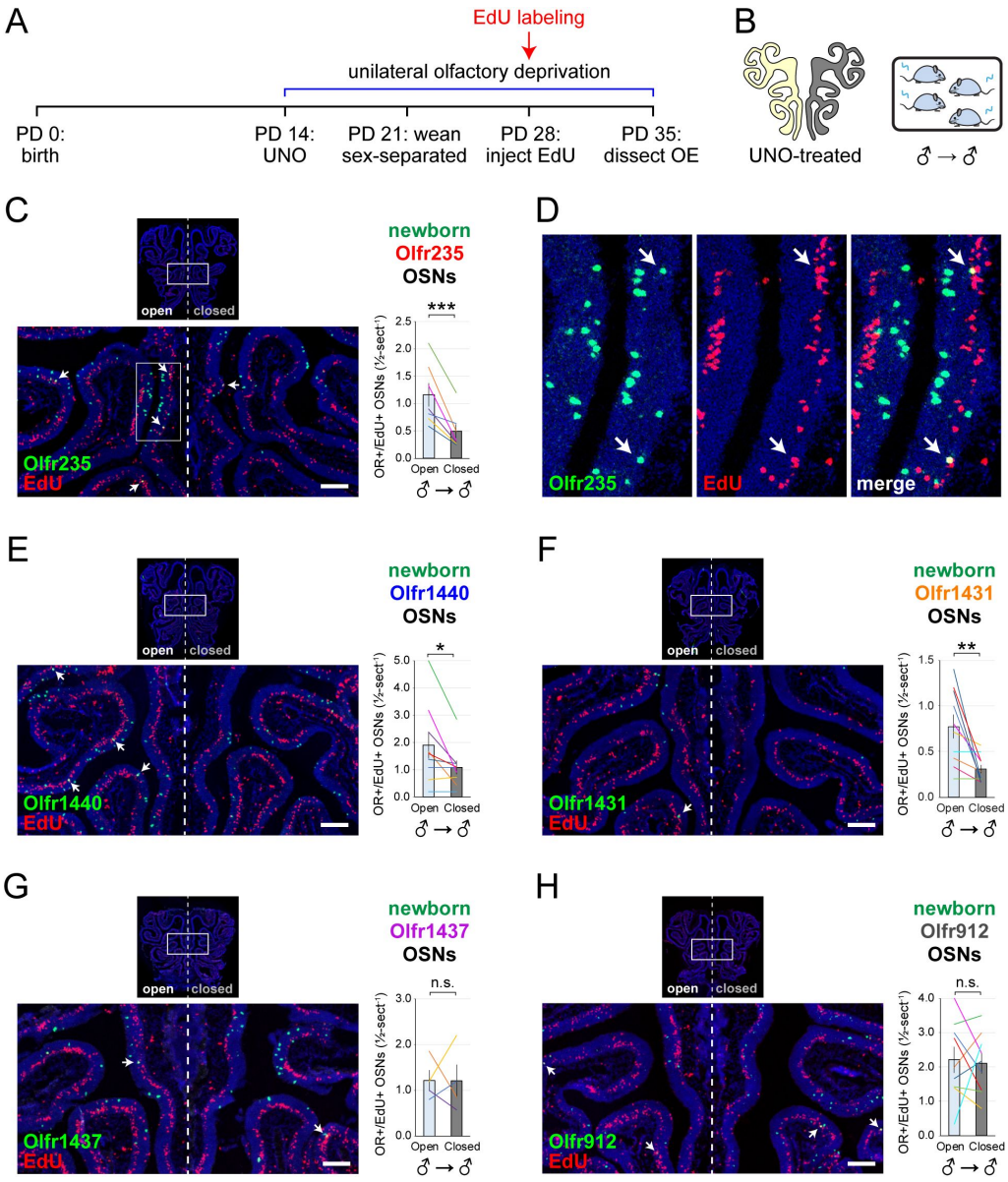


Figure 2.

EdU birthdating analyses confirm that UNO-treated adolescent male mice exhibit greater quantities of newborn OSNs of specific musk-responsive subtypes on the open side of the OE relative to the closed.

A, B. Experimental timeline (A) and conditions (B) used to generate OE tissue samples for assessing the effects of UNO on quantities of newborn OSNs of specific subtypes in adolescent male mice. Mice were UNO-treated at PD 14, weaned sex-separated at PD 21, EdU-labeled at PD 28, and sacrificed at PD 35. OEs were sectioned and analyzed using OR-specific RNA-FISH and EdU staining. C, E–H. *Left*: Representative images of OE sections from UNO-treated adolescent male mice that were exposed, at the time of EdU labeling, to male littermates (♂ → ♂), with newborn OSNs (OR+/EdU+) indicated by white arrows. *Right*: Quantifications of newborn OSNs on the open and closed sides of tissue sections spanning the anterior-posterior lengths of OEs from UNO-treated male mice reveal significant open-side biases in quantities of newborn OSNs of musk-responsive subtypes Olfr235 (C), Olfr1440 (E), and Olfr1431 (F), but not the musk-responsive subtype Olfr1437 (G) or the SBT-responsive subtype Olfr912 (H). D. Enlarged, split-channel view of boxed region in (C), with Olfr235+/EdU+ OSNs indicated by white arrows. Scale bars: 150 µm. Each line represents a distinct mouse (n = 4–10 mice [≥ 5 sections/mouse] per OSN subtype). ***P < 0.001; **P < 0.01; *P < 0.05; n.s. P > 0.05; ratio paired two-tailed t-test. Error bars: SEM. See also **Figure 2–figure supplement 1**.

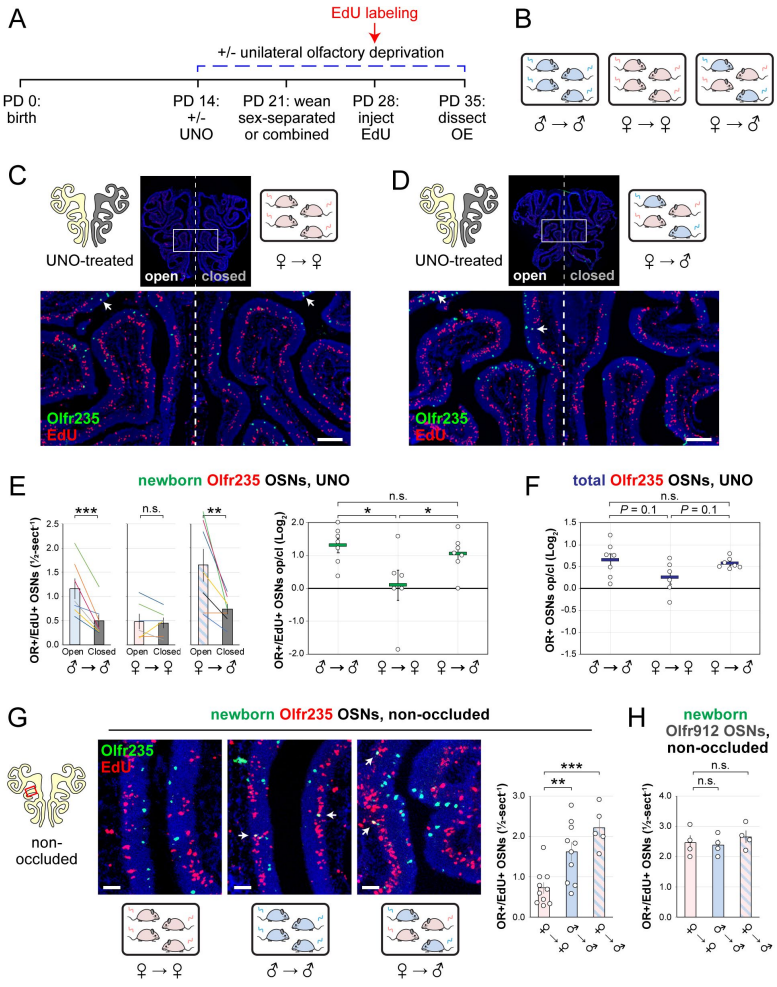


Figure 3.

Exposure of mice to male mouse odors causes selective increases in quantities of newborn OSNs of the musk-responsive subtype Olfr235.

A, B. Experimental timeline (A) and conditions (B) used to generate OE tissue samples for assessing the effects of exposure to male mouse odors on quantities of newborn OSNs of specific subtypes. Male and female mice were either UNO-treated or untreated (non-occluded) at PD 14, weaned sex-separated or sex-combined at PD 21, EdU-labeled at PD 28, and sacrificed at PD 35. OEs were sectioned and analyzed using OR-specific RNA-FISH and EdU staining. C, D. Representative images of OE sections from UNO-treated female mice that were exposed, at the time of EdU labeling, to either female (♀ → ♀) (C) or male (♀ → ♂) (D) littermates, with newborn Olfr235 OSNs (OR+/EdU+) indicated by white arrows. E, F. Quantifications of newborn (E) and total (F) Olfr235 OSNs on the open and closed sides of tissue sections spanning the anterior-posterior lengths of OEs from UNO-treated mice reveal significant open-side biases in quantities of newborn Olfr235 OSNs in male or female mice exposed to male littermates (♂ → ♂ or ♀ → ♂), but not in females exposed to only female littermates (♀ → ♀) (E-*left*), and greater UNO effect sizes for quantities of newborn (E-*right*) and total (F) Olfr235 OSNs within the OEs of mice that were exposed to male littermates. G-*left, middle*. Representative images (*middle*) corresponding to the region outlined in the schematic (*left, red box*) of OE sections from non-occluded mice that were exposed to male littermates (♂ → ♂, ♀ → ♂) or to female littermates (♀ → ♀) at the time of EdU labeling, with Olfr235+/EdU+ OSNs indicated by white arrows. G-*right*, H. Quantifications of newborn Olfr235 (G-*right*) and Olfr912 (H) OSNs in tissue sections spanning the anterior-posterior lengths of OEs reveal significantly greater quantities of newborn Olfr235 OSNs in non-occluded mice exposed, at the time of EdU labeling, to male littermates (♂ → ♂, ♀ → ♂) compared to those exposed only to female littermates (♀ → ♀), while quantities of newborn Olfr912 OSNs showed no significant differences between mice exposed to male littermates and those exposed only to females. Scale bars: 150 µm (C, D), 50 µm (G). Each line or circle represents a distinct mouse (n = 4–10 mice [≥ 5 sections/mouse] per OSN subtype and condition). ***P < 0.001; **P < 0.01; *P < 0.05; n.s. P> 0.05; ratio paired two-tailed t-test (E, *left*); one-way ANOVA test, FDR-adjusted (E, *right*, F-H). Error bars: SEM. Data in panel E for newborn OSN quantities in ♂ → ♂ OE samples correspond to **Figure 2**. See also **Figure 3–figure supplements 1-3**.

Unlike subtype Olfr235, subtypes Olfr1440 and Olfr1431 exhibited significant open-side biases in both newborn and total OSN quantities within the OEs of UNO-treated sex-separated males, sex-separated females, and sex-combined females ($P < 0.05$), with no significant differences in UNO effect size observed between the three groups (**Figure 3–figure supplement 1C-F**). As expected, the non-musk-responsive OSN subtypes Olfr912 and Olfr1463 exhibited no significant open-side biases in newborn OSN quantities in any experimental group, although Olfr912 showed significant closed-side biases in total OSNs ($P < 0.01$) (**Figure 3–figure supplement 1G-J**), consistent with the possibility that naris occlusion protects OSNs of this subtype from overstimulation [19,24]. Observations that exposure to male odors is required for occlusion-induced reductions in quantities of newborn OSNs of subtypes Olfr235 but not Olfr1440 or Olfr1431 were unexpected considering that ORs of all three subtypes show male-biased expression in mice housed sex-separated until 6 months of age (**Appendix 1–figure 1**) [19]. A conceivable explanation for these findings is that, unlike subtype Olfr235, quantities of newborn Olfr1440 and Olfr1431 OSNs are elevated by exposure to odors emitted by both male and female mice at the adolescent stage. Such differences could reflect variations in the specific odorants to which distinct musk-responsive subtypes are most sensitive [33], and which may depend on the age and sex of mice contributing to the odor environment [47–50]. If so, we predicted that the age and/or sex of mice within the odor environment at the time of EdU labeling might differentially affect the degree to which quantities of newborn OSNs of these subtypes are affected by olfactory deprivation. To test this, we compared open-side biases in newborn OSN quantities within UNO-treated adolescent males exposed to littermates alone versus those exposed to both littermates and adult parents (**Figure 3–figure supplement 3A, B**). Remarkably, newborn Olfr1431 OSNs exhibited a significantly greater ($P < 0.05$; 2-fold) UNO effect size in the presence of adult mice compared to the absence, reflecting open-side biases of 4.8-fold and 2.5-fold, respectively (**Figure 3–figure supplement 3C, D**). By contrast, UNO effect sizes for newborn OSNs of subtypes Olfr235, Olfr1440, and Olfr1437, as well as the control subtype Olfr912, were not significantly affected by exposure to adults (**Figure 3–figure supplement 3E-I**). These findings are consistent with the possibility that stimulation-dependent changes in the quantities of newborn OSNs of musk-responsive subtypes may vary depending on the age of odor-emitting mice within the environment.

The exposure of UNO-treated mice to low concentrations of a musk odorant increases open-side biases in quantities of newborn OSNs of musk-responsive subtypes

Evidence that quantities of newborn OSNs of musk-responsive subtypes can be affected by exposure to unknown components of mouse odors indicated that the birthrates of specific OSN subtypes may be accelerated by discrete odorants. If so, we predicted that the exposure of mice to musk odorants that are known to stimulate these subtypes would increase their neurogenesis rates. However, because high levels of chronic stimulation of OSNs have been found to reduce the representations of stimulated subtypes within the OSN population, presumably *via* reductions in OSN lifespan [12,13,19], we speculated that there may be a range of odorant concentrations sufficient to accelerate the birthrates of these subtypes without shortening their OSN lifespans. To assess this, we analyzed the effects of exposing UNO-treated female mice to varying concentrations of the musk odorant (*R*)-3-methylcyclopentadecanone (muscone) on quantities of newborn and total OSNs of the musk-responsive subtypes Olfr235, Olfr1440, and Olfr1431, as well as the control subtypes Olfr912 and Olfr1463, *via* a metal tea-ball from weaning (PD 21) until dissection (PD 35) (**Figure 4A, B**). Remarkably, exposure of mice to muscone was found to significantly affect the extent to which UNO treatment alters the relative quantities of both newborn and total OSNs of musk-responsive subtypes on the open and closed sides of the OE in a concentration-dependent manner (**Figure 4C**; **Figure 4–figure supplements 1**, **2**). Subtype Olfr235, for example, which showed no significant difference in newborn OSN quantities between the open and closed sides in unexposed females, exhibited significant open-side biases in females exposed to 0.1% or 1% muscone ($P < 0.01$; 2.4 and 2.0-fold, respectively), but not those exposed to 10% (1.2-fold) (**Figure 4C**-*middle*). Accordingly, mice exposed to 0.1% or 1% muscone exhibited significantly

greater UNO effect sizes for quantities of newborn Olfr235 OSNs compared to unexposed females ($P < 0.05$; 14 and 13-fold, respectively) (**Figure 4C** - *right*). Similarly, open-side biases in total Olfr235 OSNs were observed in mice exposed to 0.1% or 1% muscone, with the most significant effect observed at 0.1% ($P < 0.01$) (**Figure 4–figure supplement 2**). One possible explanation for the reduction in UNO effect sizes observed at 10% muscone relative to lower concentrations is that small amounts of odorant may enter the closed side of the OE due to transnasal and/or retronasal odor transfer *via* the nasopharyngeal canal [51] and nasopharynx, respectively [31], an effect that would be expected to increase with environmental concentrations and attenuate differences in observed effects of odor stimulation between the open and closed sides.

Compared to subtype Olfr235, open-side biases in quantities of newborn and total OSNs of subtypes Olfr1440 and Olfr1431 were more subtly affected by the concentration of muscone to which mice were exposed (**Figure 4–figure supplements 1**, **2**). We speculate that this may be explained in part by the fact that both subtypes exhibit significant open-side biases in UNO-treated female mice even in the absence of exogenous odor exposure, which may limit the influence of muscone exposure on the UNO effect size. In the case of Olfr1440, the subtype most sensitive to muscone [33,34], it is conceivable that mature OSNs are robustly stimulated on the closed side of the OE by very small amounts of odors that enter transnasally and/or retronasally. Consistent with this possibility, UNO-treated females exposed to 0.1% muscone show greater quantities of newborn Olfr1440 OSNs on both the open and closed sides of the OE compared to their unexposed counterparts (**Figure 4–figure supplement 1A-middle**). Relatedly, subtype Olfr1440 showed evidence of shortened OSN lifespans in mice exposed to 1% and 10% muscone, as reflected in the higher total OSN quantities observed on the closed side of the OE (**Figure 4–figure supplement 2B**). In the case of Olfr1431, newborn OSNs exhibited a significantly greater open-side bias in mice exposed to 1% muscone compared to unexposed controls ($P < 0.01$; 1.6-fold), but no significant difference at 0.1%, perhaps indicating that this subtype is less sensitive to muscone than other subtypes. Like subtype Olfr235, neither Olfr1440 nor Olfr1431 exhibited significant open-side biases in newborn OSN quantities in mice exposed to 10% muscone, potentially reflecting substantial transnasal and/or retronasal odor transfer to the closed side of the OE at this concentration. As expected, muscone exposure did not significantly affect open-side biases in newborn or total OSN quantities of the non-musk responsive subtypes Olfr912 or Olfr1463 (**Figure 4–figure supplements 1**, **2**). Taken together, these findings reveal that the exposure of UNO-treated mice to low concentrations of a musk odorant is sufficient to induce open-side biases in the quantities of newborn OSNs of musk-responsive subtypes without apparent adverse effects on OSN survival.

The exposure of non-occluded mice to musk odorants selectively elevates quantities of newborn OSNs of musk-responsive subtypes

Findings that the exposure of UNO-treated mice to muscone concentrations as low as 0.1% can increase open-side biases in quantities of newborn OSNs of musk-responsive subtypes suggested that the exposure of non-occluded mice to musk odorants at this concentration might selectively accelerate the birthrates of these subtypes. To test this, we compared quantities of newborn OSNs of three musk-responsive subtypes and two musk non-responsive subtypes within the OEs of non-occluded female mice exposed to a 0.1% concentration of one of two different musk odorants, muscone or 5-Cyclohexadecenone (ambretone), one of two different non-musk odorants, SBT or isoamyl acetate (IAA), or no odorant. Relative to unexposed controls, all three musk-responsive subtypes showed significantly elevated quantities of newborn OSNs in mice exposed to muscone and ambretone ($P < 0.05$), with Olfr235 exhibiting increases of 3.2 and 3.8- fold, Olfr1440 exhibiting increases of 2.3 and 2.6-fold, and Olfr1431 exhibiting increases of 1.7 and 1.5-fold, respectively (**Figure 4D-F**). Notably, these differences were not affected by the use of alternative normalization methods (**Figure 4–figure supplement 3**). By contrast, none of the three musk-responsive subtypes exhibited significant changes in newborn OSN quantities in mice exposed to the non-musk odorants SBT or IAA. Likewise, the SBT-responsive subtype Olfr912 exhibited no

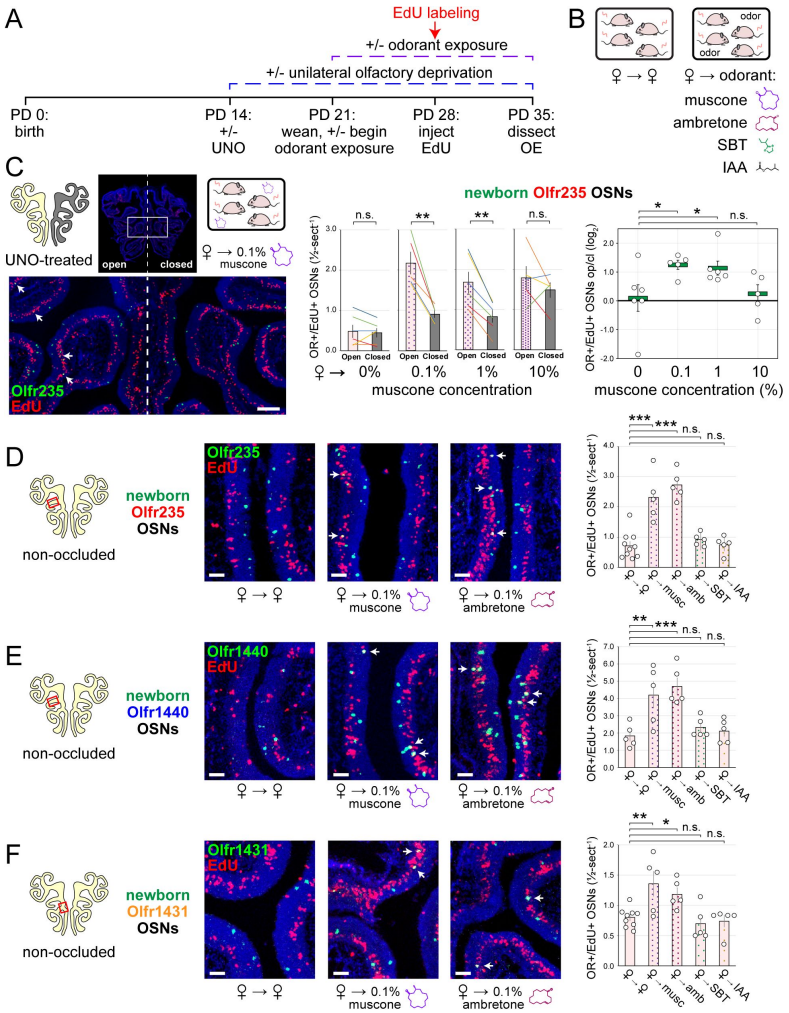


Figure 4.

Exposure of mice to musk odors causes selective increases in quantities of newborn OSNs of musk-responsive subtypes.

A, B. Experimental timeline (A) and conditions (B) used to generate OE tissue samples for assessing the effects of exposure to musk (muscone, ambretone) or non-musk (SBT, IAA) odorants on quantities of newborn OSNs of specific subtypes. Female mice were either UNO-treated or untreated (non-occluded) at PD 14, either exposed or unexposed to an exogenous musk or non-musk odorant starting at PD 21, EdU-labeled at PD 28, and sacrificed at PD 35. OEs were sectioned and analyzed using OR-specific RNA-FISH and EdU staining. C. *Left*: Representative image of an OE section from a UNO-treated female mouse that was exposed, at the time of EdU labeling, to 0.1% muscone, with newborn Olfr235 OSNs (OR+/EdU+) indicated by white arrows. *Middle, right*: Quantifications of newborn Olfr235 OSNs on the open and closed sides of tissue sections spanning the anterior-posterior lengths of OEs from UNO-treated female mice reveal significant open-side biases (*middle*) and significantly greater UNO effect sizes (*right*) in mice exposed to 0.1% or 1% (but not 10%) muscone compared to 0%, with a maximum effect size observed at a concentration of 0.1%. D–F. *Left, middle*: Representative images (*middle*) corresponding to the regions outlined in schematics (*left, red box*) of OE sections from non-occluded female mice that were exposed just to female littermates (♀ → ♀) or also to 0.1% muscone or 0.1% ambretone, with newborn Olfr235 (D), Olfr1440 (E), and Olfr1431 (F) OSNs (OR+/EdU+) indicated by white arrows. *Right*: Quantifications of newborn Olfr235 (D), Olfr1440 (E), and Olfr1431 (F) OSNs in tissue sections spanning the anterior-posterior lengths of OEs reveal that, compared to females exposed just to female littermates (♀ → ♀), significantly greater quantities of newborn OSNs of all 3 subtypes were observed in females also exposed to muscone (musc; 0.1%) or ambretone (amb; 0.1%), but not the non-musk odorants SBT (0.1%) or IAA (0.1%). Scale bars: 150 μm (C), 50 μm (D–F). Each line or circle represents a distinct mouse (n = 5–10 mice [≥ 5 OE sections/mouse] per OSN subtype and condition). ***P < 0.001; **P < 0.01; *P < 0.05; n.s. P> 0.05; ratio paired two-tailed t-test (C, *middle*); one-way ANOVA test, FDR-adjusted (C, *right*, D–F). Error bars: SEM. Data for ♀ → 0% muscone samples in panel C correspond to **Figure 3E**. Image and data for ♀ → ♀ samples (D) correspond to **Figure 3G**. See also **Figure 4–figure supplements 1–4**.

significant changes in quantities of newborn OSNs in mice exposed to muscone, ambretone, or SBT (**Figure 4–figure supplement 4C**-*left*). Moreover, two non-musk-responsive subtypes that were previously found to undergo occlusion-dependent reductions in neurogenesis [30], Olfr827 (*Or9k7*) and Olfr1325 (*Or13ae2*), showed no significant changes in quantities of newborn OSNs in mice exposed to muscone (**Figure 4–figure supplement 4C**-*middle, right*). Taken together, these findings indicate that: 1) the exposure of non-occluded mice to exogenous musk odorants selectively increases quantities of newborn OSNs of musk-responsive subtypes, 2) the exposure of mice to exogenous non-musk odorants has no effect on quantities of newborn OSNs of musk-responsive subtypes, and 3) only a fraction of subtypes have a capacity to exhibit increases in quantities of newborn OSNs following exposure to their cognate odorants.

Musk odorant-dependent increases in quantities of newborn OSNs of musk-responsive subtypes persist into adulthood

Findings that the exposure of adolescent mice to male or musk-like odors elevates quantities of newborn OSNs of musk-responsive subtypes raise the question of whether this phenomenon is limited to early life or, rather, persists into adulthood. To address this, we compared quantities of newborn OSNs of three musk-responsive subtypes and one musk non-responsive subtype within the OEs of 9-week-old (PD 65) non-occluded female mice that had been either unexposed to an exogenous odorant or exposed to 0.1% muscone starting from weaning (PD 21) and EdU-treated for 3 days starting at 8 weeks of age (PD 56-58) (**Figure 5A, B**). Remarkably, despite the generally reduced OSN birthrate in adult mice compared to adolescents, all three musk-responsive subtypes showed elevated quantities of newborn OSNs in mice exposed to muscone relative to unexposed controls, with Olfr235, Olfr1440 and Olfr1431 exhibiting 1.5-fold, 2.2- fold, and 1.9-fold increases, respectively (**Figure 5C-E**). Notably, observed increases reached statistical significance ($P < 0.05$) only for subtype Olfr1440, likely due to the higher baseline birthrate of this subtype relative to Olfr235 and Olfr1431. As expected, exposure to muscone did not significantly increase quantities of newborn OSNs of subtype Olfr912 (**Figure 5F**). These findings indicate that the capacity for odorant-dependent increases in quantities of newborn OSNs of specific subtypes persists into adulthood.

The time-independence of odor-driven increases in EdU-labeled OSNs of musk-responsive subtypes is consistent with a mechanism involving altered OSN birthrates

Odor-dependent increases in the quantities of newborn OSNs of musk-responsive subtypes could, in principle, be caused by a mechanism that selectively accelerates the rates with which these subtypes are generated or, alternatively, the rates with which they are selectively enriched following their generation (e.g., *via* enhanced survival or OR switching [52]). If differences in newborn OSN quantities are mediated by selective enrichment, they would be expected to increase in magnitude over time following EdU labeling, as a subset of newborn OSNs exhibit longer lifespans or switch their OR identities in the presence of stimulation. If, however, changes are mediated by accelerated birthrates of specific OSN subtypes, increases in newborn OSN quantities should appear shortly following EdU labeling and remain stable over time. To distinguish between these possibilities, we compared stimulation-dependent changes in quantities of newborn musk-responsive OSNs at two timepoints: 4 days post-EdU, the earliest point during OSN differentiation when OR transcripts can be consistently detected *via* RNA-FISH [30,53], and three days later (7 days post-EdU) (**Figure 6A**). In initial experiments, the time-dependence of open-side biases in quantities of EdU-labeled OSNs of musk-responsive subtypes were assessed in UNO-treated and sex-separated males, females, and females exposed to 0.1% muscone (**Figure 6B**). In sex-separated males, robust open-side biases and statistically indistinguishable UNO effect sizes were observed at 4 and 7 days-post EdU for quantities of EdU-labeled OSNs of subtypes Olfr235, Olfr1440, and Olfr1431 (**Figure 6C, D**; **Figure 6–figure supplement 1A, B**). Likewise,

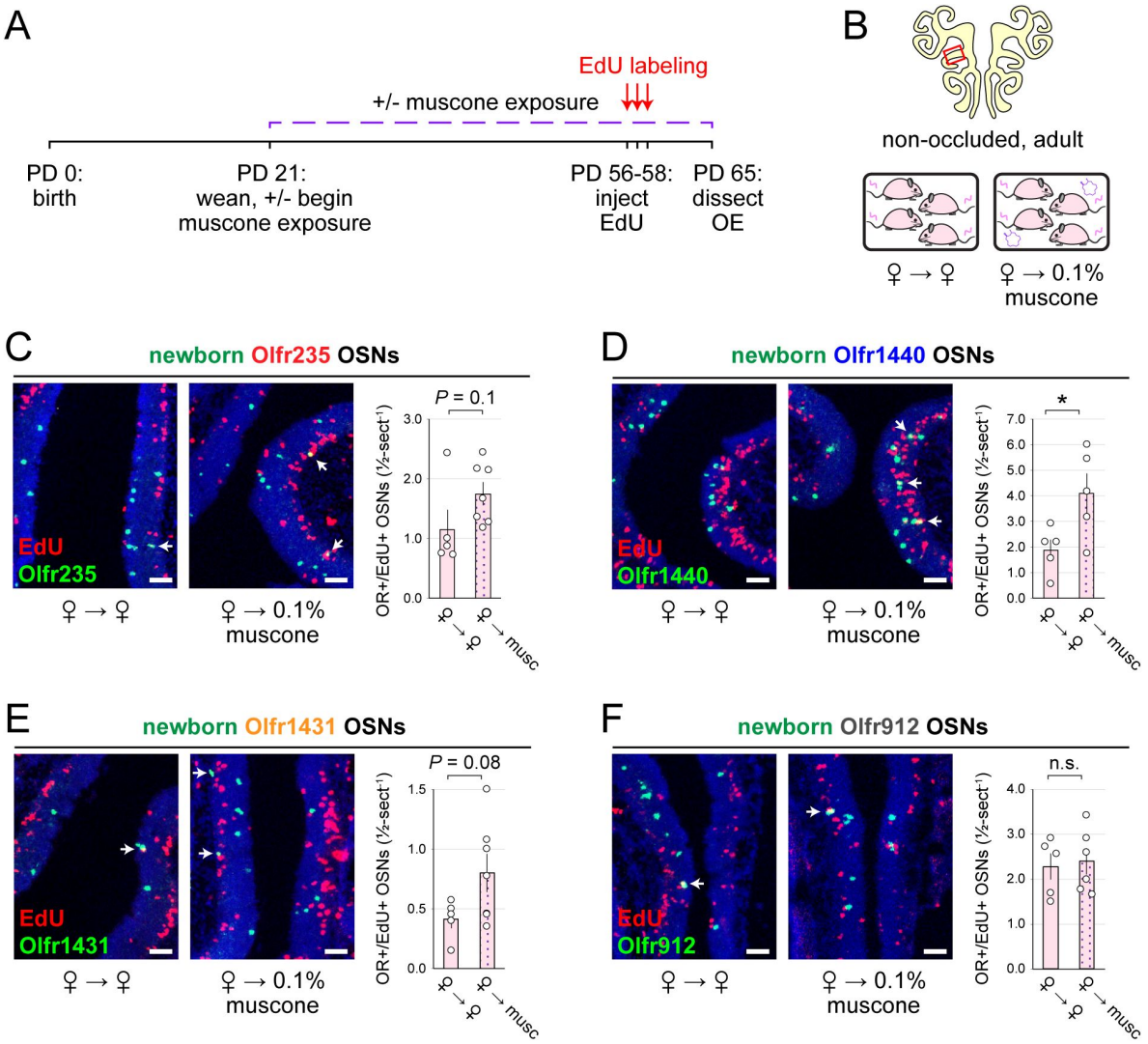


Figure 5.

Musk exposure increases quantities of newborn OSNs of musk-responsive subtypes in adulthood.

A, B. Experimental timeline (A) and conditions (B) used to generate OE tissue samples for assessing the effects of exposure to musk odors on quantities of newborn OSNs of specific subtypes in non-occluded adult mice. Mice were either exposed or unexposed to 0.1% muscone starting at PD 21, EdU-labeled at PD 56-58, and sacrificed at PD 65. OEs were sectioned and analyzed using OR-specific RNA-FISH and EdU staining. C–F. *Left*: Representative images corresponding to the region outlined in the schematic in panel B (*top*), of OEs from non-occluded adult female mice that were either exposed to just female littermates (♀ → ♀) or also to muscone (♀ → 0.1% muscone), with newborn Olfr235 (C), Olfr1440 (D), Olfr1431 (E), and Olfr912 (F) OSNs (OR+/EdU+) indicated by white arrows. *Right*: Quantifications of newborn OSNs within tissue sections spanning the anterior-posterior lengths of OE from non-occluded adult female mice reveal greater quantities of newborn OSNs of all 3 musk-responsive subtypes (Olfr235, Olfr1440, and Olfr1431) in mice exposed to muscone compared to just female littermates (♀ → ♀), while quantities of newborn OSNs of the SBT- responsive subtype Olfr912 were relatively unaffected. Scale bars: 50 µm. Each circle represents a distinct mouse (n = 5–7 mice [≥ 5 OE sections/mouse] per OSN subtype and condition). *P < 0.05; n.s. P> 0.05; unpaired two-tailed t-test. Error bars: SEM.

UNO-treated and muscone-exposed females exhibited robust open-side biases in quantities of newborn Olfr235 OSNs and statistically indistinguishable UNO effect sizes at 4 and 7 days-post EdU (**Figure 6E**; **Figure 6–figure supplement 1C**). As expected, no significant open-side biases in EdU-labeled OSN quantities or UNO effect sizes were observed at either 4 or 7 days post-EdU for subtypes Olfr912 or Olfr1463 in UNO-treated male (**Figure 6–figure supplement 1D, E**) or female mice exposed to muscone (**Figure 6–figure supplement 1F**), or for subtype Olfr235 in sex-separated females (**Figure 6–figure supplement 1G**).

Using a similar approach, we also assessed the time-dependence of differences in muscone-driven increases in quantities of EdU-labeled OSNs of specific subtypes in non-occluded female mice (**Figure 6G**-*left*). Consistent with UNO-based findings, non-occluded mice exhibited statistically indistinguishable muscone-dependent increases at 4 and 7 days post-EdU for quantities of newborn OSNs of the musk-responsive subtypes Olfr235, Olfr1440, and Olfr1431 (**Figure 6G**-*right*), as well as the control subtype Olfr912 (**Figure 6–figure supplement 1H**). Taken together, these findings support the hypothesis that odor-dependent increases in the quantities of newborn OSNs of musk responsive subtypes reflect selective changes in the birthrates of these subtypes.

Evidence that mice emit musk-like odors

Previous findings that OSNs of musk-responsive subtypes respond to male mouse odors [19] and become more highly represented within the OEs of mice exposed to males [19,24], together with evidence from the current study that the exposure of mice to male odors can accelerate the birthrates of these subtypes, indicate that mice emit odors that stimulate musk-responsive subtypes. Interestingly, mouse preputial glands have been found to have a high degree of histomorphological, transcriptomic, and molecular similarities to muskrat scent glands [54], which secrete musk odors [55]. Moreover, a previous study noted preliminary evidence that mouse preputial gland extracts contain compounds that activate Olfr1440 OSNs [33]. Based on this information, we analyzed mouse preputial gland extracts *via* gas chromatography – mass spectrometry (GC-MS) for known musk molecules, particularly those shown to stimulate Olfr235 and Olfr1440 OSNs [33]. Intriguingly, these analyses revealed the presence of molecules whose GC-MS signals are structurally consistent with known musk molecules (**Appendix 2– figure 1**). A potential match to one such signal is cycloheptadecanol, a musk molecule that is structurally similar to those known to activate Olfr235 and Olfr1440 OSNs [33]. Verification of the presence of this and other musk-like odors that correspond to signals from mouse preputial glands will require comparisons to pure standards. Notably, the approach employed here was solely focused on the preputial gland and known musk molecules. However, it is conceivable that mouse musk-responsive OSNs are naturally stimulated by molecules that are structurally unrelated to those that have been characterized to date or are emitted from sources other than preputial glands. Future studies will be needed to address these possibilities.

Discussion

The exposure of mice to discrete odorants can selectively accelerate the birthrates of OSN subtypes that they stimulate

Previous studies have established that UNO reduces the overall rate of neurogenesis on the closed side of the OE relative to the open [27–29,56]. Recently, these occlusion-induced reductions were found to reflect decelerations of the birthrates of only a fraction of the ~1200 OSN subtypes and to vary according to age and/or the olfactory environment [30], suggesting the possibility that unknown olfactory stimuli selectively promote the birthrates of these subtypes. In this study we aimed to test this by elucidating the nature of stimuli that accelerate the birthrates of specific OSN subtypes. We have presented findings that these stimuli include discrete odorants that selectively activate the same subtypes whose birthrates are accelerated. These findings build upon previous

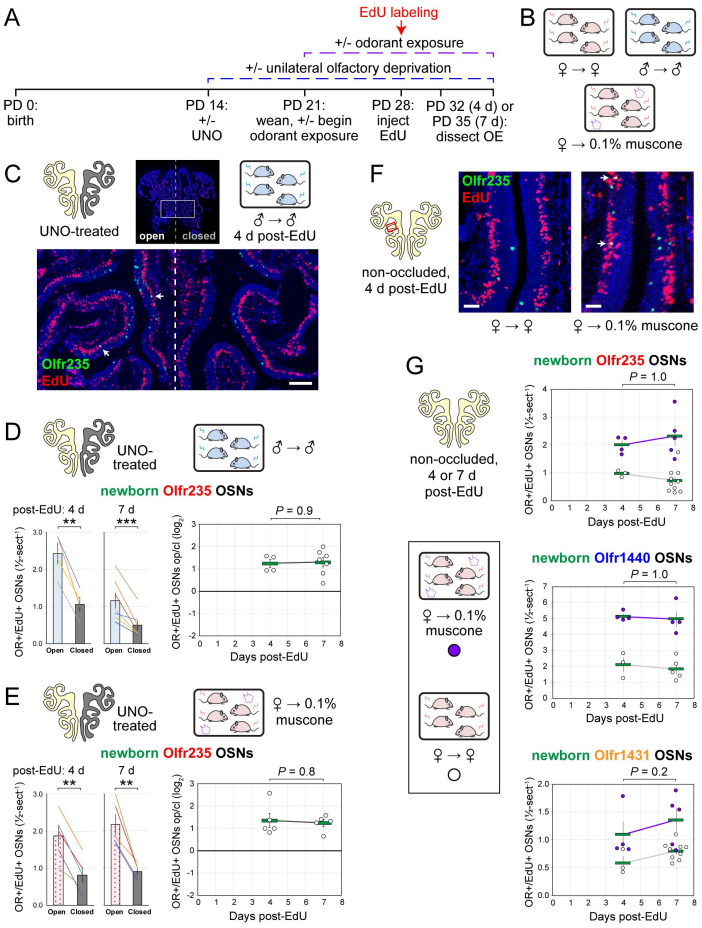


Figure 6.

Stimulation-dependent increases in quantities of newborn OSNs of musk-responsive subtypes are stable over time following birth, consistent with a mechanism involving altered neurogenesis.

A, B. Experimental timeline (A) and conditions (B) used to generate OE tissue samples for assessing the time-dependence of the effects of exposure to male or musk odors on quantities of newborn OSNs of specific subtypes. Mice were either UNO-treated or untreated (non-occluded) at PD 14, either exposed or unexposed to muscone starting at PD 21, EdU- labeled at PD 28, and sacrificed at either PD 32 (4 d post-EdU) or PD 35 (7 d post-EdU). OEs were sectioned and analyzed using OR-specific RNA-FISH and EdU staining. C. Representative image of an OE section from a UNO-treated male mouse that was exposed, at the time of EdU labeling, to male littermates (♂ → ♂) and sacrificed 4 d post-EdU, with newborn Olfr235 OSNs (OR+/EdU+) indicated by white arrows. D, E. Quantifications of newborn Olfr235 OSNs on the open and closed sides of tissue sections spanning the anterior-posterior lengths of OEs from UNO-treated male mice that were exposed to male littermates (♂ → ♂) (D) or female mice that were exposed to 0.1% muscone (♀ → 0.1% muscone) (E) and sacrificed 4 or 7 d post-EdU. Under both conditions, significant open-side biases in quantities of newborn Olfr235 OSNs were observed at both timepoints (*left*), with no statistically significant differences in UNO effect sizes observed over time (*right*). F. Representative images (*right*) corresponding to the region outlined in schematic (*left, red box*) of OE sections from non-occluded female mice that were exposed, at the time of EdU labeling, to either just their female littermates (♀ → ♀) or also to muscone (♀ → 0.1% muscone) and sacrificed 4 d post-EdU, with newborn Olfr235 OSNs (OR+/EdU+) indicated by white arrows. G. Quantifications of newborn Olfr235 (*top*), Olfr1440 (*middle*), and Olfr1431 (*bottom*) OSNs within tissue sections spanning the anterior-posterior lengths of OEs from non-occluded female mice that exposed to either just their female littermates (♀ → ♀) or also to muscone (♀ → 0.1% muscone) and sacrificed 4 or 7 d post-EdU. Greater quantities of newborn OSNs of all 3 subtypes were observed at both timepoints in muscone-exposed mice compared to controls, with no statistically significant interaction between time and newborn OSN quantities observed. Scale bars: 150 μm (C), 50 μm (F). Each circle represents a distinct mouse (n = 4–10 mice [≥ 5 OE sections/mouse] per OSN subtype and condition). ***P < 0.001; **P < 0.01; ratio paired two-tailed t-test (D, E *left*); unpaired two-tailed t-test (D, E *right*); two-way ANOVA test (factors: muscone exposure, days post-EdU) (G). Error bars: SEM. Data for 7 d post-EdU samples correspond to **Figures 2**–**4**. See also **Figure 6–figure supplement 1**.

observations that a small group of musk-responsive OSN subtypes are more highly represented in the OEs of mice exposed to male odors compared to mice isolated from them [19,24], and that some of these subtypes are also responsive to male-specific odors [19] (**Appendix 1–figure 1**). These data suggested that one or more components of mouse-emitted odors might naturally accelerate the birthrates of these OSN subtypes. Here, using scRNA-seq-based and histological approaches, we have found that UNO treatment of adolescent male mice reduces quantities of newborn OSNs of musk-responsive subtypes on the closed side of the OE relative to the open, consistent with the possibility that these subtypes have a capacity to undergo stimulation-dependent neurogenesis (**Figures 1**, **2**). Additionally, we have presented findings that the exposure of mice to male-specific odors or two different exogenous musk odorants can selectively elevate quantities of newborn OSNs of musk-responsive subtypes (**Figures 3-5**). Finally, we have described evidence that odor-driven increases in newborn OSN quantities are stable over time after EdU labeling, indicating that the observed changes reflect altered OSN birthrates as opposed to altered OSN lifespan or OR gene switching (**Figure 6**). Collectively, these findings support the hypothesis that the stimuli that regulate the birthrates of specific subtypes are discrete odors that selectively stimulate those subtypes.

How do discrete odors accelerate the birthrates of specific OSN subtypes?

Our findings that specific OSN subtypes exhibit accelerated birthrates following the exposure of mice to odors that selectively stimulate OSNs of these subtypes indicate that this process occurs *via* a mechanism that is highly specific with respect to the stimulating odors and the subtypes whose birthrates are accelerated. Considering that horizontal basal cells (HBCs) and globose basal cells (GBCs), the stem and progenitor cells that give rise to new OSNs, lack ORs and signal transduction molecules needed to detect and respond to odors, we hypothesize the existence of a signaling pathway from mature OSNs to HBCs or GBCs that alters the rates at which OSNs of specific subtypes are born. Findings from the present study and a previous one [30] indicate that this signaling capacity may be limited to only a fraction of OSN subtypes, since a majority of subtypes do not appear to exhibit stimulation-dependent neurogenesis [30]. Subtype Olfr912, for example, which detects the male-specific odor component SBT [24], exhibits no increase in birthrate upon exposure of female mice to male odors, and was therefore employed in this study as a control subtype. We speculate that the receipt of odor-derived signals by HBCs or GBCs alters OR choices or amplifies choices that have already been made. Elucidating the nature of odor-dependent signals received by HBCs/GBCs, as well as the mechanism by which these signals accelerate the birthrates of specific OSN subtypes are important areas of future investigation.

What functions does discrete odor stimulation-dependent neurogenesis of specific OSN subtypes serve?

Because OSN differentiation entails the stochastic process of singular OR choice [9,10], it has long been assumed that OSN neurogenesis is entirely stochastic with respect to OR identity. Thus, unlike other regions of the nervous system where persistent neurogenesis is known to play important adaptive roles [6–8], life-long neurogenesis within the OE is generally assumed to serve the merely homeostatic function of replacing neurons lost to turnover and injury [5]. Results of the present study, together with those of a previous one [30], challenge these assumptions by demonstrating that neurogenesis is not entirely stochastic with respect to subtype, but rather that the birthrates of a fraction of OSN subtypes can be selectively and directionally regulated by discrete odor experiences (**Figure 7**). These findings suggest the possibility that persistent neurogenesis within the OE serves an unknown adaptive function in addition to the known homeostatic one. It is conceivable, for example, that the acceleration of the birthrates of specific OSN subtypes could selectively enhance sensitivity to odors detected by those subtypes by increasing their representations within the OE [57–60]. Under this scenario, OSNs of affected subtypes might have baseline representations that lie within the dynamic range for signaling to

projection neurons under physiological concentrations of cognate odors, such that accelerated neurogenesis could enhance an animal's sensitivity to odors detected by these subtypes. This effect could have relevance to intriguing and unexplained observations in both rodents and humans that exposure to specific odors can dramatically increase sensitivity to them [61–66]. Alternatively, or in addition, OSNs produced *via* odor-dependent neurogenesis could conceivably enable the formation of new OB glomeruli and synaptic connections with projection neurons [67–70]. Under this scenario, stimulation-dependent neurogenesis of specific subtypes could alter inputs to the olfactory cortex and thereby regulate the perception of, and behavioral responses to, specific odors.

A special role for musk-responsive OSN subtypes

Results of the present study demonstrate that the birthrates of musk-responsive subtypes can be regulated by exposure to musk odors, a group of molecules that are naturally emitted by numerous mammalian species [71–73]. For some mammals, musk odors are known to function in attracting mates, marking territory, and deterring predators [55,74]. Moreover, exposure to musk odors has been reported to cause physiological changes in some mammals, including humans, suggesting that they can function as semiochemicals [75,76]. In mice, the physiological functions of musk odors, if any, are unknown, although they have been found to be selectively attractive to male mice [77]. Previous findings that exogenous musk odors activate a small number of related and evolutionarily conserved mouse ORs [32–34] and that odors emitted selectively by male mice activate OSN subtypes that express a subset of these ORs [19] suggest the possibility that mice also emit musk-like molecules. Our preliminary findings that mouse preputial gland extracts contain molecules that are structurally related to known musk odors provide additional support for this possibility.

Our findings that the exposure of mice to odors from adolescent males, but not females, accelerates the birthrate of Olfr235 OSNs, provide a mechanistic explanation for previous observations that mice exposed to male odors exhibit higher representations of this subtype [19,24]. Curiously, two other musk-responsive subtypes that displayed a higher representation in males and females housed with males, Olfr1440 and Olfr1431 [19], showed stimulation-dependent changes in newborn OSN quantities in both adolescent male and female mice, while a third such subtype, Olfr1437, exhibited no occlusion-mediated changes in quantities of newborn OSNs in adolescent mice of either sex (**Figures 1**, **2**, and data not shown). Considering the close relationship of the ORs that define musk-responsive OSN subtypes [32], these differences are intriguing. One conceivable explanation is that musk-responsive OSN subtypes vary in their sensitivity to distinct musk-like odorant molecules [33,77], which may be differentially emitted by mice in an age- and sex-dependent manner. Indeed, mouse odor profiles are known to vary considerably as a function of age and sex [47–50]. In support of this, we have found that the exposure of adolescent mice to adults selectively intensifies UNO-induced open-side biases in quantities of newborn Olfr1431 OSNs. A more complete understanding of the role of odor-stimulation-dependent neurogenesis in altering the representations of distinct musk-responsive subtypes in the mouse OE will require determining the identities and sources of the natural ligands that stimulate these subtypes and how exposure to these odorants affects OSN birthrates. Future studies will also be needed to determine the mechanism that endows musk-responsive subtypes, as well as a fraction of other subtypes whose ligands have yet to be identified [30], with the capacity to undergo stimulation-dependent neurogenesis, and whether these subtypes detect odors with special functional salience.

Methods

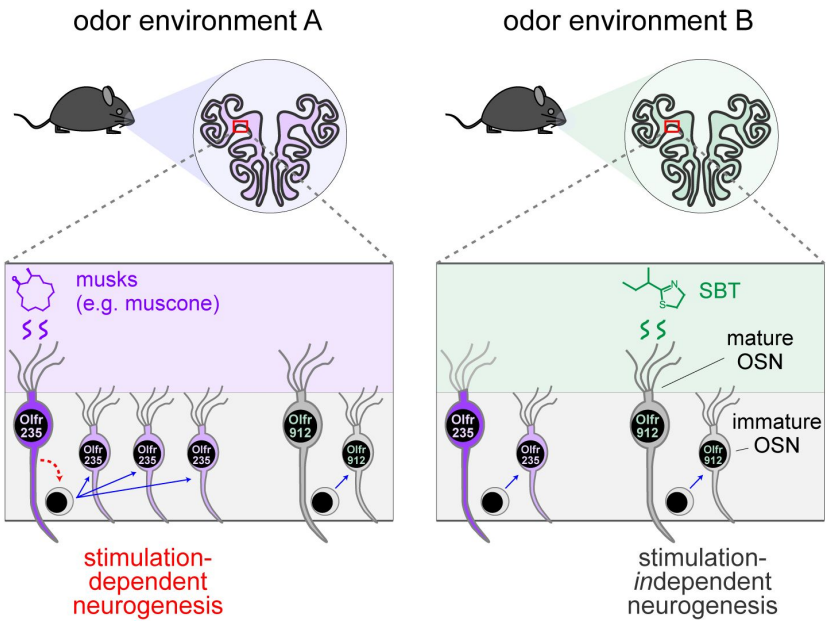
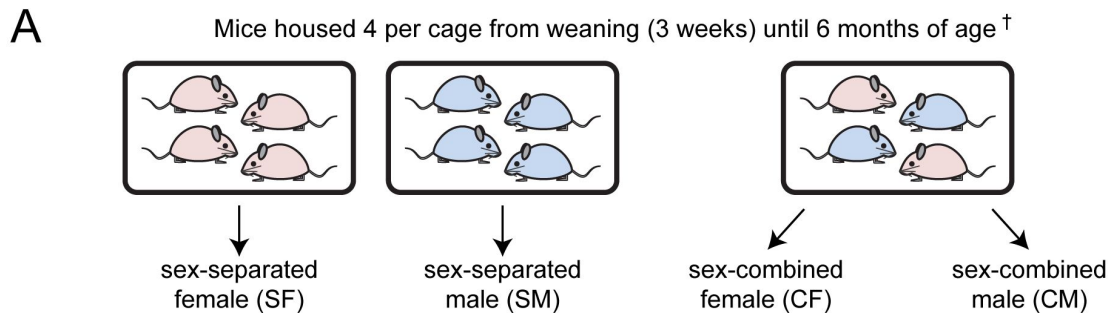


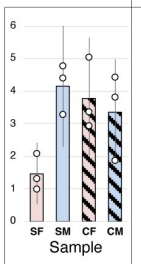
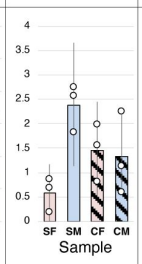
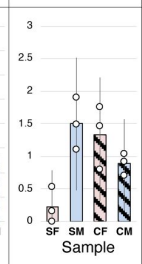
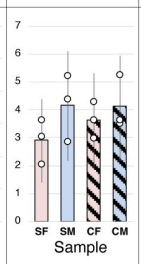
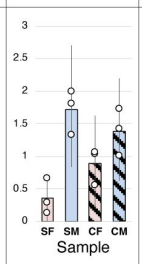
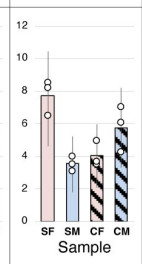
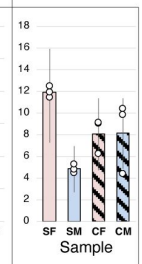
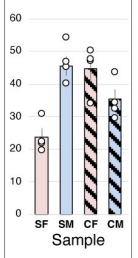
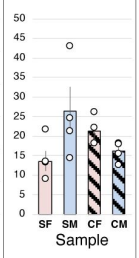
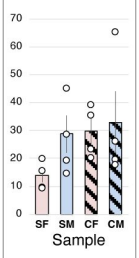
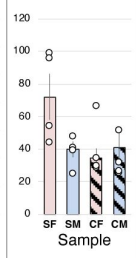
Figure 7.

Model for how specific odors selectively increase quantities of newborn OSNs of subtypes that are stimulated by them.

A fraction of OSN subtypes (e.g., Olfr235), upon stimulation by discrete odors (e.g., musks), undergo accelerated rates of neurogenesis. Most subtypes (e.g., Olfr912) do not exhibit altered rates of neurogenesis upon stimulation by odors that stimulate them (e.g., SBT). One hypothetical mechanism involves selective signaling from odor-stimulated mature OSNs of specific subtypes to neural progenitors.



B

	Olfr235 (*Or5an11*)	Olfr1437 (*Or5an1b*)	Olfr1431 (*Or5an9*)	Olfr1440 (*Or5an6*)	Olfr1434 (*Or5an1*)	Olfr1419 (*Or10q3*)	Olfr912 (*Or8b48*)	Olfr1295 (*Or4k45*)
Transcript level (fragments/ kilobase of transcript per million mapped reads; FPKM) †					expression too low			
OSN abundance (mm^{-2}) †			not determined	not determined	not determined			not determined
expression sex bias (SM vs SF)	 *†,‡	 *†,‡	 *†	 †	not determined	 *†	 *†,‡	 *†,‡
sex-selective odor-responsivity	 *†,‡	 *†,‡	not determined	not determined	not determined	none found †	 *†,‡	 *†,‡
known ligand(s)	muscone, other musks ‡,#,§	muscone, other musks ‡,#,§	muscone #	muscone, other musks #,§,¶	muscone #	not determined	SBT ‡	MTMT ‡

Appendix 1–figure 1.

Identification of OSN subtypes that are candidates for undergoing sex-specific- and/or musk odor-accelerated neurogenesis.

A. In a previous study, the OE transcript profiles of female and male mice that were housed either sex-separated or sex-combined from weaning (PD 21) until 6 months of age were profiled and compared *via* bulk RNA-seq [19,78]. B. OSN subtypes previously identified as responsive to sex-specific odors and/or musk-like odors. SBT, 2-sec-butyl-4,5-dihydrothiazole; MTMT, (methylthio)methanethiol; †, [19,78]; ‡, [24]; #, [32]; §, [33]; ¶, [34]. Error bars: 95% confidence intervals.

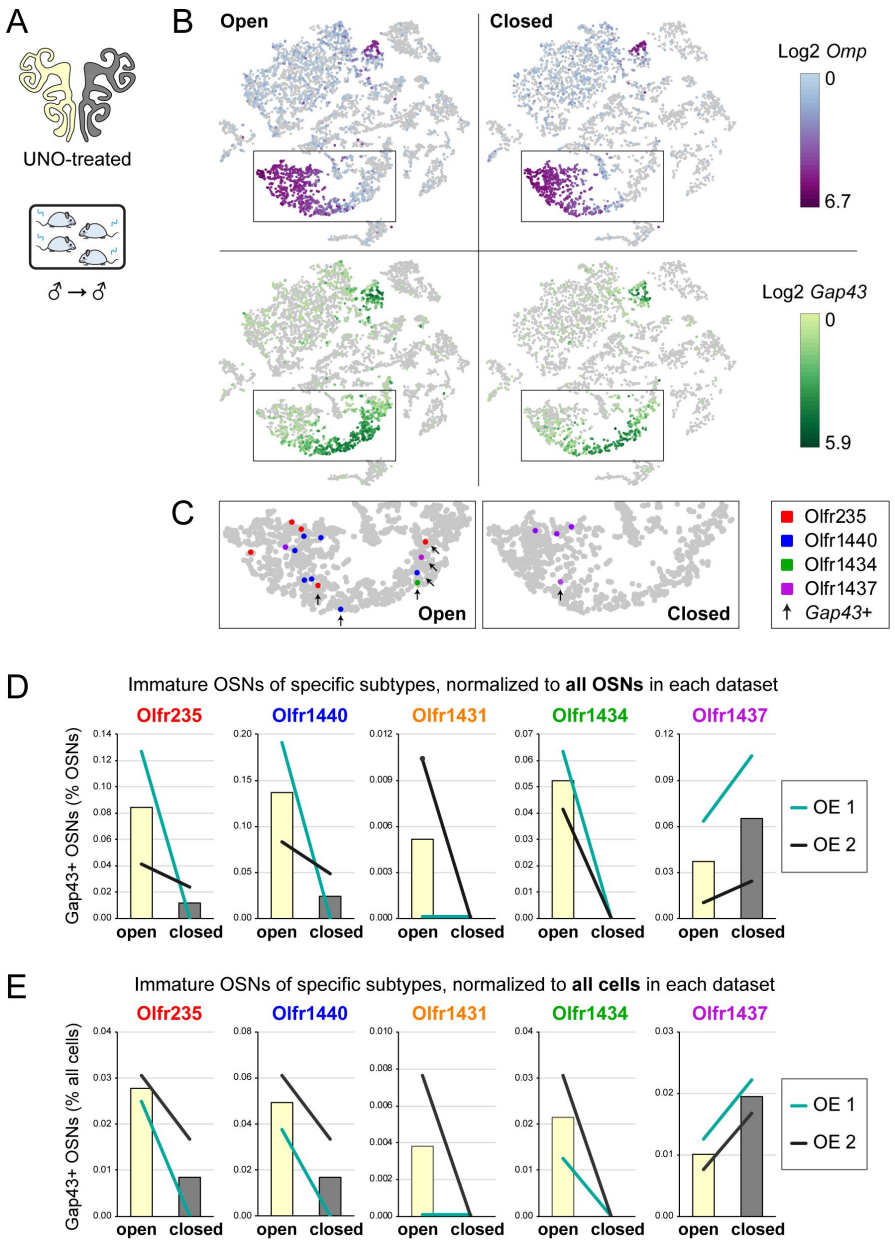


Figure 1–figure supplement 1.

scRNA-seq analyses of the open and closed sides of whole OEs from UNO-treated adolescent male mice reveal greater quantities of newborn OSNs of musk-responsive subtypes on the open side of the OE relative to the closed.

A. Experimental conditions used to generate scRNA-seq datasets for assessing the effects of UNO on quantities of newborn OSNs of specific subtypes in the OEs of adolescent male mice. B. *t*-distributed stochastic neighbor embedding (*t*-SNE) representations of all cells within scRNA-seq datasets corresponding to the open (*left*) and closed (*right*) sides of a male mouse OE (OE 1; generated in a previous study [30]), with *Omp* (mOSNs; *top*) and *Gap43* (iOSNS; *bottom*) expression shown. OE 1 datasets were generated as outlined in **Figure 1** (UNO-treated at PD 14, weaned sex-separated at P21, and sacrificed at PD 28). C. Enlarged view of the region of the *t*-SNE plot outlined in panel B, with OSNs of known musk-responsive subtypes indicated by colored dots, and immature (*Gap43+*) OSNs indicated by black arrows. D, E. Quantifications of immature (*Gap43+*) OSNs of musk-responsive subtypes as percentages of all OSNs (D) or all cells (E) within the OE 1 and OE 2 (generated in this study) datasets (*lines*), with mean values for the two datasets indicated (*bars*). In the OE 1 dataset, OSNs represent 943 of 4501 (21%) and 1573 of 7990 (20%) of all cells on the closed and open sides, respectively. In the OE 2 dataset, OSNs represent 8271 of 11919 (66%) and 9649 of 13101 (71%) of all cells on the closed and open sides, respectively.

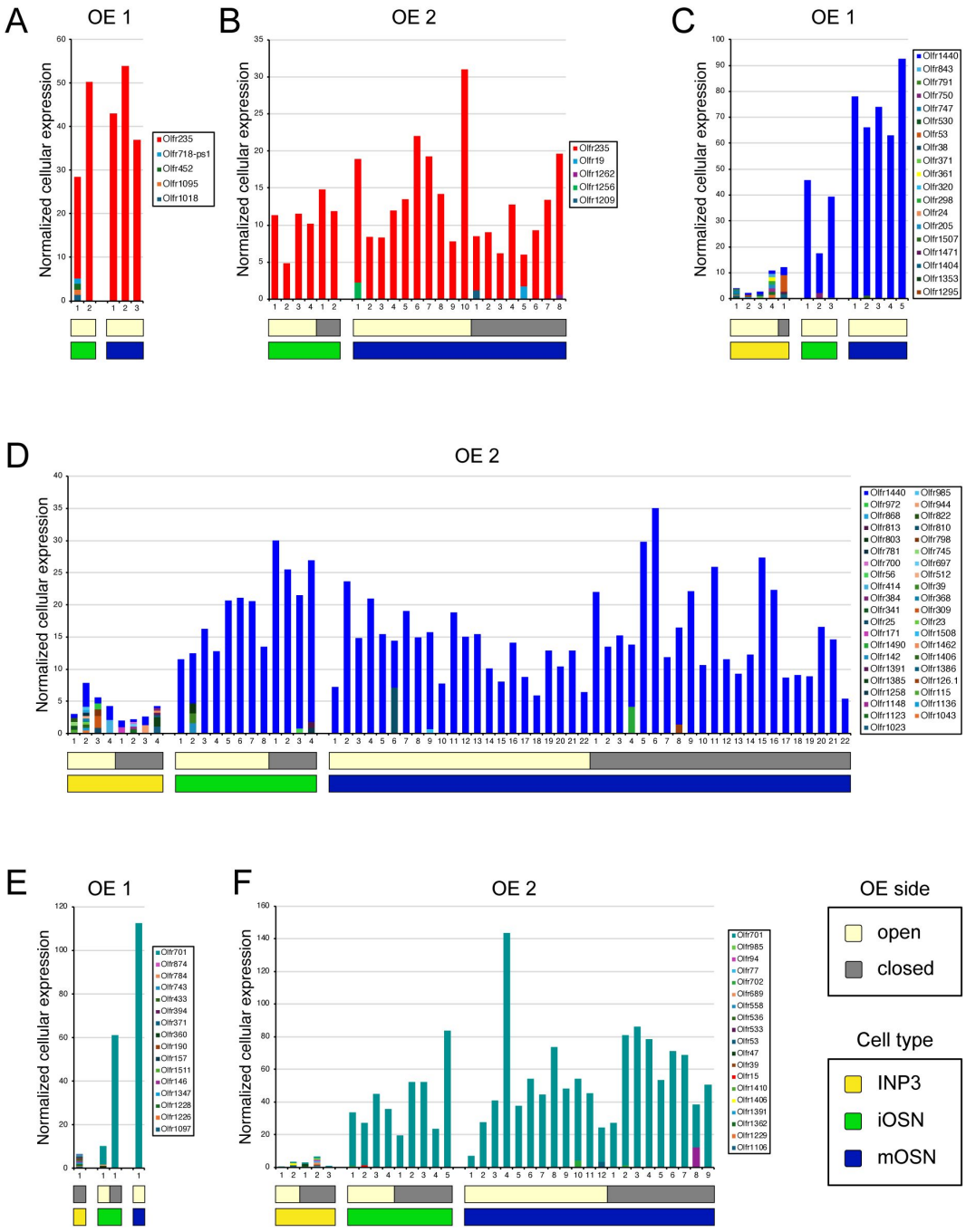


Figure 1–figure supplement 2.

Individual OSNs of musk-responsive subtypes on the open and closed sides of OEs from UNO-treated male mice exhibit typical OR expression within cells of the OSN lineage.

A-F. Cellular OR transcript levels (normalized to all transcripts within each cell) within INP3 (*horizontal dark yellow bars*), iOSN (*horizontal bright green bars*), and mOSN (*horizontal dark blue bars*) cells that contain transcripts encoding the musk responsive ORs Olfr235 (A, B) and Olfr1440 (C, D), and the randomly selected OR Olfr701 (*Or2ag2b*) from scRNA-seq datasets corresponding to 2 different male mice (OE 1, generated in a previous study [30], and OE 2, this study). As observed in previous studies [38,40,45], INP3 cells exhibit low levels of polygenic OR transcripts, while iOSNs and mOSNs exhibit high levels of monogenic OR transcripts. Moreover, OSN lineage cells of the same subtype and stage of maturity exhibit similar OR transcript levels and monogenicity within the open (*light yellow bars*) and closed sides (*gray bars*) of the OEs. Note: Olfr235 transcripts were not identified within INP3 cells of either dataset.

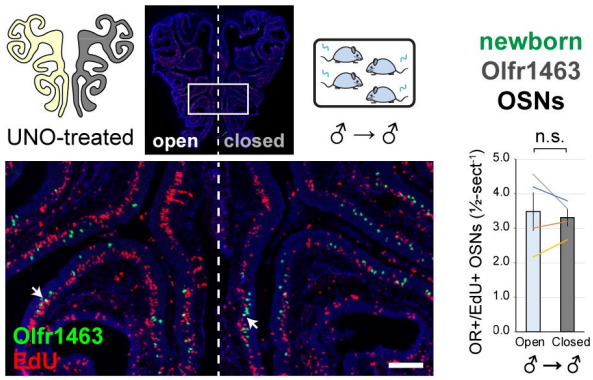


Figure 2–figure supplement 1.

EdU birthdating analyses show that UNO-treated adolescent male mice do not exhibit significantly different quantities of newborn OSNs of control subtype Olfr1463 on the open side of the OE relative to the closed.

OE tissue samples used for assessing the effects of UNO on quantities of newborn OSNs of subtype Olfr1463 were generated as outlined in **Figure 2**. OEs were sectioned and analyzed using Olfr1463-specific RNA-FISH and EdU staining. *Left*: Representative image of OE sections from a UNO-treated adolescent male mouse that was exposed, at the time of EdU labeling, to male littermates (♂ → ♂), with newborn OSNs (OR+/EdU+) indicated by white arrows. *Right*: Quantifications of newborn OSNs on the open and closed sides of tissue sections spanning the anterior-posterior lengths of OEs from UNO-treated male mice reveal no significant open-side bias in quantities of newborn OSNs of subtype Olfr1463. Scale bar: 150 μm. Each line represents a distinct mouse (n = 4 mice [≥ 5 sections/mouse]). n.s. $P > 0.05$; ratio paired two-tailed t-test. Error bars: SEM.

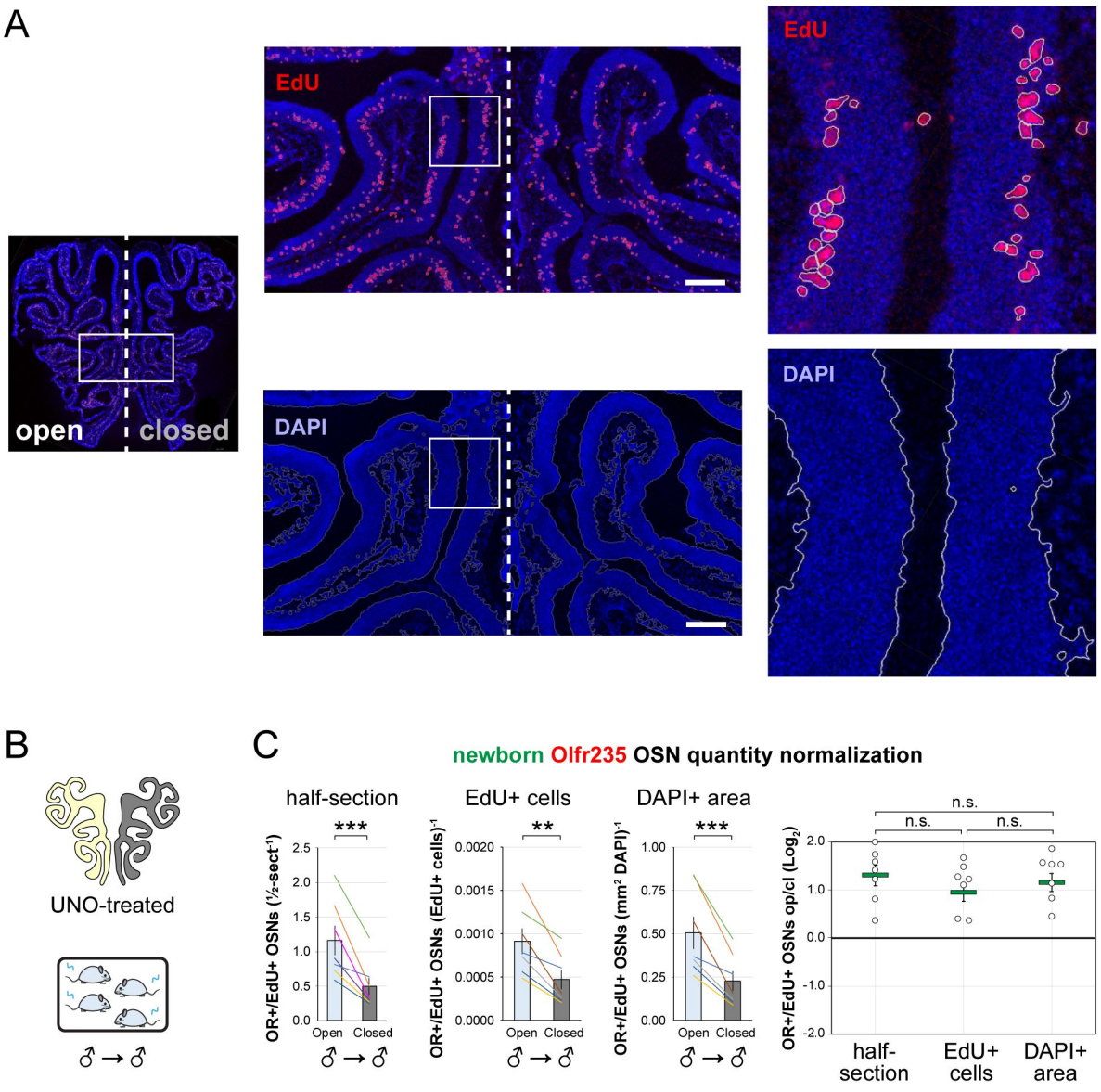


Figure 2–figure supplement 2.

Comparison of normalization methods for assessing the effects of exposure to male odors on quantities of newborn Olfr235 OSNs in the OEs of UNO-treated male mice.

A. Representative image corresponding to an EdU- and DAPI-stained OE section from an adolescent male mouse that was generated as outlined in **Figure 2**. EdU+ nuclei (*top*) and DAPI+ regions (*bottom*) are outlined in white, with medium (*middle*), and high magnification (*right*) views corresponding to the boxed regions within the preceding images. Scale bars: 150 µm. B. Experimental conditions used to generate OE tissues from UNO-treated adolescent male mice. C. Quantifications of newborn Olfr235 OSNs on the open and closed sides of tissue sections spanning the anterior-posterior lengths of OEs. OE sections were analyzed using Olfr235-specific RNA-FISH and EdU staining and newborn Olfr235 OSN quantities were normalized by half-section, number of EdU+ cells, or DAPI+ area. Each of the normalization methods yielded open-side biases of similar magnitude in quantities of newborn Olfr235 OSNs (*left*), with no significant differences in corresponding UNO effect sizes observed (*right*). Each line or circle represents a distinct mouse (n = 7–10 mice [≥ 5 sections/mouse] per condition). ***P < 0.001; **P < 0.01; *P < 0.05; n.s. P> 0.05; ratio paired two-tailed t-test (C, *left*); one-way ANOVA test, FDR-adjusted (C, *right*). Error bars: SEM. Data for newborn OSN quantities normalized by half-section correspond to **Figure 2**.

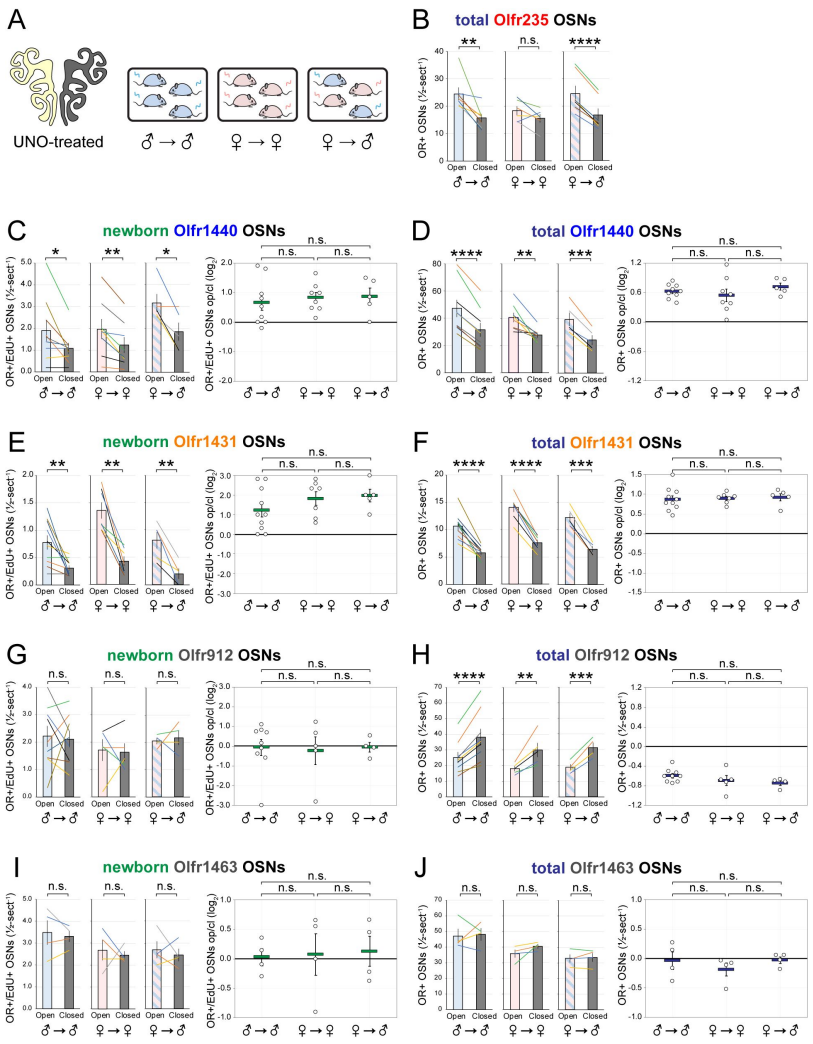


Figure 3–figure supplement 1.

Effects of exposure to male odors on differences in quantities of newborn and total OSNs of musk-responsive and control subtypes on the open and closed sides of the OEs of UNO-treated mice.

A. Experimental conditions used for tissue generation. OE tissue samples were generated according to the experimental timeline outlined in **Figure 3**, sectioned, and analyzed using OR-specific RNA-FISH and EdU staining. B. Quantifications of total (OR+) Olfr235 OSNs on the open and closed sides of tissue sections spanning the anterior-posterior lengths of OEs from UNO-treated mice reveal significant open-side biases in males and in females exposed to male littermates (♂ → ♂ or ♀ → ♂) but not in females exposed to just female littermates (♀ → ♀). C-F. Quantifications of Olfr1440 (C, D) and Olfr1431 (E, F) OSNs on the open and closed sides of tissue sections spanning the anterior-posterior lengths of OEs from UNO-treated mice reveal significant open-side biases (*left*) in quantities of newborn (OR+/EdU+; C, E) and total (D, F) OSNs of both subtypes under all odor conditions (♂ → ♂, ♀ → ♂, ♀ → ♀), with no significant differences in UNO effect sizes (*right*) observed. G, H. Quantifications of Olfr912 OSNs on the open and closed sides of tissue sections spanning the anterior-posterior lengths of OEs from UNO-treated mice reveal no significant open-side biases in quantities of newborn OSNs (G), but significant closed-side biases in total OSNs of this subtype (H) under all 3 conditions. Observed effects of UNO on total Olfr912 OSNs may be attributable to an increased rate of cell death for mature OSNs of this subtype due to overstimulation in the presence of male odors [19,24], to which all mice were exposed during at least part of the UNO period. I, J. Quantifications of Olfr1463 OSNs on the open and closed sides of tissue sections spanning the anterior-posterior lengths of OEs from UNO-treated mice reveal no significant open-side biases in quantities of newborn (I) or total (J) OSNs of this subtype under any of the conditions. Each line or circle represents a distinct mouse (n = 4–10 mice [≥ 5 sections/mouse] per OSN subtype and condition). ****P < 0.0001; ***P < 0.001; **P < 0.01; *P < 0.05; n.s. P > 0.05; ratio paired two-tailed t-test (B, C-J, *left*); one-way ANOVA test, FDR-adjusted (C-J, *right*). Error bars: SEM. Data for newborn OSN quantities in ♂ → ♂ samples (C, E, G, I) correspond to **Figure 2** and **Figure 2-figure supplement 1**.

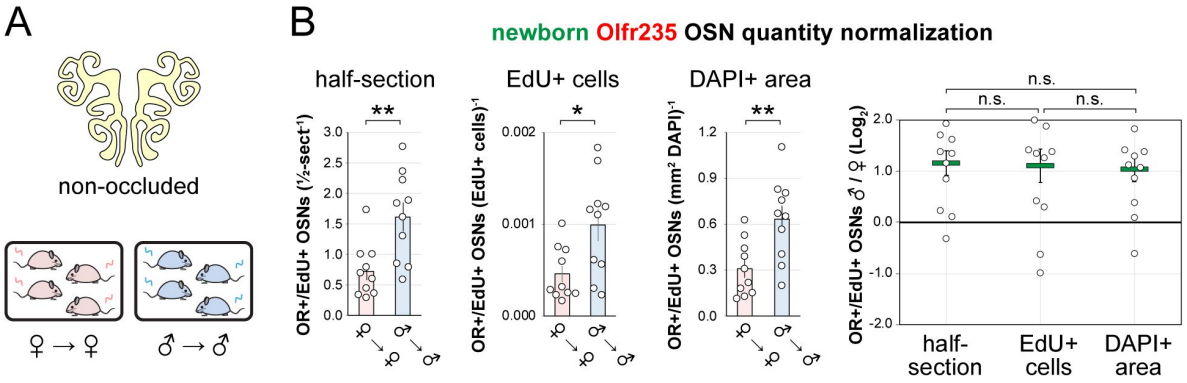


Figure 3–figure supplement 2.

Comparison of normalization methods for assessing the effects of exposure to male odors on quantities of newborn Olfr235 OSNs in the OEs of non-occluded mice.

A. Experimental conditions used to generate tissues. B. Quantifications of newborn Olfr235 OSNs within tissue sections spanning the anterior-posterior lengths of the OEs of non-occluded adolescent male and female mice that were weaned sex-separated. Newborn Olfr235 OSN quantities were normalized by half-section, number of EdU+ cells, or DAPI+ area. Each of the 3 normalization methods yielded differences of similar magnitude in quantities of newborn Olfr235 OSNs in non-occluded males exposed to male littermates (♂ → ♂) compared to females exposed to female littermates (♀ → ♀), with no significant differences observed in the corresponding odor exposure effect sizes (*right*; calculated from the ratios of normalized newborn Olfr235 OSNs within individual sex-separated male mice relative to the mean of normalized newborn Olfr235 OSNs within sex-separated females). Each line or circle represents a distinct mouse (n = 7–10 mice [≥ 5 sections/mouse] per condition). $^{**}P < 0.01$; $^{*}P < 0.05$; n.s. $P > 0.05$; ratio paired two-tailed t-test (B, *left*); one-way ANOVA test, FDR-adjusted (B, *right*). Error bars: SEM. Data for newborn OSN quantities normalized by half-section (B) correspond to **Figure 3G**.

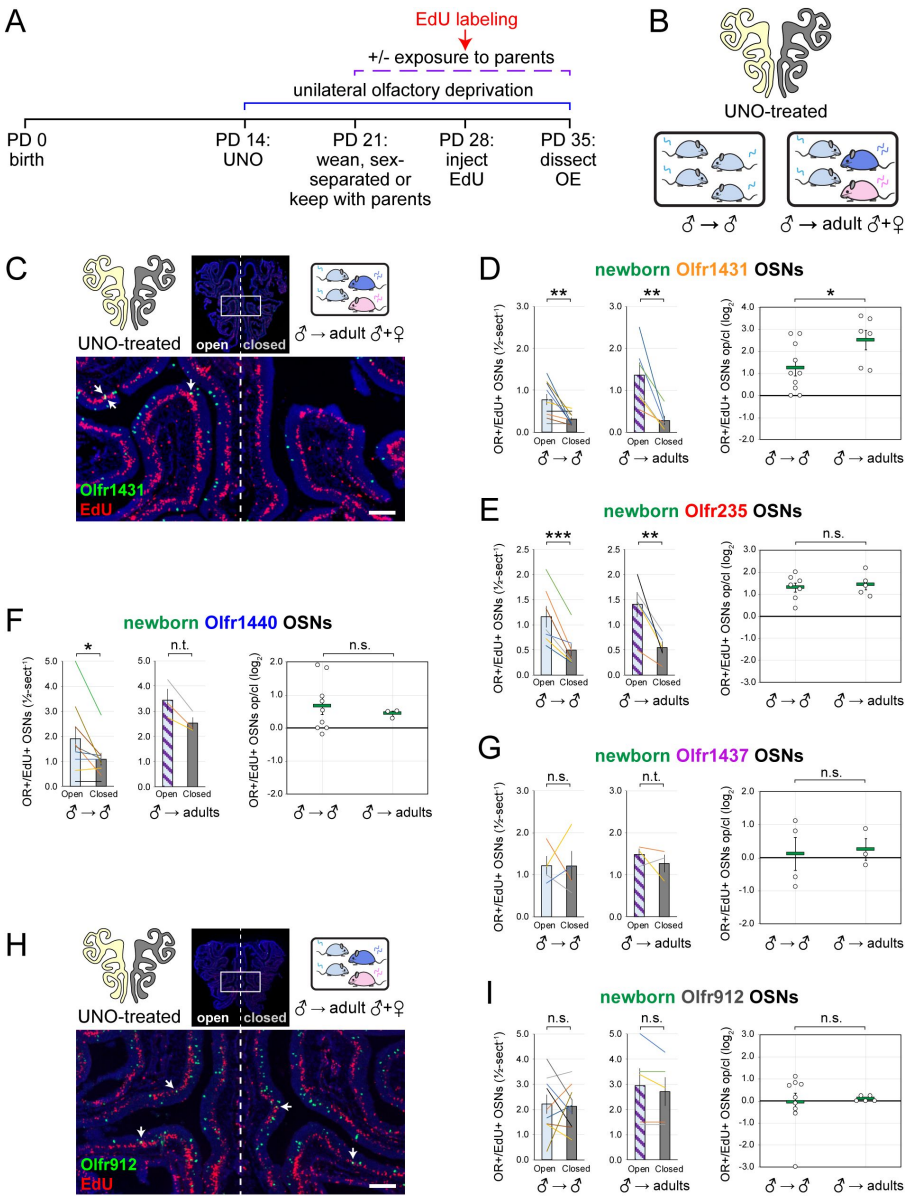


Figure 3–figure supplement 3.

UNO-induced open-side biases in quantities of newborn Olfr1431 OSNs are increased by exposure to adult mice.

A, B. Experimental timeline (A) and conditions (B) used to generate OE tissue samples for assessing the effects of exposure to adult mouse odors on quantities of newborn OSNs of specific subtypes. Male mice were UNO-treated at PD 14, weaned either sex-separated (♂ → ♂) or kept with parents (♂ → adult ♀ + ♂) at PD 21, EdU-labeled at PD 28, and sacrificed at PD 35. OEs were sectioned and analyzed using OR- specific RNA-FISH and EdU staining. C, H. Representative images of OE sections from UNO- treated adolescent male mice that were exposed, at the time of EdU labeling, to their parents, with newborn Olfr1431 (C) or Olfr912 OSNs (OR+/EdU+) indicated by white arrows. D-G, I. Quantifications of newborn Olfr1431 (D), Olfr235 (E), Olfr1440 (F), Olfr1437 (G), and Olfr912 (I) OSNs on the open and closed sides of tissue sections spanning the anterior-posterior lengths of OEs of UNO-treated mice that were exposed, at the time of EdU labeling, to just their male littermates (♂ → ♂) or also their parents (♂ → adult ♀ + ♂). Greater open-side biases (*left*) and corresponding UNO effect sizes (*right*) were observed for quantities of newborn OSNs of subtype Olfr1431 (D) in the OEs of UNO-treated male mice that were exposed to their parents compared to just littermates, while other subtypes tested were not significantly affected. Scale bars: 150 µm. Each line or circle represents a distinct mouse (n = 3–10 mice [≥ 5 sections/mouse] per OSN subtype and condition). ***P < 0.001; **P < 0.01; *P < 0.05; n.s. P> 0.05; n.t. not tested (n < 4); ratio paired two-tailed t-test (D-G, I, *left*); unpaired two-tailed t-test (D-G, I, *right*). Error bars: SEM. Data for ♂ → ♂ samples correspond to **Figure 2**.

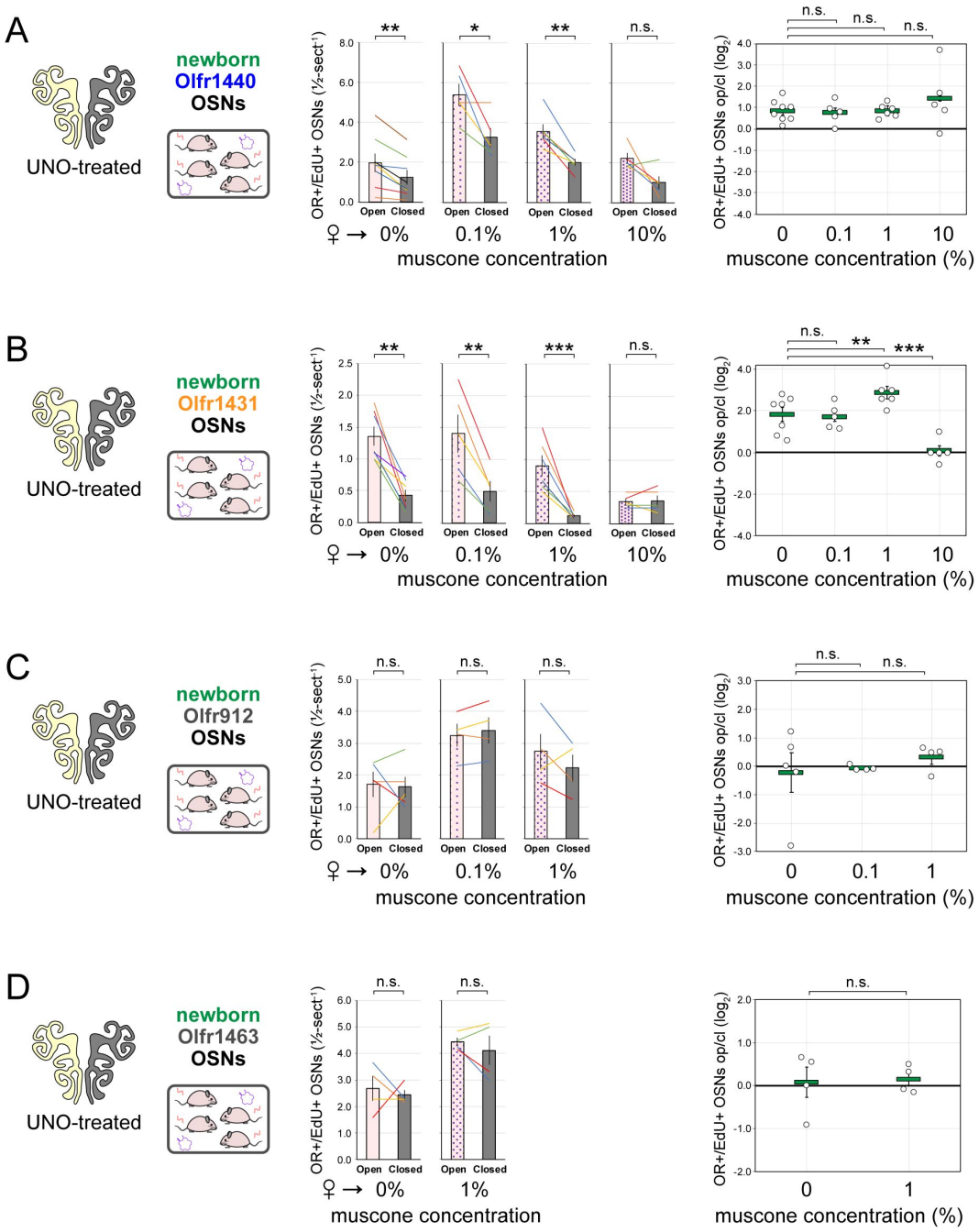


Figure 4–figure supplement 1.

Exposure of UNO-treated female mice to muscone causes concentration-dependent differences in quantities of newborn OSNs of musk responsive subtypes on the open and closed sides of the OE.

A–D. *Left*: Experimental conditions used to generate OE tissue samples for assessing the effects of exposure to muscone on quantities of newborn OSNs of specific subtypes. OE tissue samples were generated according to the experimental timeline outlined in **Figure 4**, sectioned, and analyzed using OR-specific RNA-FISH and EdU staining. *Middle, right*: Quantifications of newborn (OR+/EdU+) OSNs of the musk-responsive subtypes Olfr1440 (A) and Olfr1431 (B), and the control subtypes Olfr912 (C) and Olfr1463 (D) on the open and closed sides of tissue sections spanning the anterior-posterior lengths of the OEs of UNO-treated female mice that were exposed, at the time of EdU labeling, to 0, 0.1, 1, or 10% muscone. Each line or circle represents a distinct mouse (n = 4–8 mice [≥ 5 sections/mouse] per OSN subtype and condition). ***P < 0.001; **P < 0.01; *P < 0.05; n.s. P> 0.05; ratio paired two-tailed t-test (*middle*); unpaired two-tailed t-test (*right*). Error bars: SEM. Data for ♀ → 0% muscone samples correspond to **Figure 3–figure supplement 1**.

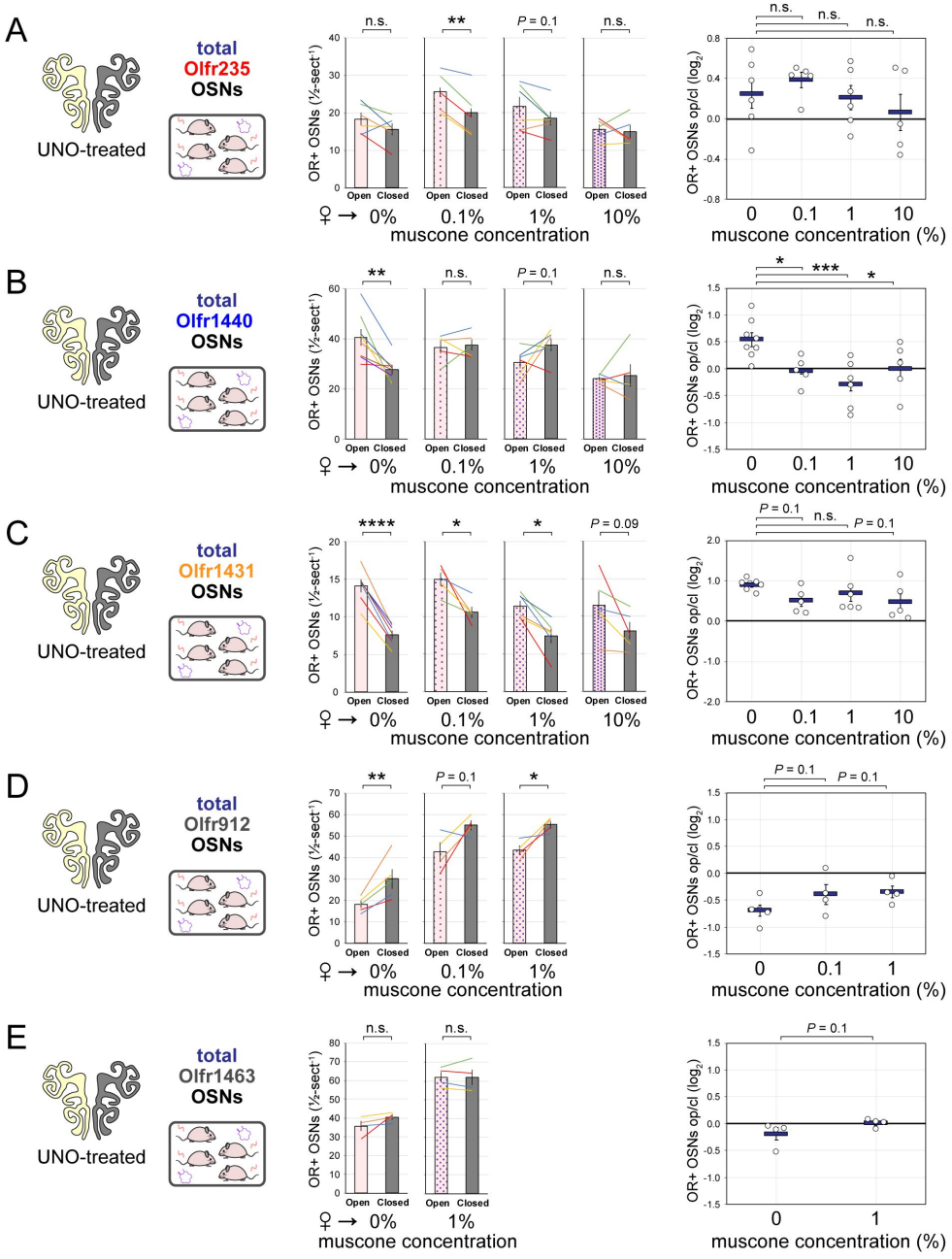


Figure 4–figure supplement 2.

Muscone exposure induces concentration-dependent differences in quantities of total OSNs of musk responsive subtypes on the open and closed sides of the OEs of UNO-treated mice.

A–E. *Left*: Experimental conditions used to generate OE tissue samples for assessing the effects of exposure to muscone on quantities of total OSNs of specific subtypes. OE tissue samples were generated according to the experimental timeline outlined in **Figure 4**, sectioned, and analyzed using OR-specific RNA-FISH and EdU staining. *Middle, right*: Quantifications of total (OR+) OSNs of the musk-responsive OSN subtypes Olfr235 (A), Olfr1440 (B) and Olfr1431 (C), and the control subtypes Olfr912 (D) and Olfr1463 (E) on the open and closed sides of tissue sections spanning the anterior-posterior lengths of the OEs of UNO-treated female mice that were exposed, at the time of EdU labeling, to 0, 0.1, 1, or 10% muscone. Note that the reduced quantities of total OSNs of subtype Olfr912 may be attributable to a reduced rate of survival for mature Olfr912 OSNs in the presence of male odors [19,24], to which mice were exposed prior to weaning. Each line or circle represents a distinct mouse (n = 4–8 mice [≥ 5 sections/mouse] per OSN subtype and condition). $^{**}P < 0.01$; $^{*}P < 0.05$; n.s. $P > 0.05$; two-tailed paired t-test (*middle*); two-tailed unpaired t-test (*right*). Error bars: SEM.

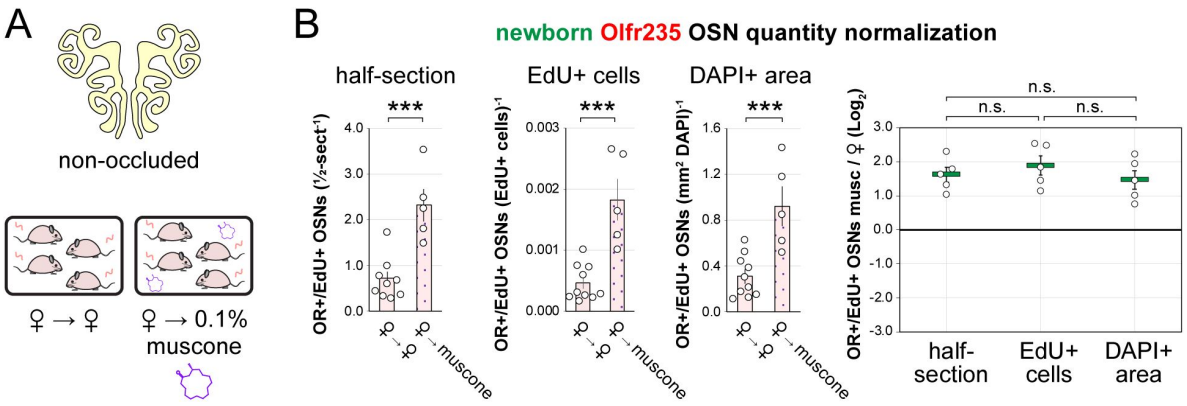


Figure 4–figure supplement 3.

Comparison of normalization methods for assessing the effects of exposure to a musk odor on subtype-specific newborn OSN quantities in the OEs of non-occluded mice.

A. Experimental conditions used to generate OE tissue samples. Samples were generated according to the experimental timeline outlined in **Figure 4**, sectioned, and analyzed using OR-specific RNA-FISH and EdU staining. B. Quantifications of newborn Olfr235 OSNs (OR+/EdU+) within tissue sections spanning the anterior-posterior lengths of the OEs of non-occluded female mice that were either exposed to 0.1% muscone (♀ → muscone) or unexposed to an exogenous odorant (♀ → ♀) starting from PD 21, EdU-labeled at PD 28, and sacrificed at PD 35. OE sections were analyzed using Olfr235-specific RNA-FISH and EdU staining and newborn Olfr235 OSN quantities were normalized by half-section, number of EdU+ cells, or DAPI+ area. Each of the normalization methods yielded significant differences in quantities of newborn Olfr235 OSNs in muscone-exposed compared to unexposed mice (B-*left*), with no significant differences observed in the corresponding odor exposure effect sizes (B-*right*; calculated from the ratios of normalized newborn Olfr235 OSNs within individual odor-exposed mice relative to the mean of normalized newborn Olfr235 OSNs within control mice). Each circle represents a distinct mouse (n = 5–10 mice [≥ 5 OE sections/mouse] per condition). ***P < 0.001; n.s. P> 0.05; unpaired two-tailed t-test (B-*left*); one-way ANOVA test, FDR-adjusted (B-*right*). Error bars: SEM. Data for newborn OSN quantities normalized by half-section correspond to **Figure 4D**.

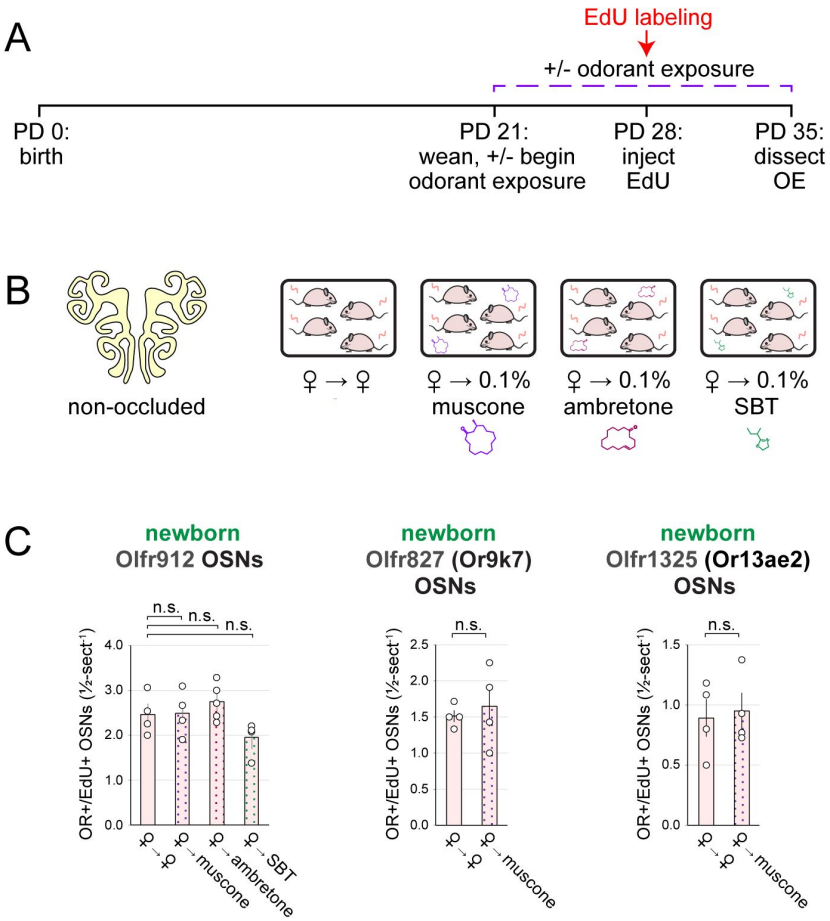


Figure 4–figure supplement 4.

Effects of exposure of mice to musk and non-musk odors on quantities of newborn OSNs of non-musk-responsive subtypes, including those previously found to undergo stimulation-dependent changes in OSN birthrate.

A, B. Experimental timeline (A) and conditions (B) used to generate OE tissue samples for assessing the effects of exposure to musk (muscone, ambretone) or non-musk (SBT) odors on quantities of newborn OSNs of specific subtypes. Non-occluded female mice were weaned sex-separated and either exposed or unexposed to an exogenous musk or non-musk odorant starting at PD 21, EdU-labeled at PD 28, and sacrificed at PD 35. OEs were sectioned and analyzed using OR-specific RNA-FISH and EdU staining. C. *Left*: Quantifications of newborn OSNs of the SBT-responsive subtype Olfr912 within tissue sections spanning the anterior-posterior lengths of OEs reveal no significant differences in non-occluded females that were exposed, at the time of EdU labeling, to a musk odorant (♀ → muscone; ♀ → ambretone) or to SBT (♀ → SBT) compared to those exposed to just female littermates (♀ → ♀). *Middle, right*: Quantifications of newborn OSNs of two subtypes previously found to undergo stimulation-dependent changes in birthrates, Olfr827 (*middle*) and Olfr1325 (*right*) [30], reveal no significant differences in non-occluded females that were exposed to a musk odorant compared to those exposed to just female littermates. Each circle represents a distinct mouse (n = 3–4 mice [≥ 5 OE sections/mouse] per OSN subtype and condition). n.s. P> 0.05; one-way ANOVA test, FDR-adjusted (C-*left*); unpaired two-tailed t-test (C-*middle, right*). Error bars: SEM. Data for newborn Olfr912 OSNs in ♀ → ♀ samples correspond to **Figure 3H**.

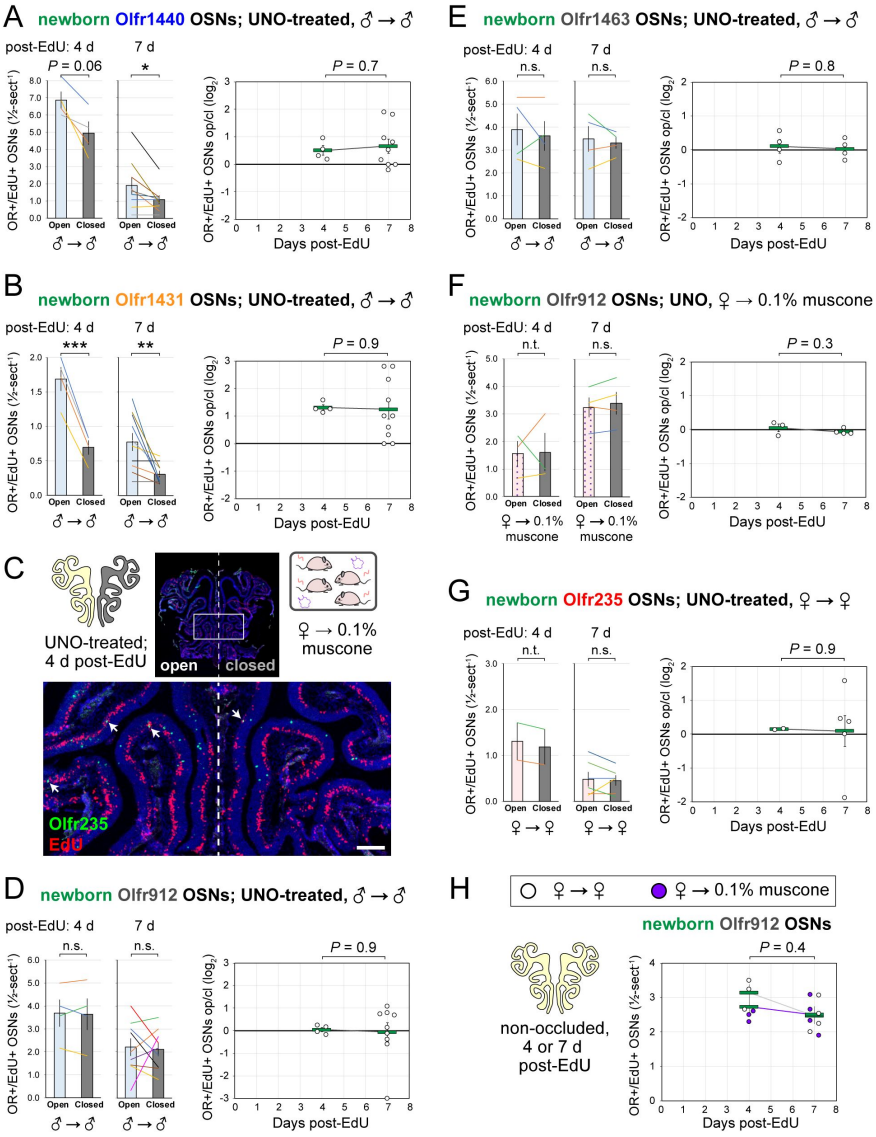


Figure 6–figure supplement 1.

Stimulation-dependent increases in quantities of newborn OSNs of musk-responsive subtypes are stable over time following neurogenesis, consistent with a mechanism involving altered birthrate.

OE tissue samples were generated according to the experimental timeline and conditions outlined in **Figure 6**, sectioned, and analyzed using OR- specific RNA-FISH and EdU staining. A, B, D–G. Quantifications of newborn Olfr1440 (A), Olfr1431 (B), Olfr912 (D, F), Olfr1463 (E), and Olfr235 (G) OSNs (OR+/EdU+) on the open and closed sides of tissue sections spanning the anterior-posterior lengths of OEs of UNO-treated males exposed to male littermates (♂ → ♂) (A, B, D, E), females exposed to muscone (♀ → 0.1% muscone) (F), or females exposed to female littermates (♀ → ♀) (G). Under all conditions, similar open-side biases in quantities of newborn OSNs were observed at both 4 and 7 d post-EdU, with no significant differences in UNO effect sizes observed over time. C. Representative image of OE sections from a UNO-treated female mouse that was exposed to muscone (♀ → 0.1% muscone) and sacrificed 4 d post-EdU, with newborn Olfr235 OSNs (OR+/EdU+) indicated by white arrows. Greater numbers of newborn Olfr235 OSNs were observed on the open side compared to the closed side at this timepoint. Scale bar: 150 μm. H. Quantifications of newborn OSNs of the SBT-responsive subtype Olfr912 within OEs of non-occluded female mice that were either exposed just to female littermates (♀ → ♀) or also to muscone (♀ → 0.1% muscone) and sacrificed 4 or 7 d post-EdU. No significant changes in newborn OSN quantity differences were observed between 4 and 7 d post-EdU. Each line or circle represents a distinct mouse (n = 2–10 mice [≥ 5 OE sections/mouse] per OSN subtype and condition). ***P < 0.001; **P < 0.01; *P < 0.05; n.s. P > 0.05; n.t. not tested (n < 4); ratio paired two-tailed t-test (A, B, D–G-*left*); unpaired two-tailed t-test (A, B, D–G-*right*); two sample ANOVA - fixed-test, using F distribution (right-tailed; H). Error bars: SEM. Data for 7 d post-EdU samples correspond to **Figures 2**–**4**.

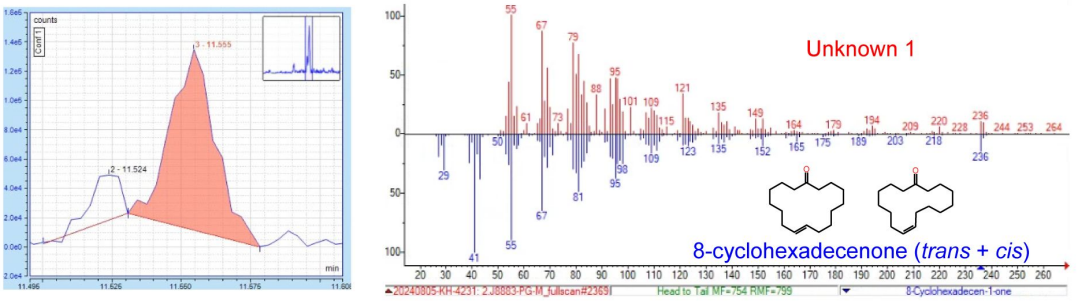
A

Predicted matches to known musks	Retention time (RT)	Predicted potential match	RT difference from standard
Unknown 1	11.55	8-cyclohexadecenone	0.06, 0.11
Unknown 2	12.27	cycloheptadecanol	not determined
Unknown 3	15.38	cyclopentadecanol	4.48

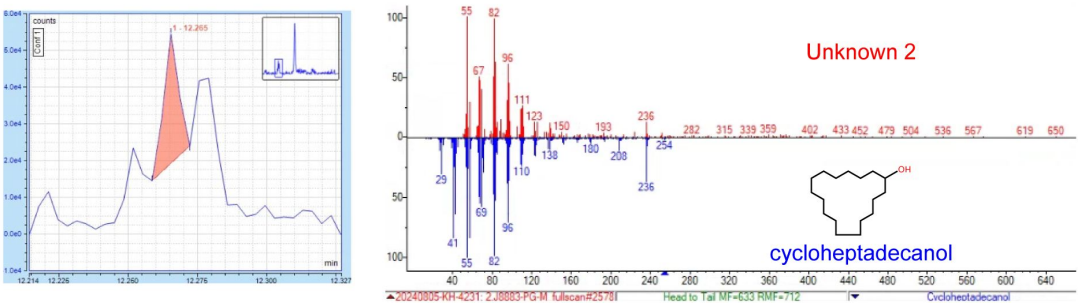
B

Standards tested	Retention time
(R)-3-methylcyclopentadecanone (muscone)	10.98
5-cyclohexadecenone (ambretone)	11.45
8-cyclohexadecenone (globanone)	11.44, 11.49
cyclopentadecanol (normuscol)	10.90

C



D



Appendix 2–figure 1.

Gas chromatography – mass spectrometry (GC-MS) analyses of mouse preputial gland extracts for molecules from with structural similarity to known musk odorants.

A, B. GC-MS signals from male mouse preputial gland extracts (A) and commercially available samples of 4 known musk compounds (B). Analyses of preputial glands revealed signals (Unknowns 1-3) with predicted potential matches to the indicated odorants based on spectral similarities (A). Experimental differences in retention times between unknowns and standards are indicated. C. *Left*: Region of the extracted ion chromatograph (m/z 236) of a preputial gland extract, with the signal corresponding to Unknown 1 highlighted in red. *Right*: Mass spectra corresponding to Unknown 1 (*red*) and a predicted match, 8-cyclohexadecenone (*blue*), a musk compound that was previously found to activate Olfr235 and Olfr1440 [33]. The retention times of Unknown 1 and 8-cyclohexadecenone differ by 0.06 and 0.11 minutes (possibly corresponding to the *cis* and *trans* isomers) (A), indicating potential structural similarity. D. *Left*: Region of the extracted ion chromatograph (m/z 236) of a preputial gland extract, with the signal corresponding to Unknown 2 highlighted in red. *Right*: Mass spectra corresponding to Unknown 2 (*red*) and a predicted match, cycloheptadecanol (*blue*). Confirmation of a match will require comparison of the observed retention time of Unknown 2 (12.27 minutes) with that of a cycloheptadecanol standard (not determined). Although Unknown 3 exhibited a predicted match to cyclopentadecanol, a musk compound previously found to activate Olfr235 and Olfr1440 [33], the observed retention time difference of 4.48 minutes (A) indicates substantial structural dissimilarity.

Appendix 3–table 1.

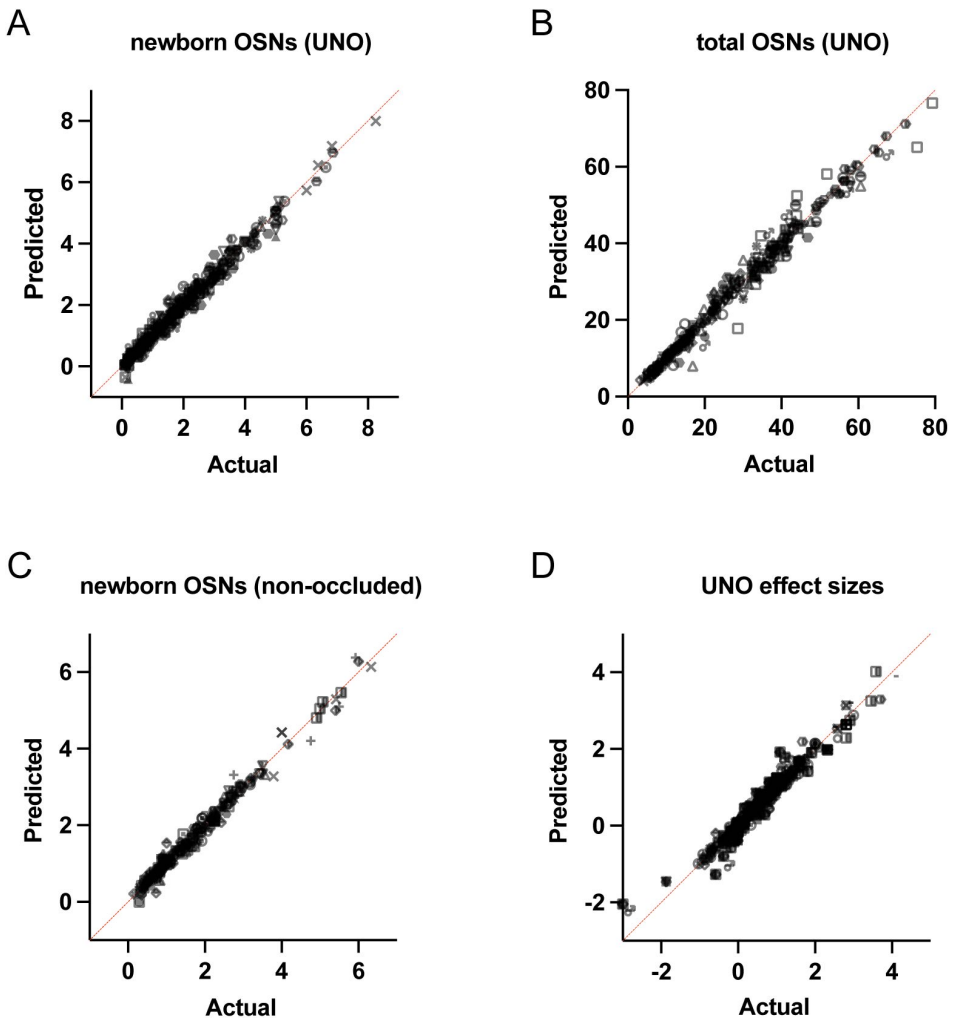
RNA-FISH probes used in this study.

Target (gene)	OE expression zone	Purpose	Gene region targeted	Genomic position relative to CDS start	Forward primer	Reverse primer	Probe length (nt)
Olfr235 (*Or5an11*)	2	OSN quantification	5'-UTR	-7217	TCAACAGCATGTTCAGGGGA	GCCTTTTTCTCACCTGGGCT	673
Olfr1440 (*Or5an6*)	2	OSN quantification	CDS	363	GACCGGTATGCTGCCATTTGTA	TTGTACCCTTAGTGGTGACAT	494
Olfr1431 (*Or5an9*)	2	OSN quantification	3'-UTR	1540	ACTGGTGAGCAAGTCATGGT	CAGTGGCCATGCTTATCAATGT	547
Olfr1437 (*Or5an1b*)	3	OSN quantification	3'-UTR	1190	TTCAGCCCTTGGTCTTGTCC	CCTGACGGTGAGAAGTGTCC	753
Olfr1419 (*Or10q3*)	3	OSN quantification	CDS	33	TTCCACTTCCGCCCCTTTTC	ACTTCTTCTGCGACATGCCT	753
Olfr912 (*Or8b48*)	3	OSN quantification	CDS/3'-UTR	483	TGCATCCTGAGACTGACTTTCT	GCTGTGTCAAATGCAAGATTTTAAA	500
Olfr1463 (*Or5b109*)	2	OSN quantification	CDS	133	GGGTTGATTCTGCTGATTCTTT	GAGCAAAGAAGAGAGTGAAGCTG	502
Olfr827 (*Or9k7*)	3	OSN quantification	CDS/3'-UTR	1050	TCTTGTGCAAGCAAAAGGTT	TGAACTCAAAGCCTGCCTCT	605
Olfr1325 (*Or13ae2*)	3	OSN quantification	CDS/3'-UTR	921	GAGGGTAAATTGTGGGGTCA	CTAAGCAGCCGATTTGGAAG	589
S100a5	1-4	UNO efficiency analysis	CDS	1	CTTCTTCCTTGGGTACATATCGTT	TGGAAGGATCCATCTGGGAGA	404

Appendix 4–table 1.

Summary of experimental conditions and numbers of biological replicates (mice) tested for each condition.

Experimental condition	UNO timepoint	Odorant exposure period	EdU treament timepoint(s)	OE dissection timepoint (d post-EdU)	Odorant concentration (%)	Number of biological replicates (mice)									
						scRNA-seq	Olfr235 (*Or5an11*)	Olfr1440 (*Or5an6*)	Olfr1431 (*Or5an9*)	Olfr1437 (*Or5an1b*)	Olfr1419 (*Or10q3*)	Olfr912 (*Or8b48*)	Olfr1463 (*Or5b109*)	Olfr827 (*Or9k7*)	Olfr1325 (*Or13ae2*)
Sex-separated males (♂→♂)	PD 14	N/A	N/A	PD 28 (N/A)	N/A	2	-	-	-	-	-	-	-	-	-
			PD 28	PD 32 (4 d)			4	4	4	-	-	4	4	-	-
				PD 35 (7 d)			7	9	10	4	6	9	4	-	-
	N/A			PD 35 (7 d)			10	-	-	-	-	4	-	-	-
Sex-separated females (♀→♀)	PD 14	N/A	PD 28	PD 32 (4 d)	N/A		2	-	-	-	-	-	-	-	-
				PD 35 (7 d)			6	8	7	-	-	5	4	-	-
	N/A		PD 28	PD 35 (7 d)			10	5	9	-	-	4	-	-	-
			PD 28	PD 32 (4 d)			3	3	3	-	-	3	-	-	-
			PD 56-58	PD 65 (7-9 d)			5	5	5	-	-	5	-	-	-
Sex-combined females (♀→♂)	PD 14	N/A	PD 28	PD 35 (7 d)	N/A		7	5	5	-	-	4	4	-	-
	N/A						5	-	-	-	-	4	-	-	-
Adult-exposed males (♂→adult ♂+♀)	PD 14	N/A	PD 28	PD 35 (7 d)	N/A		5	3	6	3	3	5	-	-	-
Muscone-exposed females (♀→musc)	PD 14	PD 21-35	PD 28	PD 32 (4 d)	0.1		5	5	5	-	-	3	-	-	-
				PD 35 (7 d)	0.1		5	5	5	-	-	4	-	-	-
				PD 35 (7 d)	1		6	6	6	-	-	4	4	-	-
				PD 35 (7 d)	10		5	5	5	-	-	-	-	-	-
	N/A	PD 21-35	PD 28	PD 35 (7 d)	0.1		5	5	5	-	-	4	-	4	4
			PD 28	PD 32 (4 d)	0.1		4	4	4	-	-	4	-	-	-
		PD 21-65	PD 56-58	PD 65 (7-9 d)	0.1		7	5	7	-	-	6	-	-	-
Ambretone-exposed females (♀→amb)	N/A	PD 21-35	PD 28	PD 35 (7 d)	0.1		5	5	5	-	-	5	-	-	-
SBT-exposed females (♀→SBT)	N/A	PD 21-35	PD 28	PD 35 (7 d)	0.1		5	5	5	-	-	4	-	-	-
Isoamyl acetate-exposed females (♀→IAA)	N/A	PD 21-35	PD 28	PD 35 (7 d)	0.1		5	5	5	-	-	-	-	-	-



Appendix 4–figure 1.

Quantile-quantile (QQ) plots for comparison of actual data to a theoretically normal distribution.

A. Analysis of quantities of newborn OSNs on the open and closed sides of the OEs of UNO-treated mice. B. Analysis of quantities of total OSNs on the open and closed sides of the OEs of UNO-treated mice. C. Analysis of quantities of newborn OSNs within the OEs of non-occluded mice. D. Analysis of UNO effect sizes of newborn and total OSNs on the open and closed sides of the OEs of UNO-treated mice. In each plot, predicted values (assuming sampling from a Gaussian distribution) are plotted on the vertical axis, while actual values are plotted on the horizontal axis. Points generally follow the line of identity, indicating that the data reflect a Gaussian (normal) distribution.

Experimental model and subject details

All procedures involving mice were carried out in accordance with NIH standards and approved by the University of Colorado Anschutz Medical Campus Institutional Animal Care and Use Committee (IACUC). For all experiments described, tissue samples were obtained from male or female C57Bl/6J mice that were 28, 35, or 65 days of age at the time of sacrifice. Except for adult-exposed mice, which remained with their parents until sacrifice, all mice were weaned at PD 21 and group-housed either sex-separated (4 females or 4 males per cage) or sex-combined (2 females and 2 males per cage) in standard cages.

Method details

- Unilateral naris occlusion (UNO). P14 pups were administered buprenorphine (extended release; 1 mg/kg) subcutaneously using a 31-gauge needle, anesthetized using isoflurane (completeness of anesthesia confirmed through a tail pinch), and then immediately subjected to electrocautery for ~5 seconds on the right nostril under a dissecting microscope. During electrocautery care was taken to avoid contact of the electrocautery unit with any non-superficial tissues. Pups were examined daily following the procedure to ensure complete blockage of the right nostril through scar formation (typically ~3–5 days after the procedure) and normal development and activity.
- Preparation of single cells from open and closed sides of OEs of UNO-treated mice for single-cell RNA sequencing (scRNA-seq) analyses. Generation of the OE 2 dataset (this study) was performed as described previously for dataset OE 1[30], with modifications. Briefly, the OE was dissected from a PD 28 male mouse that had been UNO-treated at PD 14 and weaned sex-separated at PD 21 (**Figure 1A, B**). The OE was separated at the midline using a clean scalpel and each half was separately minced in 750 µL Hibernate AB Complete media (HAB; BrainBits, HAB100) using a razor blade. For each half, the minced tissue and HAB, along with an additional 750 µL of HAB, were transferred to a 15-mL conical tube using a 1000-µL wide-bore pipette tip and allowed to settle for 1-min, after which the HAB supernatant was removed and transferred to a new 15-mL conical tube. Minced OE halves were each suspended in 1.5 mL of papain solution (2 mg/mL papain [BrainBits; PAP] in Hibernate A-minus Calcium medium [HA-Ca; BrainBits, HACA100]) and incubated for 20 minutes at 37°C, after which the papain solution was removed and discarded. To each digested OE half, the previously-saved HAB supernatant was added, 750-µL at a time, and used to triturate the tissue using a 1000-µl wide-bore pipette tip with 10–15 passes to achieve ~85% tissue dissociation, after which any large pieces of tissue debris were allowed to settle for ~20s. Suspended cells were transferred and combined into a new 15-mL conical tube, centrifuged (300 RCF; 3 min, 4°C), resuspended in 1 ml pre-warmed neuronal culturing medium (BrainBits; NbActiv1-100, filtered using a 40-µm Flowmi cell strainer (Bel-Art; H13680-0040), quantified using trypan blue staining and hemocytometry, centrifuged (300 RCF; 3 min, 4°C), resuspended in 1x PBS, stained using the LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen, L34957) according to the manufacturer's instructions, centrifuged (300 RCF; 3 min, 4°C), resuspended in 1 mL of 1% bovine serum albumin in 1x PBS, centrifuged (300 RCF; 3 min, 4°C), and resuspended in 400 µL of sorting buffer (1x PBS, 1mM EDTA, 25 mM Hepes, pH 7, 1% FBS). Live (non-fluorescent) single cells were sorted using an Astrios Cell Sorter (Beckman Coulter) into a 1.5-mL collection tube containing 20 µL of collection buffer (10% FBS in NbActiv1 media), and used for single cell capture and sequencing.
- Single-cell capture, library preparation, and sequencing. Immediately following sorting, single cells were processed and sequenced within the Genomics Core at the University of Colorado, Anschutz Medical Campus. Following quantification *via* hemocytometry, ~19,000 cells per sample were targeted for capture using a Chromium X controller (10X Genomics)

and used to prepare scRNA-seq libraries using the 3' HT Kit (10X Genomics) according to the manufacturer's instructions. Libraries were sequenced to a targeted depth of 40,000 reads/cell using a NovaSeq 6000 instrument (Illumina).

- Odorant exposure. A 1-mL aliquot of 0.1, 1, or 10% odorant solution [(R)-3-methylcyclopentadecanone (muscone; Ambeed, Inc., A275816); 5-Cyclohexadecenone (ambretone; TCI Ltd., C0874; 0.1%); 2-(sec-Butyl)-4,5-dihydrothiazole (SBT; Ambeed, Inc., A578012); or isoamyl acetate (IAA; TCI Ltd., A0033)] in propylene glycol was transferred to a compactly folded piece of absorbent paper (KimTech), which was enclosed within a metal tea ball that was hung within a standard mouse cage. Mice (4 per cage) were housed within odorant-containing cages from weaning (PD 21) until sacrifice (PD 35 or PD 65), with the odorants refreshed every other day.
- 2-Deoxy-5-ethynyluridine (EdU) injections. EdU (Carbosynth; NE08701) was administered intraperitoneally to C57Bl/6J mice at PD 28 or PD 56–58 (2 injections/day, spaced 3 h apart, of 10 mg/mL EdU in PBS; 50 mg EdU/kg mouse body weight/injection) [46].
- RNA-fluorescent *in situ* hybridization (RNA-FISH) probe design and generation. FISH probes were designed to span 500-1000 base pairs and were targeted to CDS and/or UTR regions of each mRNA transcript (**Appendix 3–table 1**). Probes were designed to minimize predicted cross-hybridization with off-target transcripts, as assessed using BLAST (NCBI) alignment to the mouse genome. To further minimize off-target cross-hybridization, probes aligning to 3 distinct transcript regions were generated for each target mRNA, and the probe yielding the best signal strength and homogeneity selected for use. Target probe sequences were amplified by PCR from whole OE cDNA using target-specific primers (**Appendix 3– table 1**), inserted into the pCRII-TOPO vector (ThermoFisher), and confirmed by Sanger sequencing. DIG-labeled antisense riboprobes were generated from 1 µg of linearized plasmid templates using T7 or Sp6 RNA polymerases (NEB) and DIG-11-UTP (Roche), treated with DNaseI (Promega) to remove the template DNA, purified via ethanol precipitation, and dissolved in 30-µL of water. A detailed description of this procedure was published previously [46].
- One-color RNA fluorescent *in situ* hybridization (RNA-FISH) combined with EdU staining *via* click chemistry. OEs were dissected from experimental mice (age PD 35 or PD 65), placed in a cryomold containing OCT, flash-frozen in liquid-nitrogen-cooled isopentane, and stored at −80 °C until sectioning. Tissues were cut into 12-µm thick cryo-sections, placed onto slides, and stored at −80 °C until staining. Slide-mounted sections were warmed (37 °C, 5 min), equilibrated in phosphate-buffered saline (PBS; pH 7.2; 3 min, room temperature [RT]), fixed in paraformaldehyde (PFA; 4% in PBS; 10 min, RT), washed in PBS (3 min, RT), permeabilized with Triton-X-100 (0.5% in PBS; 10 min, RT) followed by sodium dodecyl sulfate (1% in PBS; 3 min, RT), washed in PBS (3 × 3 min, RT), incubated in acetylation solution (triethanolamine [0.1 M; pH 7.5], acetic anhydride [0.25%]; 10 min, RT), washed in PBS (3 × 3 min, RT), incubated in hybridization solution (formamide [50%], SSC [5×], Denhardts [5×], yeast tRNA [250 µg/mL], herring sperm DNA [500 µg/mL], heparin [50 µg/mL], EDTA [2.5 mM], Tween-20 [0.1%], CHAPS [0.25 %]; 30 min, RT), hybridized with a DIG-labeled antisense RNA probe (1:750 in hybridization solution; 16 hr, 65 °C), washed with SSC (5×; 1 × 5 min, 65 °C), washed with SSC (0.2×; 4 × 20 min, 65 °C), incubated in H_2O_2 (3% in TN [Tris-HCl (0.1 M; pH 7.5), 0.15 M NaCl]; 30 min, RT), washed in TNT (Tween-20 [0.05%] in TN; 5 × 3 min, RT), incubated in TNB (Blocking Reagent [Perkin Elmer; 0.05% in TN]; 30 min, RT), incubated with anti-DIG-POD antibody (Roche; 1:1000 in TNB; 12 hr, 4 °C), and washed in TNT (3 × 20 min, RT). Fluorescent signals corresponding to the target transcript were generated using the Tyramide Signal Amplification (TSA) Plus Fluorescein Kit (Perkin Elmer) according to the manufacturer's instructions. Slides were washed in 3% BSA in PBS (2 × 5 min, RT, with gentle rocking), incubated with EdU reaction solution (4 mM $CuSO_4$, 4 µM Sulfo-Cyanine 3 Azide [Lumiprobe], 100 mM sodium ascorbate [prepared fresh], in PBS; 30 min, RT, in darkness), and washed with 3% BSA in PBS (2 × 3 min, RT).

Slides were washed in TNT (2 × 3 min, RT), incubated in DAPI (300 nM in TN; 3 min, RT), washed in TNT (1 × 3 min, RT), and mounted using Vectashield (Vector Laboratories). A detailed description of this procedure was published previously [46].

- Image acquisition and processing. Images were acquired using a Zeiss LSM 900 with Airyscan 2 microscope with an automated stage and Zen Blue software (Zeiss). Mosaic images were stitched and each fluorescence channel was adjusted individually to enhance contrast using Zen Blue software. Representative images were exported in jpg format, and rotated and cropped using Adobe Photoshop. A detailed description of this procedure was published previously [46].
- Quality criteria for sectioned OEs. For UNO-treated mice, UNO efficiency was assessed by visual inspection of mouse nostrils prior to dissection. Additionally, OE cryosections from UNO-treated mice were stained *via* one-color RNA-FISH for *S100a5* transcript levels. For each section analyzed, *S100a5* mRNA intensities were evaluated within paired regions on the two sides of each OE section. OEs from UNO-treated mice were excluded from further analysis if the mean fluorescence intensity corresponding to *S100a5* transcripts was not noticeably (~10-fold) greater on the open side of the OE compared to the closed side. All sections from both UNO-treated and non-occluded mice were assessed for left-right symmetry and intactness. Individual OE sections were excluded if they lacked a high degree of symmetry or were less than 90% intact. No sections were otherwise excluded. A detailed description of this procedure was published previously [46].
- Gas chromatography – mass spectrometry (GC-MS) analysis of mouse preputial gland (PG) extracts. Immediately following euthanasia, three PGs were dissected from 5-week-old male mice, transferred to separate 1.5-mL microcentrifuge tubes, and flash-frozen on dry ice. Frozen PG tissues were shipped on dry ice to the Analytical Resource Core at Colorado State University for processing, essentially as described previously [79]. Each gland was ground in liquid nitrogen using a mortar and pestle, transferred to a glass extraction vial containing 200 µL of dichloromethane, vortexed and sonicated for 30 min, and centrifugated at 3,000 g for 1 min. Supernatants were transferred to glass autosampler vials and stored at −20 °C until analysis. For GC-MS analyses, 1 µL of dichloromethane extract was injected into a Trace 1310 gas chromatography instrument coupled to a Thermo ISQ-LT mass spectrometer, at a 5:1 split ratio. The inlet was held at 285 °C. Separation was achieved on a 30m DB-5MS column (J&W, 0.25 mm ID, 0.25 µm film thickness). Oven temperature was held at 80 °C for 0.5 min, ramped at 15°C/min to 330°C, and held at 330 °C for 8 min. Helium carrier gas flow was held at 1.2 mL/min. Temperatures of transfer line and ion source were held at 300 °C and 260 °C, respectively. Fullscan mode (50-650 m/z) was used on all the samples. The injection order of samples was randomized. Standards: (R)-3-Methylcyclopentadecanone (Muscone) was purchased from Ambeed, Inc. (A275816), 5-Cyclohexadecenone (Ambretone) was from TCI America (C0874), 8-Cyclohexadecenone (Globanone) was from Perfumer's Apprentice (ac-1510-sz1), and Cyclopentadecanol was from TCI America (C1528). GC-MS data were processed using Chromeleon 7.2.10 software (Thermo Scientific).

Quantification and statistical analysis

- scRNA-seq analysis of UNO-induced changes in subtype-specific quantities of newborn OSNs. Newborn OSNs of specific subtypes were quantified within scRNA-seq datasets generated from the open and closed sides of the OEs of 2 different male C57Bl/6J mice that were UNO-treated at PD 14 and sacrificed at PD 28 (**Figure 1**): OE 1 (generated previously [30]; *https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE157119*), and OE 2 (this study; *https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE278693*). Newborn OSNs of specific subtypes were identified by their expression of the iOSN-specific marker *Gap43* (Log_2 UMI > 1) and a specific OR gene (Log_2 UMI > 2) (**Figure 1**) and quantified within the open and closed sides of the OE 1 and OE 2 datasets using Loupe Browser software (8.0.0; 10X

Genomics). Subtype-specific quantities of newborn OSNs in each OE half were normalized based on the total number of cells or OSNs (*Gap43* Log_2 UMI > 1 or *Omp* Log_2 UMI > 3) within the corresponding dataset.

- scRNA-seq analysis of OR transcripts within individual INP3, iOSN, and mOSN cells on the open and closed sides of the OEs of UNO-treated mice. Individual INP3, iOSN, and mOSN cells expressing transcripts of a specific OR gene were identified based on their expression of *Tex15* [38,43] (Log_2 UMI > 2) and the OR (Log_2 UMI > 0), their expression of *Gap43* (Log_2 UMI > 1) and the OR (Log_2 UMI > 2), or their expression the OR (Log_2 UMI > 2) and lack of expression of *Gap43* (Log_2 UMI ≤ 1), respectively, using Loupe Browser software (8.0.0). The cellular transcript levels of all ORs within INP3, iOSN, and mOSN cells expressing a specific musk-responsive (Olfr235, Olfr1440) or non-musk responsive (Olfr701) OR within the open and closed datasets of OE 1 and OE 2 samples were calculated and exported using Loupe Browser software (8.0.0).
- Histological quantification of newborn and total OSNs of specific subtypes. Quantities of newborn and total OSNs within the OEs of individual mice were determined from images of a series of at least 5 coronal tissue sections stained for EdU and a specific OR mRNA and spanning the anterior-posterior length of each OE. Quantifications were performed separately on the two sides of each OE section, with the experimenter blinded to the sample identities. Newborn cells of specific subtypes were identified based on robust nuclear EdU staining (Cy3) (> 2-fold above background) that was overlapped at least 50% by OR mRNA staining (FITC). A detailed description of this procedure was published previously [46].
- Comparison of methods for normalizing quantities of newborn OSNs of specific subtypes. For all histological quantifications of newborn and total OSNs of specific subtypes in this study, OSN quantities were normalized to the number of half-sections analyzed. This approach has been used in multiple previous studies for quantifying newborn (OR+/EdU+) and total (OR+) OSN abundances [17,19,30,46]. To verify the rigor of this method, newborn Olfr235 OSN quantities were also normalized by total quantities of newborn OSNs (approximated by total EdU+ cells) or total quantities of cells (approximated by DAPI+ area) for three experimental comparisons in this study: the open versus the closed sides of OEs from UNO-treated male mice (**Figure 2–figure supplement 2**), the OEs from non-occluded females versus males (**Figure 3–figure supplement 2**), and the OEs of non-occluded females exposed just to female littermates versus females also exposed to 0.1% muscone (**Figure 4– figure supplement 4**). For each condition, EdU+ cells and DAPI+ areas were identified and quantified within images of OE sections stained for Olfr235 mRNA and EdU using the surface and stats tools, respectively, within Imaris software (version 10.2).
- Statistics. To assess deviation from normality of the histological quantifications of newborn and total OSNs of specific subtypes used for comparisons in this study, all datasets were tested using the Shapiro-Wilk test for non-normality and the P values obtained are included within Supplementary file 1. Of the 274 datasets tested, 253 have Shapiro-Wilk P values > 0.05, indicating that the vast majority (92%) do not show evidence of significant deviation from a normal distribution. A general lack of deviation of the datasets in this study from a normal distribution is further supported by quantile-quantile (QQ) plots (**Appendix 4–figure 1**). Moreover, results of both parametric and non-parametric statistical tests of comparisons in this study are, in general, in good agreement (Supplementary file 2). Statistical analyses of differences in OSN quantities between the open and closed sides of OEs from UNO-treated mice were performed using ratio paired two-tailed *t*-tests (parametric) and Wilcoxon matched-pairs signed rank two-tailed tests (non-parametric). For these tests, the open and closed sides of each OE were paired, enabling testing of differences between the two sides independent of OSN number and staining variance between sections. Statistical analyses of differences in OSN quantities in the OEs of non-occluded mice or UNO effect sizes in UNO- treated mice subjected to two different experimental conditions were performed using unpaired two-tailed *t*-tests

(parametric) and Mann-Whitney two-tailed tests (non-parametric). Statistical analyses of differences in OSN quantities in the OEs of non-occluded mice or UNO effect sizes in UNO-treated mice subjected more than two different experimental conditions were performed using one-way ANOVA tests, FDR-adjusted using the 2-stage linear step-up procedure of Benjamini, Krieger and Yekutieli (parametric) and Kruskal-Wallis tests, FDR-adjusted using the 2-stage linear step-up procedure of Benjamini, Krieger and Yekutieli (non-parametric). For comparisons of differences in quantities of newborn OSNs of musk-responsive subtypes at 4 and 7 days post-EdU between non-occluded mice exposed and unexposed to muscone, a two sample ANOVA - fixed-test, using F distribution (right-tailed) was used. Data presented in figures represent mean +/- SEM. For all statistical analyses, a significance threshold of $P < 0.05$ was used. All statistical tests were performed using Prism 10 software (Graphpad). Results of all statistical tests performed in this study are summarized in Supplementary file 2. P values reported within the text and figures are based on parametric tests.

- Sample-size estimation. Results from previous studies [19,30] were used to determine an appropriate sample size for comparing the number of total (OR+) and newborn (OR+/EdU+) OSNs on the open and closed sides of the OE. Previously, it was found that for an OR with a typical expression frequency (~0.1%) and an effect size of ~2-fold, 12 OE sections taken from four different animals were sufficient to find a highly statistically significant difference ($P < 0.001$; paired two-tailed t test). For comparisons between different animals, results from previous studies [19,30] were also used to determine an appropriate sample size. Previously, we had found that for an OR with a typical expression frequency (~0.1%) and an effect size of ~2-fold, 20 OE sections taken from four different animals was sufficient to find a highly statistically significant difference between different animals ($P < 0.01$; two-tailed unpaired t test).

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Reviewer #1 (Public review):

Summary

Olfactory sensory neurons (OSNs) in the olfactory epithelium detect myriads of environmental odors that signal essential cues for survival. OSNs are born throughout life and thus represent one of the few neurons that undergo life-long neurogenesis. Until recently, it was assumed that OSN neurogenesis is strictly stochastic with respect to subtype (i.e. the receptor the OSN chooses to express). However, a recent study showed that olfactory deprivation via naris occlusion selectively reduced birthrates of only a fraction of OSN subtypes and indicated that these subtypes appear to have a special capacity to undergo changes in birthrates in accordance with the level of olfactory stimulation. These previous findings raised the interesting question of what type of stimulation influences neurogenesis, since naris occlusion does not only reduce the exposure to potentially thousands of odors, but also to more generalized mechanical stimuli via preventing airflow.

In this study, the authors set out to identify the stimuli that are required to promote the neurogenesis of specific OSN subtypes. Specifically, they aim to test the hypothesis if discrete odorants selectively stimulate the same OSN subtypes whose birthrates are affected. This would imply a highly specific mechanism in which exposure to certain odors can "amplify" OSN subtypes responsive to those odors suggesting that OE neurogenesis serves, in part, an adaptive function.

To address this question, the authors focused on a family of OSN subtypes that had previously been identified to respond to musk-related odors and that exhibit higher transcript levels in the olfactory epithelium of mice exposed to males compared to mice isolated from males. First, the authors confirm via a previously established cell birth dating assay in unilateral naris occluded mice that this increase in transcript levels actually reflects a stimulus-dependent birthrate acceleration of this OSN subtype family. In a series of experiments (in unilateral occluded and non-occluded mice) using the same birth dating assay, they show that several subtypes of this OSN family, but not other "control" subtypes exhibit increased birthrates in response to adolescent male exposure, but not to female exposure.

In the core experiment of the study, they expose unilaterally naris occluded and non-occluded mice to two musk-related odors and two "control" odors (that do not activate musk-responsive OSN subtypes) to test if these odors specifically accelerate the birth rates of OSN types that are responsive to these odors. This experiment reveals that (for the tested odors and OSN subtypes) indeed birthrates are only affected by discrete odorants that stimulate these OSN subtypes (with a complex relationship between birth rate acceleration and odor concentrations) suggesting that OE neurogenesis may serve, in part, as an adaptive function

Strength:

The scientific question is valid and opens an interesting direction. The previously established cell birth dating assay in naris occluded and non-occluded mice is well performed and

accompanied by several control experiments addressing potential other interpretations of the data.

In this revised version, the authors added several new experiments addressing the previous concern that only the effect of one specific odor (muscone) on musk-responsive OSN subtypes had been tested to make the general claim that discrete odors specifically accelerate the birth rate of OSN subtypes they stimulate. Now the authors demonstrate that another musk-related odor (ambretone) also induces this effect and that other non-musk odors do not. In addition, they show that two other OSN subtypes that do not respond to musk-related odors are not affected. These experiments further substantiate the above claim.

Weakness:

(1) The main research question of this study was to test if discrete odors specifically accelerate the birth rate of OSN subtypes they stimulate, i.e. does muscone only accelerate the birth rate of OSNs that express muscone-responsive ORs, or vice versa is the birthrate of muscone-responsive OSNs only accelerated by odors they respond to?
As mentioned under "strength" the authors added several experiments to further substantiate their claim. While these controls are very important to show that the observed effect is indeed specific for musk-related odors on musk-responsive OSN subtypes, these experiments still only focus on one closely related family of musk-responsive OSN subtypes. To understand if this phenomenon is a more generalized mechanism and plays a role for other OSN subtypes beyond this small family of related receptors, further experiments showing this effect for other OSN subtypes are critical.

(2) Previous concerns (#2, #4, #5 and #6) about a lack of increase in UNO effect size for olfr1440 under any muscone concentrations, strong fluctuations of newborn neurons on the closed side as well as a seemingly contradicting statement that overstimulation possibly reflects reduced survival have been addressed by adding potential explanations to the text.

In addition, the previous remark (#3) that certain phrases gave the misleading impression that musk-related odors are indeed excreted into male mouse urine at certain concentrations was addressed not only by re-phrasing, but by performing additional experiments. Although these did not deliver clear results (because of technical difficulties), interesting possibilities are discussed.

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Reviewer #3 (Public review):

Summary

Neurogenesis in the mammalian olfactory epithelium continues throughout the animal's lifespan, replacing damaged or dying olfactory sensory neurons. It has been tacitly assumed that the replacement of olfactory receptor (OR) subtypes occurs randomly. However, anecdotal evidence has suggested otherwise. In this study, Santoro and colleagues challenge this assumption by systematically exploring three key questions: Is there enrichment of specific OR subtypes during neurogenesis? Is this enrichment influenced by sensory stimuli? Does enrichment result from differential generation of OR types or differential cell death regulated by neural activity? The authors present convincing evidence that muscone stimulus selectively enhances the neurogenesis of OSNs expressing muscone receptors, suggesting that the selection of ORs in regenerating neurons is not random.

Strengths

This study is the first in formulating and systematically testing the selective promotion hypothesis. It is a comprehensive and systematic examination of multiple musk receptors under conditions of unilateral naris occlusion and various stimuli. The controls are properly done. The quality of in situ hybridization and immunofluorescent staining is high, allowing the authors to effectively estimates the number of OR types. The data convincingly demonstrate that increased expression of musk receptors in response to male odor or muscone stimulation.

Weaknesses

The revised version has addressed most initial weaknesses. However, some inconsistencies remain, raising questions about the proposed model. For instance, in the unilateral naris occlusion experiment, although average expression of non-musk receptors shows no significant change, receptors seem to fall into two groups with one subset increasing and another decreasing (Fig. 1E). This suggests naris occlusion may regulate OR expression independently of odor-induced activity, a possibility that remains unaddressed.

There is curiosity regarding receptors for the male odor SBT, olfr912, and olfr1295, which exhibit increased expression on the occluded side. The explanation that SBT exposure shortens these neurons' lifespan lacks substantiation and is inconsistent with later data. For example, Figure 3-figure supplement 3 I does not show olfr912 changes on the UNO side, and Figure 4-figure supplement 4 shows no significant decrease in olfr912 expression in SBT-exposed mice. Additionally, single-cell RNASeq experiments did not examine olfr912 and olfr1295 expression.

While the study convincingly demonstrates muscone's selective stimulation of olfr235-expressing OSNs, it lacks exploration of the mechanisms underlying this specificity. The discussion of signaling pathways remains too generic.

In summary, while the study offers significant insights into neurogenesis and OR subtype enrichment, further investigation into underlying mechanisms and addressing existing inconsistencies would strengthen its conclusions.

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Author response:

The following is the authors' response to the original reviews

> ***Reviewer #1 (Public Review):***
>
> *Summary:*
>
> *Olfactory sensory neurons (OSNs) in the olfactory epithelium detect myriads of environmental odors that signal essential cues for survival. OSNs are born throughout life and thus represent one of the few neurons that undergo life-long neurogenesis. Until recently, it was assumed that OSN neurogenesis is strictly stochastic with respect to subtype (i.e. the receptor the OSN chooses to express).*
>
> *However, a recent study showed that olfactory deprivation via naris occlusion selectively reduced birthrates of only a fraction of OSN subtypes and indicated that these subtypes appear to have a special capacity to undergo changes in birthrates in accordance with the level of olfactory stimulation. These previous findings raised the interesting question of what type of stimulation influences neurogenesis, since naris occlusion does not only*

> *reduce the exposure to potentially thousands of odors but also to more generalized mechanical stimuli via preventing airflow.*
>
> *In this study, the authors set out to identify the stimuli that are required to promote the neurogenesis of specific OSN subtypes. Specifically, they aim to test the hypothesis that discrete odorants selectively stimulate the same OSN subtypes whose birthrates are affected. This would imply a highly specific mechanism in which exposure to certain odors can "amplify" OSN subtypes responsive to those odors suggesting that OE neurogenesis serves, in part, an adaptive function.*
>
> *To address this question, the authors focused on a family of OSN subtypes that had previously been identified to respond to musk-related odors and that exhibit higher transcript levels in the olfactory epithelium of mice exposed to males compared to mice isolated from males. First, the authors confirm via a previously established cell birth dating assay in unilateral naris occluded mice that this increase in transcript levels actually reflects a stimulus-dependent birthrate acceleration of this OSN subtype family. In a series of experiments using the same assay, they show that one specific subtype of this OSN family exhibits increased birthrates in response to juvenile male exposure while a different subtype shows increased birthrates to adult mouse exposure. In the core experiment of the study, they finally exposed naris occluded mice to a discrete odor (muscone) to test if this odor specifically accelerates the birth rates of OSN types that are responsive to this odor. This experiment reveals a complex relationship between birth rate acceleration and odor concentrations showing that some muscone concentrations affect birth rates of some members of this family and do not affect two unrelated OSN subtypes.*

In addition to the results nicely summarized by the reviewer, which focus on experiments to examine the effects of odor stimulation on unilateral naris occluded (UNO) mice, an important part of the present study are experiments on non-occluded (i.e., non-UNO-treated) mice. These experiments show: 1) that the exposure of non-occluded mice to odors from adolescent male mice selectively increases quantities of newborn OSNs of the musk-responsive subtype Olfr235 (Figure 3G, H; previously Figure 6), 2) the exposure of non-occluded female mice to 2 different musk odorants (muscone, ambretone) selectively increases quantities of newborn OSNs of 3 musk responsive subtypes: Olfr235, Olfr1440 and Olfr1431 (Figure 4D-F; previously Figure 6), and 3) the exposure of non-occluded adult female mice to a musk odorants selectively increases quantities of newborn OSNs of musk responsive subtypes (Figure 5; previously Fig. S7). We have reorganized the revised manuscript to more prominently and clearly present the experimental design and findings of these experiments. We have also made changes to clarify (via schematics) the experimental conditions used (i.e., UNO, non-UNO, odor exposure) in each experiment.

> *Strengths:*
>
> *The scientific question is valid and opens an interesting direction. The previously established cell birth dating assay in naris occluded mice is well performed and accompanied by several control experiments addressing potential other interpretations of the data.*
>
> *Weaknesses:*
>
> *(1) The main research question of this study was to test if discrete odors specifically accelerate the birth rate of OSN subtypes they stimulate, i.e. does muscone only accelerate the birth rate of OSNs that express muscone-responsive ORs, or vice versa is the birthrate of muscone-responsive OSNs only accelerated by odors they respond to?*

> *This question is only addressed in Figure 5 of the manuscript and the results only partially support the above claim. The authors test one specific odor (muscone) and find that this odor (only at certain concentrations) accelerates the birth rate of some musk-responsive OSN subtypes, but not two other unrelated control OSN subtypes. This does not at all show that musk-responsive OSN subtypes are only affected by odors that stimulate them and that muscone only affects the birthrate of musk-responsive OSNs, since first, only the odor muscone was tested and second, only two other OSN subtypes were tested as controls, that, importantly, are shown to be generally stimulus-independent OSN subtypes (see Figure 2 and S2).*
>
> *As a minimum the authors should have a) tested if additional odors that do not activate the three musk-responsive subtypes affect their birthrate b) choose 2-3 additional control subtypes that are known to be stimulus-dependent (from their own 2020 study) and test if muscone affects their birthrates.*

We appreciate these suggestions. Within the revised manuscript, we have described and included the results from several new experiments:

(1) As noted by the reviewer, we had previously tested the effects of exposure to only one exogenous musk odorant, muscone, on quantities of newborn OSNs of the musk-responsive subtypes Olfr235, Olfr1440, and Olfr1431. To test whether the effects observed with muscone exposure occur with other musk odorants, we assessed the effects of exposure to ambretone (5-cyclohexadecenone), a musk odorant previously found to robustly activate musk-responsive OSNs (Sato-Akuhara et al., 2016; Shirasu et al., 2014), on quantities of newborn OSNs of 3 musk-responsive subtypes Olfr235, Olfr1440, and Olfr1431, as well as the SBT-responsive subtype Olfr912, in the OEs of non-occluded female mice. Exposure to ambretone was found to significantly increase quantities of newborn OSNs of all 3 musk-responsive subtypes (Figure 4D-F) but not the SBT-responsive subtype (Figure 4–figure supplement 4C-left), indicating that a variety of musk odorants can accelerate the birthrates of musk responsive subtypes.

(2) To verify that exogenous non-musk odors do not increase quantities of newborn OSNs of musk responsive OSN subtypes (point a, above), we quantified newborn OSNs of 3 musk-responsive subtypes, Olfr235, Olfr1440, and Olfr1431, in non-occluded female mice that were exposed to the non-musk odorants SBT or IAA. As expected, neither of these odorants significantly affected the birthrates of the subtypes tested (Figure 4D-F).

(3) To confirm that exogenous musk odors do not accelerate the birthrates of non-musk responsive OSN subtypes that were previously found to undergo stimulation-dependent neurogenesis (point b, above), we quantified newborn OSNs of 2 such subtypes, Olfr827 and Olfr1325, in non-occluded female mice that were exposed to muscone. As expected, exposure to muscone did not significantly affect the birthrates of either of these subtypes (Figure 4–figure supplement 4C-middle, right).

(4) To provide additional confirmation that only some OSN subtypes have a capacity to exhibit increases in newborn OSN quantities in the presence of odors that activate them, we compared quantities of newborn OSNs of the SBT-responsive subtype Olfr912 in non-occluded females that were either exposed to 0.1% SBT versus unexposed controls. As expected, exposure of SBT caused no significant increase in quantities of newborn Olfr912 OSNs (Figure 4–figure supplement 4C-left).

> *(2) The finding that Olfr1440 expressing OSNs do not show any increase in UNO effect size under any muscone concentration (Figure 5D, no significance in line graph for UNO effect sizes, middle) seems to contradict the main claim of this study that certain odors specifically increase birthrates of OSN subtypes they stimulate. It was shown in several*

> *studies that olfr1440 is seemingly the most sensitive OR for muscone, yet, in this study, muscone does not further increase birthrates of OSNs expressing olfr1440. The effect size on birthrate under muscone exposure is the same as without muscone exposure (0%).*

In contrast, the supposedly second most sensitive muscone-responsive OR olfr235 shows a significant increase in UNO effect size between no muscone exposure (0%) and 0.1% as well as 1% muscone.

Findings that quantities of newborn Olfr1440 OSNs do not show a significantly greater UNO effect size in the OEs from mice exposed to muscone compared to control mice was also somewhat surprising to us. We think that there are two potential explanations for this result: 1) Unlike subtype Olfr235, subtype Olfr1440 exhibits a significant open-side bias in newborn OSN quantities in UNO-treated adolescent females even in the absence of exposure to muscone. We speculate that this subtype (as well as subtype Olfr1431) is stimulated by odors that are emitted by female mice at the adolescent stage, and/or by another environmental source. This may limit the influence of muscone exposure on the UNO effect size. 2) There is compelling evidence that odors within the environment can enter the closed side of the OE transnasally [via the nasopharyngeal canal (Kelemen, 1947)] and/or retronasally (via the nasopharynx) in UNO-treated mice [reviewed in (Coppola, 2012)]. Thus, it is conceivable that chronic exposure of UNO-treated mice to muscone results in the eventual entry on the closed side of the OE of muscone at concentrations sufficient to promote neurogenesis. If Olfr1440 is more sensitive to muscone than Olfr235 [e.g., (Sato-Akuhara et al., 2016; Shirasu et al., 2014)], OSNs of this subtype may be especially sensitive to small amounts of odors that enter the closed side of the OE transnasally and/or retronasally. These explanations are supported by the following results:

- UNO-treated females exposed to 0.1% muscone show higher quantities of newborn Olfr1440 OSNs on both the open and closed sides of the OE in muscone exposed females compared to their unexposed counterparts (Figure 4–figure supplement 1A-middle). Similar results were also observed for newborn Olfr235 OSNs (Figure 4C-middle), albeit to a lesser extent, perhaps due to the lower sensitivity of this subtype to muscone.

- In non-occluded female mice, exposure to 0.1% muscone was found to significantly increase quantities of newborn Olfr1440 OSNs, as well as newborn Olfr235 and Olfr1431 OSNs (Figure 4D-F in revised manuscript; Figure 6 in original version). Similar results were also observed upon exposure to ambretone, another musk odor (Figure 4D-F). These experiments strongly support the hypothesis that musk odors selectively increase birthrates of OSN subtypes that they stimulate.

We have addressed these points within the results section of the revised manuscript.

> *(3) The authors introduce their choice to study this particular family of OSN subtypes with first, the previous finding that transcripts for one of these musk-responsive subtypes (olfr235) are downregulated in mice that are deprived of male odors. Second, musk-related odors are found in the urine of different species. This gives the misleading impression that it is known that musk-related odors are indeed excreted into male mouse urine at certain concentrations. This should be stated more clearly in the introduction (or cited, if indeed data exist that show musk-related odors in male mouse urine) because this would be a very important point from an ethological and mechanistic point of view.*
>
> *In addition, this would also be important information to assess if the chosen muscone concentrations fall at all into the natural range.*

These are important points, which have addressed within the revised manuscript:

(1) Within the introduction, we have now stated that the emission of musk odors by mice has not been documented. We have also added extensive discussions of what is known about the emission of musk odors by mice in a new subsection within Results, as well as within the Discussion section. Most prominently, we have cited one study (Sato-Akuhara et al., 2016) that noted unpublished evidence for the emission of Olfr1440-activating compounds from male preputial glands: “Indeed, our preliminary experiments suggest that there are unidentified compounds that activate MOR215-1 in mouse preputial gland extracts.” Another study, which used histomorphology, metabolomic and transcriptomic analyses to compare the mouse preputial glands to muskrat scent glands, found that the two glands are similar in many ways, including molecular composition (Han et al., 2022). However, the study did not identify known musk compounds within mouse preputial glands.

(2) Based on the reviewer’s feedback and our own curiosity, we used GC-MS to analyze both mouse urine and preputial gland extracts for the presence of known musk odorants, particularly those known to activate Olfr235 and Olfr1440 (Sato-Akuhara et al., 2016). Although we were unable to find evidence for known musk odorants in mouse urine extracts (possibly due to insufficient sensitivity of the assay employed), we found that preputial gland extracts contain GC-MS signals that are structurally consistent with known musk odorants. A limitation of this approach, however, is that the conclusive identification of specific musk odorants in extracts derived from mouse urine and tissues requires comparisons to pure standards, many of which we could not readily obtain. For example, we were unable to obtain a pure sample of cycloheptadecanol, a musk molecule with a predicted potential match to a signal identified within preputial gland extracts. Another limitation is that although several known musk odorants have been found to activate Olfr235 and Olfr1440 OSNs, it is conceivable that structurally distinct odorants that have not yet been identified might also activate them. The findings from these experiments have been included in a new figure within the revised manuscript (Appendix 2–figure 1).

> *Related: If these are male-specific cues, it is interesting that changes in OR transcripts (Figure 1) can already be seen at the age of P28 where other male-specific cues are just starting to get expressed. This should be discussed.*

We agree that the observed changes in quantities of newborn OSNs of musk-responsive subtypes in mice exposed to juvenile male odors deserves additional discussion. We have included a more extensive discussion of this observation in both the Results and Discussion sections of the revised manuscript.

> *(4) Figure 5: Under muscone exposure the number of newborn neurons on the closed sides fluctuates considerably. This doesn't seem to be the case in other experiments and raises some concerns about how reliable the naris occlusion works for strong exposure to monomolecular odors or what other potential mechanisms are at play.*

We agree that the variability in quantities of newborn OSNs of musk-responsive subtypes on the closed side of the OE of UNO-treated mice deserves further discussion. As noted above, we suspect that these fluctuations are due, at least in part, to transnasal and/or retronasal odor transfer via the nasopharyngeal canal (Kelemen, 1947) and nasopharynx, respectively [reviewed in (Coppola, 2012)], which would be expected to result in exposure of the closed OE to odor concentrations that rise with increasing environmental concentrations. In support of this, quantities of newborn Olfr235 and Olfr1440 OSNs increase on both the open and closed sides with increasing muscone concentration (except at the highest concentration, 10%, in the case of Olfr1440) (Figure 4C-middle, Figure 4–figure supplement 1A-middle). It is conceivable that reductions in newborn Olfr1440 OSN quantities observed in the presence of 10% muscone reflect overstimulation-dependent reductions in survival. Our findings from UNO-based experiments are consistent with expectations that naris occlusion does not completely

block exposure to odorants on the closed side, particularly at high concentrations. However, they also appear consistent with the hypothesis that exposure to musk odors promotes the neurogenesis of musk-responsive OSN subtypes.

Considering the limitations of the UNO procedure, it is important to note that the present study also includes experimental exposure of non-occluded animals to both male odors (Figure 3G, H) and exogenous musk odorants (Figures 4D-F). Findings from the latter experiments provide strong evidence that exposure to multiple musk odorants (muscone, ambretone) causes selective increases in the birthrates of multiple musk-responsive OSN subtypes (Olfr235, Olfr1440, Olfr1431).

We have included within the Results section of the revised manuscript a discussion of how observed effects of muscone exposure of UNO-treated mice may be influenced by transnasal/retronasal odor transfer to the closed side of the OE.

> *(5) In contrast to all other musk-responsive OSN types, the number of newborn OSNs expressing olfr1437 increases on the closed side of the OE relative to the open in UNO-treated male mice (Figure 1). This seems to contradict the presented theory and also does not align with the bulk RNAseq data (Figure S1).*

Subtype Olfr1437 is indeed an outlier among musk-responsive subtypes that were previously found to be more highly represented in the OSN population in 6-month-old sex-separated males compared to females (Appendix 1–figure 1)(C. van der Linden et al., 2018; Vihani et al., 2020). Somewhat unexpectedly, our findings from scRNA-seq experiments show slightly greater quantities of immature Olfr1437 OSNs on the closed side of the OE in juvenile males (Figure 1D, E of the revised manuscript, which now includes data from a second OE). Perhaps more informatively considering the small number of iOSNs of specific subtypes in the scRNA-seq datasets, EdU birthdating experiments show no difference in newborn Orlfr1437 OSN quantities on the 2 sides of the OE from UNO-treated juvenile males (Figure 2G). It is unclear to us why subtype Olfr1437 does not show open-side biases in newborn OSN quantities in juvenile male mice, but potential explanations include:

- Age: Findings based on bulk RNA-seq that musk responsive OSN subtypes are more highly represented in mice exposed to male odors analyzed mice that were 6 months old (C. van der Linden et al., 2018) or > 9 months old (Vihani et al., 2020) at the time of analysis. By contrast, the present study primarily analyzed mice that were juveniles (PD 28) at the time of scRNA-seq analysis (Figure 1) or EdU labeling (Figure 2G). It is conceivable that different musk-responsive subtypes are selectively responsive to distinct odors that are emitted at different ages. In this scenario, odors that increase the birthrates of Olfr235, Olfr1440, and Olfr1431 OSNs may be emitted starting at the juvenile stage, while those that increase the birthrate of Olfr1437 OSNs may be emitted in adulthood. In potential support of this, juvenile males exposed to their adult parents at the time of EdU labeling showed a slightly greater (although not statistically significantly different) UNO effect size in quantities of newborn Olfr1437 OSNs compared to controls (Figure 3–figure supplement 3).

- Capacity for stimulation-dependent neurogenesis: It is also conceivable that, unlike other musk-responsive OSN subtypes, Olfr1437 OSNs lack the capacity for stimulation-dependent neurogenesis (like the SBT-responsive subtype Olfr912, for example). If so, this would imply that increased representations of Olfr1437 OSNs observed in mice exposed to male odors for long periods (C. van der Linden et al., 2018; Vihani et al., 2020) may be due to male odor-dependent increases in the lifespans of Olfr1437 OSNs.

Within the Discussion section of the revised manuscript, we have discussed the findings concerning Olfr1437.

> *(6) The authors hypothesize in relation to the accelerated birthrate of musk-responsive OSN subtypes that "the acceleration of the birthrates of specific OSN subtypes could selectively enhance sensitivity to odors detected by those subtypes by increasing their representation within the OE". However, for two other OSN subtypes that detect male-specific odors, they hypothesize the opposite "By contrast, Olfr912 (Or8b48) and Olfr1295 (Or4k45), which detect the male-specific non-musk odors 2-sec-butyl-4,5-dihydrothiazole (SBT) and (methylthio)methanethiol (MTMT), respectively, exhibited lower representation and/or transcript levels in mice exposed to male odors, possibly reflecting reduced survival due to overstimulation."*
>
> *Without any further explanation, it is hard to comprehend why exposure to male-derived odors should, on one hand, accelerate birthrates in some OSN subtypes to potentially increase sensitivity to male odors, but on the other hand, lower transcript levels and does not accelerate birth rates of other OSN subtypes due to overstimulation.*

We agree that this point deserves further explanation. Within the revised manuscript, we have expanded the Introduction and Results to describe evidence from previous studies that exposure to stimulating odors causes two categories of changes to specific OSN subtypes: elevated representations or reduced representations within the OSN population. In one study (C. J. van der Linden et al., 2020), UNO treatment was found to cause a fraction of OSN subtypes to exhibit lower birthrates and representations on the closed side of the OE relative to the open. By contrast, another fraction of OSN subtypes exhibited higher representations on the closed side of the OEs of UNO-treated mice, but no difference in birthrates between the two sides. The latter subtypes were found to be distinguished by their receipt of extremely high levels of odor stimulation, suggesting that reduced odor stimulation via naris occlusion may lengthen their lifespans. In support of the possibility that Olfr912 (and Olfr1295), which detect SBT and MTMT, respectively (Vihani et al., 2020), which are emitted specifically by male mice (Lin et al., 2005; Schwende et al., 1986), UNO treatment was previously found to increase total Olfr912 OSN quantities on the closed side compared to the open side in sex-separated males (C. van der Linden et al., 2018), a finding confirmed in the present study (Figure 3–figure supplement 1H).

Taken together, findings from previous studies as well as the current one indicate that olfactory stimulation can accelerate the birthrates and/or reduced the lifespans of OSNs, depending on the specific subtypes and odors within the environment. As we have now indicated in the Discussion, we do not yet know what distinguishes subtypes that undergo stimulation-dependent neurogenesis, but it is conceivable that they detect odors with a particular salience to mice. Thus, observations that some odorants (e.g., musks) cause stimulation-dependent neurogenesis while others do not (e.g., SBT) might reflect an animal's specific need to adapt its sensitivity to the former. Alternatively, it is conceivable that stimulation-dependent reductions in representations of subtypes such as Olfr912 and Olfr1295 reflect a fundamentally different mode of plasticity that is also adaptive, as has been hypothesized (C. van der Linden et al., 2018; Vihani et al., 2020).

> ***Reviewer #1 (Recommendations For The Authors):***
>
> *To support the main claim, several controls are necessary as mentioned under point 1 of the public review.*
>
> *As outlined in our responses to the public review, new experiments within the revised manuscript indicate the following:*
>
> *(1) Accelerated birthrates of 3 different musk responsive OSN subtypes (Olfr235, Olfr1440, Olfr1431) are observed in non-occluded mice following exposure to multiple exogenous musk odorants (muscone, ambretone) (Figure 4D-F).*

> *(2) Exposure of non-occluded mice to non-musk odors (SBT, IAA) does not accelerate the birthrates of musk responsive OSN subtypes (Olfr235, Olfr1440, Olfr1431) (Figure 4D-F).*
>
> *(3) Exposure of mice to exogenous musk odors (muscone, ambretone) does not accelerate the birthrates of non-musk responsive OSN subtypes (e.g., Olfr912), including those previously found to undergo stimulation-dependent neurogenesis (Olfr827, Olfr1325) (Figure 4–figure supplement 4C).*
>
> *(4) Only a fraction of OSN subtypes have a capacity to undergo accelerated neurogenesis in the presence of odors that activate them (e.g., Olfr912 birthrates are not accelerated by SBT exposure) (Figure 4–figure supplement 4C-left).*
>
> *In addition, this study could be considerably improved by showing that the proposed mechanism applies beyond a single OSN subtype (olfr235), especially since the most sensitive OR subtype (expressing olfr1440) does not align with the main claim. The introduction states that this is difficult because the ligands for many ORs are unknown including all subtypes previously found to undergo stimulation-dependent neurogenesis referring to your 2020 study. While this reviewer agrees that the lack of deorphanization is a significant hurdle in the field, the 2020 study states that about 4% of all ORs (which should equal >40 ORs) show a stimulus-dependent down-regulation on the closed side, not only the 7 ORs which are closer examined (Figure 1). It would tremendously improve the impact of the current study to show that the proposed effect applies also to one of these other >40 ORs.*

We appreciate this question, as it alerted us to some shortcomings in how our findings were presented within the original manuscript. We respectfully disagree that only findings regarding subtype Olfr235 align with the main hypothesis of this study, which is that discrete odors can selectively promote the neurogenesis of sensory neuron subtypes that they stimulate. Specifically, we would like to draw attention to experiments on non-occluded female mice exposed to exogenous musk odorants (muscone, ambretone; revised Figures 4D-F; previously, Figure 6). Findings from these experiments provide compelling evidence that exposure to musk odorants causes selective increases in the birthrates of three different musk-responsive OSN subtypes: Olfr235, Olfr1440, and Olfr1431. Thus, we would suggest that results from the present study already show that the proposed mechanism applies to more than the just Olfr235 subtype. However, we agree with what we think is the essence of the reviewer's point: that it is important to determine the extent to which this mechanism applies to OSN subtypes that are responsive to other (i.e., non-musk) odorants. While, as noted by the reviewer, our previous study identified several OSN subtypes that undergo stimulation-dependent neurogenesis (as well as many others that predicted to do so)(C. J. van der Linden et al., 2020), we are not aware of ligands that have been identified with high confidence for those subtypes. Although we are in the process of conducting experiments to identify additional odor/subtype pairs to which the mechanism described in this study applies, the early-stage nature of these experiments precludes their inclusion in the present manuscript.

> *The ethological and mechanistic relevance of the current study could be significantly improved by showing that musk-related odors that activate olfr235 are actually found in male mouse urine (and additionally are not found in female mouse urine). Otherwise, the implicated link between the acceleration of OSN birthrates by exposure to male odors and acceleration by specific monomolecular odors does not hold, raising the question of any natural relevance (e.g. the proposed adaptive function to increase sensitivity to certain odors).*

As noted in our responses to the public review, we have addressed this important point within the revised manuscript as follows:

(1) We have included an extensive discussion of what is known about the emission of musk-like odors by mice.

(2) We have used GC-MS to analyze both mouse urine and preputial gland extracts for the presence of known musk compounds. Although inconclusive, we report that preputial glands contain signals that are structurally consistent with known musk compounds. The findings of these experiments have been included in the revised manuscript (new Appendix 2–figure 1), along with a discussion of their limitations.

> ***Reviewer #2 (Public Review):***
>
> *In their paper entitled "In mice, discrete odors can selectively promote the neurogenesis of sensory neuron subtypes that they stimulate" Hossain et al. address lifelong neurogenesis in the mouse main olfactory epithelium. The authors hypothesize that specific odorants act as neurogenic stimuli that selectively promote biased OR gene choice (and thus olfactory sensory neuron (OSN) identity). Hossain et al. employ RNA-seq and scRNA-seq analyses for subtype-specific OSN birthdating. The authors find that exposure to male and musk odors accelerates the birthrates of the respective responsive OSNs. Therefore, Hossain et al. suggest that odor experience promotes selective neurogenesis and, accordingly, OSN neurogenesis may act as a mechanism for long-term olfactory adaptation.*

We appreciate this summary but would like to underscore that a mechanism involving biased OR gene choice is just one of two possibilities proposed in the Discussion section to explain how odorant stimulation of specific subtypes accelerates the birthrates of those subtypes.

> *The authors follow a clear experimental logic, based on sensory deprivation by unilateral naris occlusion, EdU labeling of newborn neurons, and histological analysis via OR-specific RNA-FISH. The results reveal robust effects of deprivation on newborn OSN identity. However, the major weakness of the approach is that the results could, in (possibly large) parts, depend on "downregulation" of OR subtype-specific neurogenesis, rather than (only) "upregulation" based on odor exposure. While, in Figure 6, the authors show that the observed effects are, in part, mediated by odor stimulation, it remains unclear whether deprivation plays an "active" role as well. Moreover, as shown in Figure 1C, unilateral naris occlusion has both positive and negative effects in a random subtype sample.*

In our view, the present study involves two distinct and complementary experimental designs: 1) odor exposure of UNO-treated animals and 2) odor exposure of non-occluded animals. Here we address this comment with respect to each of these designs:

(1) For experiments performed on UNO-treated animals, we agree that observed differences in birthrates on the open and closed sides of the OE reflect, largely, a deceleration (i.e., downregulation) of the birthrates of these subtypes on the closed side relative to the open (as opposed to an acceleration of birthrates on the open side). Our objective in using this design was to test the extent to which specific OSN subtypes undergo stimulation-dependent neurogenesis under various odor exposure conditions. According to the main hypothesis of this study, a lower birthrate of a specific OSN subtype on the closed side of the OE compared to the open is predicted to reflect a lower level of odor stimulation on the closed side received by OSNs of that subtype. However (and as described in our responses to reviewer #1), a limitation of this design is that environmental odorants, especially at high concentrations, are likely to stimulate responsive OSNs on the closed side of the OE in addition to the open side due to transnasal and/or retronasal air flow.

(2) Experiments performed on non-occluded animals were designed to provide critical complementary evidence that specific OSN subtypes undergo accelerated neurogenesis in the presence of specific odors. Using this design, we have found compelling evidence that:

- Exposure of non-occluded mice to male odors causes the selective acceleration of the birthrate of Olfr235 OSNs (Figure 3G, H).

- Exposure of non-occluded female mice to two different musk odorants (muscone and ambretone) selectively accelerates the birthrates three different musk responsive subtypes: Olfr235, Olfr1440, and Olf1431 (Figure 4D-F and Figure 4–figure supplement 4C).

We have reorganized the revised manuscript to more clearly present the most important experimental findings using these two experimental designs. We have also highlighted (via schematics) the experimental conditions (e.g., UNO, non-occlusion, odor exposure) used for each experiment.

> *Another weakness is that the authors build their model (Figure 8), specifically the concept of selectivity, on a receptor-ligand pair (Olfr912 that has been shown to respond, among other odors, to the male-specific non-musk odors 2-sec-butyl-4,5-dihydrothiazole (SBT)) that would require at least some independent experimental corroboration. At least, a control experiment that uses SBT instead of muscone exposure should be performed.*

We agree that this important concern deserves additional control experiments and discussion. We have addressed this concern within the revised manuscript as follows:

- Within the Results section, we have added multiple new control experiments (detailed in response to Reviewer #1), including the one recommended above. As suggested, we quantified newborn OSNs of the SBT-responsive subtype Olfr912 in non-occluded females that were either exposed to 0.1% SBT or unexposed controls. Exposure of SBT was found to cause no significant increase in quantities of newborn Olfr912 OSNs (newly added Figure 4–figure supplement 4C-left). These findings further support the model in Figure 7 (previously Figure 8) that only a fraction of OSN subtypes have a capacity to undergo accelerated neurogenesis in the presence of odors that activate them.

- Also within the Results section, we have made efforts to better highlight relevant control experiments that were included in the original version, particularly those showing that quantities of newborn Olfr912 OSNs are not affected by UNO in mice exposed to male odors (Figure 2H and Figure 3–figure supplement 1G; previously Figure 2F and Figure 3H) or by exposure of non-occluded females to male odors (Figure 3H; previously Figure 6E). Since Olfr235 is responsive to component(s) of male odors (C. van der Linden et al., 2018; Vihani et al., 2020), these results indicate that this subtype does not have the capacity of stimulation-dependent neurogenesis, which is consistent with our previous findings that only a fraction of subtypes have this capacity (C. J. van der Linden et al., 2020).

> *In this context, it is somewhat concerning that some results, which appear counterintuitive (e.g., lower representation and/or transcript levels of Olfr912 and Olfr1295 in mice exposed to male odors) are brushed off as "reflecting reduced survival due to overstimulation." The notion of "reduced survival" could be tested by, for example, a caspase3 assay.*

This is a point that we agree deserves further discussion. Please see the explanation that we have outlined above in response to Reviewer #1.

Within the revised manuscript, we have expanded the Introduction to describe evidence from previous studies that exposure to stimulating odors causes two categories of changes to

specific OSN subtypes: elevated representations or reduced representations within the OSN population. We outline evidence from previous studies that Olfr912 and Olfr1295 belong to the latter category, and that the representations of these subtypes are likely reduced by male odor overstimulation-dependent shortening of OSN lifespan.

> *Important analyses that need to be done to better be able to interpret the findings are to present (i) the OR+/EdU+ population of olfactory sensory neurons not just as a count per hemisection, but rather as the ratio of OR+/EdU+ cells among all EdU+ cells; and (ii) to the ratio of EdU+ cells among all nuclei (UNO versus open naris). This way, data would be normalized to (i) the overall rate of neurogenesis and (ii) any broad deprivation-dependent epithelial degeneration.*

We have addressed this concern in two ways within the revised manuscript:

(1) We have noted within the Methods section that the approach of using half-sections for normalization has been used in multiple previous studies for quantifying newborn (OR+/EdU+) and total (OR+) OSN abundances (Hossain et al., 2023; Ibarra-Soria et al., 2017; C. van der Linden et al., 2018; C. J. van der Linden et al., 2020). Additionally, within the figure legends and Methods, we have more thoroughly described the approach used, including that it relies on averaging the quantifications from at least 5 high-quality coronal OE tissue sections that are evenly distributed throughout the anterior-posterior length of each OE and thereby mitigates the effects of section size and cell number variation among sections. In the case of UNO treated mice, the open and closed sides within the same section are paired, which further reduces the effects of section-to section variation. We have found that this approach yields reproducible quantities of newborn and total OSNs among biological replicate mice and enables accurate assessment of how quantities of OSNs of specific subtypes change as a result of altered olfactory experience, a key objective of this study.

(2) To assess whether the use of alternative approaches for normalizing newborn OSN quantities suggested by the reviewers would affect the present study's findings, we compared three methods for normalizing the effects of exposure to male odors or muscone on quantities of newborn Olfr235 OSNs in the OEs of both UNO-treated and non-occluded mice: 1) OR+/EdU+ OSNs per half-section (used in this study), 2) OR+/EdU+ OSNs per total number of EdU+ cells (reviewer suggestion (i)), and 3) OR+/EdU+ OSNs per unit of DAPI+ area (an approximate measure of nuclei number; reviewer suggestion (ii)). The three normalization methods yielded statistically indistinguishable differences in assessing the effects of exposure of either UNO-treated or non-occluded mice to male odors (newly added Figure 2–figure supplement 2 and Figure 3–figure supplement 2), or of exposure of non-occluded mice to muscone (newly added Figure 4–figure supplement 3). Based on these findings, and the considerable time that would be required to renormalize all data in the manuscript, we have chosen to maintain the use of normalization per half-section.

> *Finally, the paper will benefit from improved data presentation and adequate statistical testing. Images in Figures 2 - 7, showing both EdU labeling of newborn neurons and OR-specific RNA-FISH, are hard to interpret. Moreover, t-tests should not be employed when data is not normally distributed (as is the case for most of their samples).*

We have made extensive changes within the revised manuscript to increase the clarity and interpretability of the figures, including:

(1) Addition of a split-channel, high-magnification view of a representative image that shows the overlap of FISH and EdU signals (Figure 2D).

(2) Addition of experimental schematics and timelines corresponding to each set of experiments.

In the revised manuscript, several changes to the statistical tests have been made, as follows:

(1) To assess deviation from normality of the histological quantifications of newborn and total OSNs of specific subtypes in this study, all datasets were tested using the Shapiro-Wilk test for non-normality and the P values obtained are included in Supplementary file 1 (figure source data). Of the 274 datasets tested, 253 were found to have Shapiro-Wilk P values > 0.05, indicating that the vast majority (92%) do not show evidence of significant deviation from a normal distribution.

(2) A general lack of deviation of the datasets in this study from a normal distribution is further supported by quantile-quantile (QQ) plots, which compare actual data to a theoretically normal distribution (Appendix 4–figure 1). The datasets analyzed were separated into the following categories:

a. Quantities of newborn OSNs in UNO treated mice (Appendix 4-figure 1A)

b. Quantities of total OSNs in UNO treated mice (Appendix 4-figure 1B)

c. Quantities of newborn OSNs in non-occluded mice (Appendix 4-figure 1C)

d. UNO effect sizes for newborn or total OSNs (Appendix 4-figure 1D)

(3) Results of both parametric and non-parametric statistical tests of comparisons in this study have been included in Supplementary file 2 (statistical analyses). In general, the results from parametric and non-parametric tests are in good agreement.

(4) Statistical analyses of differences in OSN quantities in the OEs of non-occluded mice or UNO effect sizes in UNO-treated mice subjected more than two different experimental conditions have now been performed using one-way ANOVA tests, FDR-adjusted using the 2-stage linear step-up procedure of Benjamini, Krieger and Yekutieli.

> ***Reviewer #2 (Recommendations for the Authors):***
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> *The manuscript by Hossain et al. would benefit from a thorough revision. Here, we outline several points that should be addressed:*
>
> *Figure 3E - I & Figure 4E&F: Red lines that connect mean values are misleading.*

Within the revised manuscript, the UNO effect size graphs have been modified for clarity, including removal of the lines between mean values except for those comparing changes over time post EdU injection (Figure 6 and Figure 6-figure supplement 1). For these latter graphs, we think that lines help to illustrate changes in effect sizes over time.

> *Figure 3E - I: UNO effect sizes (right) should be tested via ANOVA.*

In the revised manuscript, statistical analyses of UNO effect sizes in UNO-treated mice subjected more than two different experimental conditions were done using one-way ANOVA tests, FDR-adjusted using the 2-stage linear step-up procedure of Benjamini, Krieger and Yekutieli (Figure 2-figure supplement 2; Figure 3; Figure 3-figure supplement 1; Figure 4; Figure 4-figure supplements 1, 2). The same tests were used for analysis of differences in OSN quantities in the OEs of non-occluded mice subjected more than two different experimental conditions (Figure 3; Figure 3-figure supplement 2; Figure 4; Figure 4-figure supplements 3, 4). For comparisons of differences in quantities of newborn OSNs of musk-responsive subtypes at 4 and 7 days post-EdU between non-occluded mice exposed and unexposed to muscone, a two sample ANOVA - fixed-test, using F distribution (right-tailed) was used (Figure 6; Figure 6-figure supplement 1).

> *Images in Figures 2 - 7, showing both EdU labeling of newborn neurons and OR-specific RNA-FISH: Colabeling is hard / often impossible to discern. Show zoom-ins and better explain the criteria for "colabeling" in the methods.*

In the revised manuscript an enlarged and split-channel view of an image showing multiple newborn Olfr235 OSNs (OR+/EdU+) has been added (Figure 2D). A detailed description of the criteria for OR+/EdU+ OSNs is provided in Methods under the section “Histological quantification of newborn and total OSNs of specific subtypes.”

> *Figure 1C: add Olfr912.*

As a control group for iOSN quantities of musk-responsive subtypes in Figure 1, we selected random subtypes that are expressed in the same zones: 2 and 3. Olfr912 OSNs were not included because this subtype was not randomly chosen, nor is it expressed the same zones (Olfr912 is expressed in zone 4). We also note that the scRNA-seq analysis was done to allow an initial exploration of the hypothesis that some OSN subtypes with that are more highly represented in mice exposed to male odors show stimulation-dependent neurogenesis. Considering that the scRNA-seq datasets contain only small numbers of iOSNs of specific subtypes, we think they are more useful for analyzing changes in birthrates within groups of subtypes (e.g., musk responsive, random) rather than individual subtypes.

> *The time of OE dissection is different for data shown in Figure 1 (P28) as compared to other figures (P35). Please comment/discuss.*

Within the Results section of the revised manuscript, we have now clarified that the PD 28 timepoint chosen for EdU birthdating in the histological quantification of newborn OSNs of specific subtypes is analogous to the PD 28 timepoint chosen for identification of immature (Gap43-expressing) OSNs in the scRNA-seq samples. In the case of EdU birthdating, it is necessary to provide a chase period of sufficient length to enable robust and stable expression of an OR, which defines the subtype. A chase period of 7 days was chosen based on a previous study (C. J. van der Linden et al., 2020). Hence, a dissection date of PD 35 was chosen.

> *Figure 3F&G: please discuss the female à female effects*

In the Results and Discussion sections of the revised manuscript, we discuss our observation that the Olfr1440 and Olfr1431 subtypes show significantly higher quantities of newborn OSNs on the open side compared to closed sides in UNO-treated females. We speculate that these subtypes may receive some odor stimulation in juvenile females, perhaps via musk or related odors emitted by females themselves or from elsewhere within the environment.

> *Figure 4E (and other examples): male à male displays two populations (no effect versus effect); please explain/speculate.*

For some UNO effect sizes, there appears to be high degree of variation among mice, and, in some cases, this diversity appears to cause the data to separate into groups. We assessed whether this diversity might reflect mice that came from different litters, but this is not the case. Rather, we speculate that the observed diversity most likely reflects low representations of newborn OSNs of some subtypes and/or under specific conditions. The data referred to by the reviewer (now Figure 3–figure supplement 3D), for example, shows UNO effect sizes for quantities of newborn Olfr1431 OSNs, which has the lowest representation among the musk-responsive subtypes analyzed in this study.

> *Figure 5C-E: It is unclear why strong muscone concentrations (10%) have no effect, whereas no muscone sometimes (D&E) has an effect.*

As discussed in response to comments from Reviewer #1, we speculate that fluctuations in UNO effect sizes in muscone-exposed mice, particularly at high muscone concentrations, may be due, at least in part, to transnasal and/or retronasal air flow [reviewed in (Coppola, 2012)], which would be expected to result in exposure of the closed side of the OE to muscone concentrations that increase with increasing environmental concentrations. In support of this, quantities of newborn Olfr235 (Figure 4C-middle) and Olfr1440 (Figure 4–figure supplement 1A-middle) OSNs increase on both the open and closed sides with increasing muscone concentration (except at the highest concentration, 10%, in the case of Olfr1440). We speculate that reductions in newborn Olfr1440 OSN quantities observed in the presence of 10% muscone may reflect overstimulation-dependent reductions in survival.

As emphasized above, our study also includes experiments on non-occluded animals (Figures 3, 4, 5). Findings from these experiments provide additional evidence that exposure to multiple musk odorants (muscone, ambretone) causes selective increases in the birthrates of multiple musk-responsive OSN subtypes (Olfr235, Olfr1440, Olfr1431).

We have included an extensive interpretation of UNO-based experiments, including their limitations, within the Results section of the revised manuscript.

> *Figure S1: please explain the large error bars regarding "Transcript level".*

We have clarified that the error bars in this figure, which is now Appendix 1–figure 1, correspond to 95% confidence intervals.

> *The figure captions could be improved for ease of reading.*

Figure captions have been revised for increased clarity.

> *Figure 4: Include Olfr235 data for consistency.*

All OSN subtypes analyzed for the effects of exposure to adult mice on UNO-induced open-side biases in quantities of newborn OSNs have been included in a single figure, which is now Figure 3–figure supplement 3.

> *Figure S6F&G: Do not run statistics on n = 2 (G) or 3 (F) samples.*

We have removed statistical test results from comparisons involving fewer than 4 observations.

> ***Reviewer #3 (Public Review):***
>
> *Summary:*
>
> *Neurogenesis in the mammalian olfactory epithelium persists throughout the life of the animal. The process replaces damaged or dying olfactory sensory neurons. It has been tacitly that replacement of the OR subtypes is stochastic, although anecdotal evidence has suggested that this may not be the case. In this study, Santoro and colleagues systematically test this hypothesis by answering three questions: is there enrichment of specific OR subtypes associated with neurogenesis? Is the enrichment dependent on sensory stimulus? Is the enrichment the result of differential generation of the OR type or from differential cell death regulated by neural activity? The authors provide some solid*

evidence indicating that musk odor stimulus selectively promotes the OR types expressing the musk receptors. The evidence argues against a random selection of ORs in the regenerating neurons.

Strengths:

The strength of the study is a thorough and systematic investigation of the expression of multiple musk receptors with unilateral naris occlusion or under different stimulus conditions. The controls are properly performed. This study is the first to formulate the selective promotion hypothesis and the first systematic investigation to test it. The bulk of the study uses in situ hybridization and immunofluorescent staining to estimate the number of OR types. These results convincingly demonstrate the increased expression of musk receptors in response to male odor or muscone stimulation.

Weaknesses:

A major weakness of the current study is the single-cell RNASeq result. The authors use this piece of data as a broad survey of receptor expression in response to unilateral nasal occlusion. However, several issues with this data raise serious concerns about the quality of the experiment and the conclusions. First, the proportion of OSNs, including both the immature and mature types, constitutes only a small fraction of the total cells. In previous studies of the OSNs using the scRNASeq approach, OSNs constitute the largest cell population. It is curious why this is the case. Second, the authors did not annotate the cell types, making it difficult to assess the potential cause of this discrepancy. Third, given the small number of OSNs, it is surprising to have multiple musk receptors detected in the open side of the olfactory epithelium whereas almost none in the closed side. Since each OR type only constitutes ~0.1% of OSNs on average, the number of detected musk receptors is too high to be consistent with our current understanding and the rest of the data in the manuscript. Finally, unlike the other experiments, the authors did not describe any method details, nor was there any description of quality controls associated with the experiment. The concerns over the scRNASeq data do not diminish the value of the data presented in the bulk of the study but could be used for further analysis.

We are grateful to the reviewer for raising these important questions.

In the revised manuscript, we have clarified that the scRNA-seq dataset presented in the original version of the manuscript (now called dataset OE 1) was published and described in detail in a previous study (C. J. van der Linden et al., 2020). The reviewer is correct that the proportion of OSNs within that dataset was lower in that dataset than in other datasets that have been published more recently (using updated methods). We think this is likely because of the way that the cells were processed (e.g., from cryopreserved single cells followed by live/dead selection). However, because the open and closed sides were processed identically, we do not expect the ratios of OSNs of specific subtypes to be greatly affected. Hence, the differences observed for specific OSN subtypes on the open versus closed sides are expected to be valid.

As the reviewer notes, there is a surprisingly large difference between the number of OSNs of musk-responsive subtypes on the open and closed sides within the OE 1 dataset. This difference is a key piece of information that led us to formulate the hypothesis in the study: that musk responsive subtypes are born at a higher rate in the presence of male/musk odor stimulation. And while it is true that, on average, each subtype represents ~0.1% of the population, it is known that there is wide variance in representations among different subtypes [e.g., (Ibarra-Soria et al., 2017)]. The frequencies of the musk responsive subtypes among all OSNs on the open side of OE 1 (0.3% for Olfr235, 0.4% for olfr1440, 0.06% for Olfr1434, 0% for olfr1431, and 1% for Olfr1437) are in line with previous findings.

To confirm that the scRNA-seq findings from dataset OE 1 are not an artifact of the cell preparation methods used, we generated a second scRNA-seq dataset, OE 2, which has been added to the revised manuscript (Figure 1). The OE 2 dataset was prepared according to the same experimental timeline as OE 1, but the cells were captured immediately after dissociation and live/dead sorting via FACS. As expected, most cells within OE 2 dataset are OSNs (77% on the open side, 66% on the closed). Importantly, like the OE 1 dataset, the OE 2 dataset shows higher quantities of iOSNs of musk responsive subtypes on the open side of the OE compared to the closed (normalized for either total cells or total OSNs) (Figure 1–figure supplement 1D, E).

> *A weakness of the experiment assessing musk receptor expression is that the authors do not distinguish immature from mature OSNs. Immature OSNs express multiple receptor types before they commit to the expression of a single type. The experiments do not reveal whether mature OSNs maintain an elevated expression level of musk receptors.*

While it is established that multiple ORs are coexpressed at a low level during OSN differentiation (Bashkirova et al., 2023; Fletcher et al., 2017; Hanchate et al., 2015; Pourmorady et al., 2024; Saraiva et al., 2015; Scholz et al., 2016; Tan et al., 2015), this has been found to occur primarily at the immediate neuronal precursor 3 (INP3) stage (Bashkirova et al., 2023; Fletcher et al., 2017), which is characterized by expression of *Tex15* (Fletcher et al., 2017; Pourmorady et al., 2024) and precedes the immature OSN (iOSN) stage, which is characterized by expression of *Gap43* (Fletcher et al., 2017; McIntyre et al., 2010; Verhaagen et al., 1989). Within the scRNA-seq datasets in the present study, iOSNs of specific subtypes are identified based on robust expression of *Gap43* (Log^2 UMI > 1) and a specific OR gene (Log^2 UMI > 2), as described in the figures and methods. Thus, the cells defined as iOSNs are expected to express a single OR gene and this expression should be maintained as iOSNs transition to mOSNs. To confirm these predictions, we carried out a detailed analysis of OR expression at three different stages of OSN differentiation: INP3, iOSN, and mOSN (Figure 1–figure supplement 2). The cells chosen for analysis express the musk-responsive ORs Olfr235 or Olfr1440 or a randomly chosen OR Olfr701, in addition to markers that define INP3, iOSN, or mOSN cells. As expected, individual iOSNs and mOSNs of musk-responsive subtypes were found to exhibit robust and singular OR expression on the open and closed sides of OEs from UNO-treated mice. Moreover, and as observed previously, INP3 cells coexpress multiple OR transcripts at low levels. A detailed description of how the analysis was performed is included in the Methods section under Quantification and statistical analysis.

Within the histology-based quantifications, newborn OSNs are identified based on their robust RNA-FISH signals corresponding to a specific OR transcript and an EdU label. Considering the EdU chase time of 7 days, most EdU-positive cells are expected to have passed the INP3 stage and be iOSNs or mOSNs. Moreover, considering the low level of OR expression within INP3 cells, it is unlikely OR transcripts are expressed at a high enough level to be detectable and/or counted at this stage and thereby affect newborn OSN quantifications.

> *There are also two conceptual issues that are of concern. The first is the concept of selective neurogenesis. The data show an increased expression of musk receptors in response to male odor stimulation. The authors argue that this indicates selective neurogenesis of the musk receptor types. However, it is not clear what the distinction is between elevated receptor expression and a commitment to a specific fate at an early stage of development. As immature OSNs express multiple receptors, a likely scenario is that some newly differentiated immature OSNs have elevated expression of not only the musk receptors but also other receptors. The current experiments do not distinguish the two alternatives. Moreover, as pointed out above, it is not clear whether mature OSNs maintain the increased expression. Although a scRNASeq experiment can clarify it, the authors, unfortunately, did not perform an in-depth analysis to determine at which point*

> *of neurogenesis the cells commit to a specific musk receptor type. The quality of the scRNASeq data unfortunately also does not lend confidence for this type of analysis.*

The addition of a second scRNA-seq dataset within the revised manuscript (Figure 1), combined with the new scRNA-seq-based analyses of OR expression in INP3, iOSN, and mOSN cells (Figure 1-figure supplement 2), provide strong evidence that iOSNs and mOSNs robustly express a single OR gene and that cellular expression is stable from the iOSN to the mOSN stage. These analyses do not support a scenario in which odor stimulation causes upregulated expression of multiple ORs and thereby causes apparent increases in quantities of newly generated OSNs that express musk-responsive ORs. Rather, the data firmly support a mechanism in which odor stimulation increases quantities of newly generated OSNs that have stably committed to the robust expression of a single musk-responsive OR.

> *A second conceptual issue, the idea of homeostasis in regeneration, which the authors presented in the Introduction, needs clarification. In its current form, it is confusing. It could mean that a maintenance of the distribution of receptor types, or it could mean the proper replacement of a specific OR type upon the loss of this type. The authors seem to refer to the latter and should define it properly.*

We have revised the Introduction section to clarify our use of the term homeostatic in one instance (paragraph 4) and replace it with more specific language in a second instance (paragraph 5).

> ***Reviewer #3 (Recommendations For The Authors):***
>
> *Concerns over scRNASeq data. It appears that the samples may have included non-OE tissues, which reduced the representation of the OSNs. This experiment may need to be repeated to increase the number of OSNs.*

As outlined in the response to the public comments, we think that the low proportion of OSNs in the OE 1 data set reflects how the cells were prepared and processed. We have now included a second scRNA-seq dataset to address this concern.

> *Cell types should be identified in the scRNASeq analysis, and the number of cells documented for each cell type, at least for the OSNs. The data should be made available for general access.*

We have now clarified that the OE 1 dataset was published as part of a previous study (C. J. van der Linden et al., 2020) and was made publicly available as part of that study (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE157119). All cell types in the newly generated OE 2 dataset have been annotated (Figure 1) and this dataset has also been made publicly available (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE278693). The numbers and percentages of OSNs within OE 1 and OE 2 datasets have been added to the legend of Figure 1-figure supplement 1.

> *The specific OR types should be segregated for mature and immature OSNs. The percentage of a specific OR type should be normalized to the total number of OSNs, rather than the total cells. The current quantification is misleading because it gives the false sense that the muscone receptors represent ~0.1% of cells when the proportion is much higher if only OSNs are considered.*

In the revised manuscript, quantities of iOSNs (*Gap43*+ cells) of specific subtypes within the OE 1 and OE 2 scRNA-seq datasets are graphed as percentages of both all OSNs (Figure 1E, Figure 1–figure supplement 1D) and all cells (Figure 1–figure supplement 1E). As a percentage

of all OSNs, average quantities of iOSNs of musk responsive subtypes on the open side of the OE range from 0.005% (for Olfr1431) to 0.14% (for Olfr1440) (Figure 1E).

Within the feature plots for the two datasets, the differentiation stages of indicated OSNs have been clearly defined within the figures and figure legends. For the OE 1 dataset, iOSNs are differentiated from mOSNs by arrows (Figure 1–figure supplement 1C). For the OE 2 dataset (Figure 1D), only immature OSNs are shown for simplicity.

> *Technical details of the scRNASeq should be documented. In the feature plot of musk-response receptors (Figure. 1D), it is better to use the actual quantity of expression rather than binarized representation (with or without an OR). If one needs to use on/off to determine the number of cells for a given OR type, then the criteria of selection should be given.*

Technical details of generation of the scRNA-seq datasets have been documented in the "Method details" section (for the OE 2 dataset) and in the method section of our previous publication of the OE 1 dataset (C. J. van der Linden et al., 2020). Details of the scRNA-seq analyses, including the criteria used to define immature OSNs of specific subtypes, are documented within the "Quantification and statistical analysis" section.

Within the feature plots, we have decided to show OSNs of a given subtype in a binary fashion using specific colors for the sake of simplicity (Figure 1D, Figure 1-figure supplement 1C). To address the reviewer's cooncern, we have added a new figure that provides detailed information about OR transcript expression (levels and genes) within iOSNs and mOSNs of two different musk responsive subtypes and a randomly chosen subtype (Figure 1-figure supplement 2).

> *An in-depth analysis of the onset of OR expression in the GBC, INP, immature, and mature OSNs should be performed. It is also important to determine how many other receptors are detected in the cells that express the musk receptors. The current scRNASeq data may not be of sufficiently high quality and the experiment needs to be repeated. It is also important for the authors to take measures to eliminate ambient RNA contamination.*

The revised manuscript includes a second scRNA-seq dataset (OE 2; Figure 1). Details of how both the original (OE 1) and new datasets were generated have been documented within the Methods sections of the corresponding publications [(C. J. van der Linden et al., 2020); present study]. For both datasets, live/dead selection of cells was performed, which was expected to reduce ambient RNA.

The revised manuscript also includes a new figure that provides detailed information about OR transcript expression within INP3, iOSN and mOSN cells that express one of two different musk responsive ORs or a randomly chosen OR (Figure 1-figure supplement 2). These data reveal, as reported previously (Bashkirova et al., 2023; Fletcher et al., 2017; Pourmorady et al., 2024), that low levels of multiple OR transcripts are detected in INP3 (*Tex15*+) cells. By contrast, iOSN (Gap43+) and mOSN (Omp+) cells robustly express a single OR, with little or no expression of other ORs.

> *Quantification of cells for Figure 2-7 should be changed. Instead of using cell number per 1/2 section, the data should be calculated using density (using the area of the epithelium or normalized to the total number of cells (based on DAPI staining). This is because multiple sections are taken from the same mouse along the A-P axis. These sections have different sizes and numbers of cells.*

As noted in response to a similar concern of Reviewer #2, this has been addressed in two ways within the revised manuscript:

(1) We have noted within the Methods section that the approach of using half-sections for normalization has been used in multiple previous studies for quantifying newborn (OR+/EdU+) and total (OR+) OSN abundances (Hossain et al., 2023; Ibarra-Soria et al., 2017; C. van der Linden et al., 2018; C. J. van der Linden et al., 2020). Additionally, within the figure legends and Methods, we have more thoroughly described the approach used, including that it relies on averaging the quantifications from at least 5 high-quality coronal OE tissue sections that are evenly distributed throughout the anterior-posterior length of each OE and thereby mitigates the effects of section size and cell number variation among sections. In the case of UNO treated mice, the open and closed sides within the same section are paired, which further reduces the effects of section-to section variation. We have found that this approach yields reproducible quantities of newborn and total OSNs among biological replicate mice and enables accurate assessment of how quantities of OSNs of specific subtypes change as a result of altered olfactory experience, a key objective of this study.

(2) To assess whether the use of alternative approaches for normalizing newborn OSN quantities suggested by the reviewers would affect the present study's findings, we compared three methods for normalizing the effects of exposure to male odors or muscone on quantities of newborn Olfr235 OSNs in the OEs of both UNO-treated and non-occluded mice: 1) OR+/EdU+ OSNs per half-section (used in this study), 2) OR+/EdU+ OSNs per total number of EdU+ cells (reviewer suggestion (i)), and 3) OR+/EdU+ OSNs per unit of DAPI+ area (an approximate measure of nuclei number; reviewer suggestion (ii)). The three normalization methods yielded statistically indistinguishable differences in assessing the effects of exposure of either UNO-treated or non-occluded mice to male odors (newly added Figure 2–figure supplement 2 and Figure 3–figure supplement 2), or of exposure of non-occluded mice to muscone (newly added Figure 4–figure supplement 3). Based on these findings, and the considerable time that would be required to renormalize all data in the manuscript, we have chosen to maintain the use of normalization per half-section.

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