Title: Fear conditioning biases olfactory stem cell receptor fate

Authors: Clara W. Liff [1], Yasmine R. Ayman[1], Eliza C.B. Jaeger[1], Hudson S. Lee[1], Alexis Kim[1,2], Angélica Viña Albarracín[1], Bianca Jones Marlin[1,2,3]*

Affiliations:

[1]Mortimer B. Zuckerman Mind Brain and Behavior Institute, Columbia University; New York, 10027, USA.

[2]Howard Hughes Medical Institute, Columbia University, New York, NY, USA.

[3]Department of Psychology, Columbia University; New York, 10032, USA.

[4]Department of Neuroscience, Columbia University; New York, 10032, USA.

*Corresponding author. Email: bjm2174@columbia.edu

Abstract: The main olfactory epithelium initiates the process of odor encoding. Recent studies have demonstrated intergenerationally inherited changes in the olfactory system in response to fear conditioning, resulting in increases in olfactory receptor frequencies and altered responses to odors. We investigated changes in the morphology of the olfactory epithelium in response to an aversive stimulus. Here, we achieve volumetric cellular resolution to demonstrate that olfactory fear conditioning increases the number of odor-encoding neurons in mice that experience odor-shock conditioning (F0), *as well as their offspring* (F1). We provide evidence that increases in F0 were due to biased stem cell receptor choice. Thus, we reveal dynamic regulation of the olfactory epithelium receptor composition in response to olfactory fear conditioning, providing insight into the heritability of acquired phenotypes.

One-Sentence Summary: Odor-shock pairing is inherited by naïve offspring and biases neurogenesis in the nose.

Main Text: Aversive olfactory conditioning in mice results in the persistent avoidance of the conditioned odor, and the olfactory sensory neurons (OSNs) responsive to this odor increase in number in the sensory epithelium (*1*). Strikingly, this increase in the number of specific sensory neurons was observed not only in trained F0 males, but also in their offspring (F1), despite the fact that the progeny had never been exposed to the conditioned odor (*2–4*). This phenomenon, intergenerational epigenetic inheritance, invokes the transfer of information from one generation to the next without alterations to the sequence of the genome.

Transgenerational epigenetic inheritance, the transfer of information beyond the F1 generation, is responsible for several examples of non-Mendelian transmission in plants, fission yeast, and worms (*5–9*). Molecular genetics has provided a detailed mechanistic understanding of the transmission of epigenetic information from parent to offspring in these organisms (*10*, *11*). Olfactory conditioning in the parent may provide future generations with an adaptive advantage: enhanced sensitivity to aversive sensory features in the environment of the parent. Intergenerational epigenetic inheritance of olfactory properties in mice poses an elusive problem

as to how signals responsible for the increase in specific OSNs in the sensory epithelium are transmitted from the nose, to the gamete, and then to the offspring.

Olfactory perception is initiated by the recognition of odorants by a large repertoire of receptors in the sensory epithelium. Individual sensory neurons in mice express only one of 1,400 different receptor genes (*12*). The choice of a receptor is stochastic and is mediated by an unusual mechanism of transactivation that delivers the necessary transcription factors to only one allele of a single receptor gene in a sensory neuron (*13–15*). Neurons expressing a given receptor are distributed within zones of the epithelium but project with precision to spatially invariant glomeruli in the olfactory bulb. Each odorant can interact with multiple distinct receptors, resulting in the activation of a unique ensemble of glomeruli. The recognition of an odor requires the integration of information from multiple glomeruli to the mitral and tufted cells in the olfactory bulb, and then to downstream olfactory convergent areas (*16–19*). If the stochastic choice of a single receptor in each neuron can be biased by salient odor associations in the environment, this would afford a mechanism to alter receptor representations in the epithelium.

The mechanisms responsible for the increase in the number of OSNs following aversive conditioning are more readily addressed in the sensory epithelium of F0 mice than in the F1 progeny. Elucidation of these local signaling events may then provide insight into the more distant transmission of information to the gametes. The mature olfactory sensory epithelium undergoes constant neurogenesis throughout the life of vertebrates. In mice, the lifespan of a mature OSN is estimated to be 30 days, and new sensory neurons are continually generated by the division of basal stem cells, transit amplification, and ultimately differentiation to the mature OSN (*20*). Continual balanced neurogenesis suggests that increases in OSNs upon aversive conditioning could result from the increased birth or enhanced survival of a specific OSN population.

In this study, we performed quantification of OSNs after aversive olfactory learning, corroborating the results of an increase in the OSNs expressing a receptor responsive to the conditioned odor. Moreover, enhanced numbers of these specific OSNs are also observed in the F1 generation. Contrary to previous studies, we do not observe the inheritance of odor-evoked aversion to the conditioned odor in the F1 generation using our behavioral paradigm. We demonstrate that this phenomenon is persistent, as increases in specific OSNs continue for at least 63 days after conditioning. We further demonstrate that a biased increase in specific OSNs after learning is likely to result from the enhanced birth of specific OSNs, suggesting that biased receptor choice underlies this phenomenon in the parent and is epigenetically inherited by their offspring.

Olfactory fear conditioning leads to an increase in conditioned-odor-responsive cells in parents (F0)

In initial experiments, we asked whether we could observe changes in the abundance of receptors responsive to conditioned odors after aversive olfactory learning. The odorant receptor M71 is responsive to the odorant acetophenone, whereas neurons expressing MOR23 are activated by lyral. Homozygous mice modified at the M71 or MOR23 loci to also express GFP allowed for a determination of receptor abundance. These mice were subjected to an aversive olfactory conditioning paradigm in which acetophenone, lyral, or propanol, co-terminating with 0.75mA

foot shock, were presented 5 times daily for 3 consecutive days (Fig. 1B,C.). The unpaired control group also received odor presentations but experienced a 60-second delay prior to foot shock (Fig. 1C.). Only mice in which odorant and shock were paired exhibited conditioned aversive behavior (Fig. S1B,C,D. Tukey's multiple comparisons. F0 acetophenone unpaired vs. paired P=<0.0001. n=10,15. F0 lyral unpaired vs. paired P=<0.000. n=17,20. F0 propanol unpaired vs. paired P<0.0001. n=16,20.). Mouse nasal turbinates were surgically extracted 21 days after the initiation of training and subjected to iDISCO+ tissue clearing to visualize M71- and MOR23-expressing OSNs in transparent intact olfactory epithelia (Fig. 1B,D,E.) (*21*). We then imaged the cleared epithelia using light sheet microscopy and counted the number of M71 or MOR23 OSNs in a fixed volume of tissue using automated spot detection software (Fig. 1G,I.).

Importantly, both M71 and MOR23 OSNs are expressed in the same zone of the epithelium, enabling consistent imaging and counting protocols for both OSN populations. Male and female M71-IRES-tauGFP$^{+/+}$ (M71GFP) mice paired with acetophenone exhibited a 33% increase in the number of M71 OSNs 21 days after the initiation of aversive conditioning when compared to unpaired controls (Fig. 1H. One-way ANOVA. P<0.0001. Tukey's multiple comparisons. Naïve vs. F0 paired P<0.0001. F0 unpaired vs. F0 paired P<0.0001. n=12,11,12.). Male and female MOR23-IRES-tauGFP$^{+/+}$ (MOR23GFP) mice conditioned with lyral exhibited a 39% increase in MOR23 OSNs when compared to unpaired controls (Fig. 1J. One-way ANOVA. P<0.0001. Tukey's multiple comparisons. Naïve vs. F0 paired P=<0.001. F0 unpaired vs. F0 paired P<0.0001. n=7,9,9.). We performed a series of control experiments to demonstrate that fear conditioning does not lead to a global increase in the number of OSNs per cubic volume in the sensory epithelium. The odorant propanol does not activate M71 sensory neurons (*1*, *22*). When propanol was employed as the conditioned odor in both the paired and unpaired training paradigms, we observed no difference in the number of M71 OSNs between the two groups of mice (Fig. 1F. Student's unpaired t-test. Unpaired vs. paired P=0.3009. n=6,7.). These results indicate that olfactory fear conditioning results in a specific increase in the number of cells responsive to the conditioned odor and does not lead to a global increase in all OSNs.

Conditioned-odor-responsive cell increase is sustained through at least 9 weeks of cell turnover

We observe an increase in M71 OSNs 21 days after aversive conditioning with acetophenone. We next asked if this increase persists at later time points (Fig. 2A.). At 42 days, we observe a persistent 20% increase (Fig. 2B. Tukey's multiple comparisons. 42d unpaired vs. paired P=0.0476. n=8,8.) in the number of M71 OSNs in paired versus unpaired animals and a 30% increase at 63 days (Fig. 2B. Tukey's multiple comparisons. 63d unpaired vs. paired P=0.0011. n=4,6.). Since the half-life ($t_{1/2}$) of the mouse olfactory sensory epithelium (the amount of time required for half of the epithelium to regenerate) is approximately 26 days (*23*), then at 42 days, approximately 67% will have been replaced by newly born neurons, and at 63 days, approximately 81% will have been replaced. The observation that these changes persist at least 63 days after aversive conditioning, together with the reported 26-day half-life for the main olfactory epithelium, suggests that a signaling mechanism must persist despite the fact that the entire sensory epithelium present at the time of conditioning will eventually be regenerated.

Fear conditioning-induced increases in conditioned-odor-responsive cells is heritable (F1)
We next asked whether the increase in the number of specific OSNs observed following conditioning is inherited by naïve offspring. Ten days after the initiation of aversive training, we bred F0 males from both the paired and unpaired groups with naïve M71GFP$^{+/+}$ or MOR23GFP$^{+/+}$ female mice. Each mating pair was separated ten days after co-housing to ensure that the offspring were never exposed to the conditioned father. Main olfactory epithelia were then collected from the offspring of these mating pairs at 8 weeks of age. The F1 mice were never exposed to acetophenone or lyral, nor had they undergone aversive conditioning. We nonetheless observed a 36% increase in M71 OSNs in both male and female offspring whose fathers experienced paired aversive conditioning with acetophenone when compared with the F1 of fathers that experienced the unpaired training paradigm (Fig. 1H. Tukey's multiple comparisons. F1 unpaired vs. F1 paired P<0.0001. n=12,14.). A similar relative increase of 27% was observed in MOR23 OSNs in offspring of fathers that experienced paired aversive conditioning with lyral compared to F1 of unpaired fathers (Fig. 1J. Tukey's multiple comparisons. F1 unpaired vs. F1 paired P=0.0368. n=6,6.). These results demonstrate the intergenerational epigenetic inheritance of an olfactory phenotype, namely an increase in specific OSNs in naïve F1 offspring following aversive conditioning in F0.

Previous behavioral studies demonstrated that offspring from fathers that experienced aversive olfactory conditioning exhibit enhanced sensitivity to the conditioned odor in both odor potentiated startle and aversive odor association assays (*2*). Therefore, we asked whether we could detect an aversive behavioral response to either acetophenone or lyral in the F1 population after aversive training in F0 fathers. In initial experiments, we performed aversive conditioning with either acetophenone or lyral in F0 males and females. Five days after the initiation of training, we placed mice in a 3-chamber arena with the conditioned odor on one side and a control odor (propanol) on the other. F0 mice in the paired group actively avoided the conditioned odor, whereas mice in the unpaired group exhibited no aversion to the conditioned odors (Fig. S1B,C. Tukey's multiple comparisons. F0 acetophenone unpaired vs. paired P=0.0008. n=10,15. F0 lyral unpaired vs. paired P=0.0057. n=10,15.). Control mice spent roughly equal time exploring the propanol and conditioned odor chambers, whereas the paired mice spent approximately 67% (lyral paired) to 75% (acetophenone paired) of the time exploring the propanol chamber. Importantly, the offspring of F0 fathers that experienced aversive training with acetophenone exhibited no apparent avoidance of the conditioned odor. F1 mice spent equal time in both chambers (Fig. S1B. Tukey's multiple comparisons. F1 acetophenone unpaired vs. paired P=0.9551. n=4,6.). We hypothesize that a higher number of conditioned odor-responsive OSNs in F1, despite no avoidance to the F0 paired odor, may position animals to learn avoidance behaviors in fewer trials or at lower odor concentrations. We note an unexplained result with the offspring of male mice conditioned with propanol. Propanol was behaviorally neutral in the F1 offspring from fathers that experienced paired aversive training with propanol (Fig. S1D. Tukey's multiple comparisons. Naïve vs. F1 paired P=0.6624. n=25,12.). However, offspring from fathers that had undergone unpaired training exhibited an attraction to propanol (Fig. S1D. Tukey's multiple comparisons. Naïve vs. F1 unpaired P=0.0001. n=25,5.). We note our avoidance paradigm may not be as sensitive to modest behavioral responses. Taken together, these results demonstrate that aversive odorant conditioning in the F0 population elicits active avoidance, but suggest that this behavioral phenotype is not transmitted to F1 progeny.

Olfactory fear conditioning biases olfactory receptor choice toward conditioned-odor-responsive cell-specific identities

The olfactory epithelium undergoes neurogenesis for the life of the organism. This continual renewal of OSNs suggests a possible mechanism for the observed increase in specific neuron populations responsive to conditioned odors. The increase in M71 and MOR23 cells following aversive training could result from a biased increase in either the birth or survival of specific OSNs. In initial experiments, we examined the relative number of M71 and MOR23 OSNs born during and after aversive training. We injected mice at the onset of training with 5-Ethynyl-2′-deoxyuridine (EdU), a thymidine analog that incorporates into newly synthesized DNA and labels newborn cells. Animals were injected during each of the 3 days of training and for 2 subsequent days (Fig. 3A.). Epithelia were examined to determine the number of EdU-labeled M71 and MOR23 OSNs 21 days after the initiation of paired and unpaired aversive training (Fig. 3A.). Since EdU has a half-life of approximately 35 minutes (*24*), analysis of EdU 16 days after the cessation of EdU exposure reflects a pulse-chase, allowing us to quantify a subset of the neurons born during the 5 days following the initiation of aversive conditioning (Fig. 3B.).

The number of newborn M71 cells (EdU-labeled) out of total M71 cells is 1.24 ± 0.29% in naïve mice, 2.91 ± 0.56% after the unpaired paradigm, and 7.61 ± 0.53% following paired aversive training with acetophenone (Fig. 3D. One-way ANOVA. $P<0.0001$. Tukey's multiple comparisons. Naïve vs. paired $P<0.0001$. Unpaired vs. paired $P<0.0001$. n=6,6,6.). When lyral is used as the conditioned odor, the number of newborn MOR23 cells out of total MOR23 cells is 0.29 ± 0.06% in naïve mice, 0.55 ± 0.09% after the unpaired paradigm, and 1.11 ± 0.21% following paired aversive training (Fig. 3E. One-way ANOVA. $P=0.0120$. Tukey's multiple comparisons. Naïve vs. paired $P=0.0154$. Unpaired vs. paired $P=0.0653$. n=4,6,8.). These observations suggest that aversive learning results in a significant increase in the number of newborn M71 and MOR23 cells when comparing the paired and unpaired paradigms. The observation that the percentage of newborn M71 cells is 4-5 times that of MOR23 may simply reflect differences in the birth rates of the two cell populations.

We scored the number of EdU-labeled M71 and MOR23 OSNs 16 days after the cessation of EdU exposure. Since the differentiation of a newborn cell to a mature olfactory neuron requires about 7-10 days (*20*, *25*), these data strongly suggest that aversive training results in a specific increase in the birth of new cells responsive to the conditioned odor. Experiments suggest that an enhanced rate of survival is not responsible for the observed increase in specific OSNs. If EdU is administered prior to the onset of training, an increase in the number of EdU+ cells would reflect enhanced survival rather than increased birth. However, daily exposure to EdU for 5 days 12 days prior to conditioning does not reveal a relative increase in the frequency of EdU+ M71 or MOR23 cells when comparing the paired and unpaired paradigms (data not shown). Taken together, these data strongly suggest that the specific increase in cells responsive to the conditioned odor is a consequence of a relative increase in the birth of new cells expressing the M71 and MOR23 receptors.

Discussion

We used tissue-clearing techniques and light-sheet microscopy to demonstrate an increase in olfactory sensory neurons (OSNs) expressing the receptor for an aversively conditioned odor. Moreover, enhanced cell numbers to the conditioned odor were observed in naïve offspring.

These data are in accord with findings that employ other cellular visualization techniques (*2–4*). In F0, this increase is stable for 63 days, a time by which the vast majority of the cells present during aversive training have been replaced by newborn sensory neurons. The increase in F0 results, in part, from the contribution of newborn neurons responsive to the conditioned odor, demonstrating a biasing of olfactory receptors during OSN development. The sustained increase in F0, along with the inheritance in F1, suggests that there is a stable signal that is responsible for the induction, maintenance, and inheritance of the increase in OSNs responsive to the paired odor.

The stochastic choice of olfactory receptors may provide an opportunity to alter the representation of receptors in order to allow an organism to adapt to the environment. Changes in the number of OSNs may lead to an increase in sensitivity of the paired odor. A change in OSN number may also lead to increased inputs to downstream sensory areas. Such perceptual changes have been reported in the motor, visual, olfactory, and auditory systems, where topographical arrangements at the primary sensory cortex are modulated in certain fear conditioning paradigms in mammals (*26–31*), although this has not yet been demonstrated intergenerationally. We observed an increase in conditioned odor-responsive neurons in both the F0 and F1 populations. Only the F0, however, exhibited avoidance behavior. We speculate that the increase in neurons responsive to the conditioned odor could enhance the sensitivity to, or the discrimination of, the paired odor in F0 and F1. This would enable the F1 population to learn that odor predicts shock with fewer training cycles or less odorant when trained with the conditioned odor. An area of exploration is how a long latency between training and testing (e.g., 42 or 63 days, timepoints at which we observe a sustained increase in cell number) affects avoidance behavior. These studies will lend insight to the behavioral differences observed in F1. These findings set a foundation to uncover the mechanism by which olfactory receptor bias is communicated within the main olfactory epithelium, to the germline, and, moreover, maintained during the development of offspring. What remains to be uncovered are the mechanisms to bias the choice of specific receptors in the main olfactory epithelium and how the information governing the biasing of receptor choice is transferred to the gametes.

In mice, the paternal transmission of epigenetic information has been observed following metabolic disturbances, social stress, and exposure to drugs and toxins (*32*). High-fat or low-protein diets, as well as caloric restriction in the father, results in metabolic disturbances in the offspring, even after *in vitro* fertilization (*33*, *34*). Parental stressors, such as chronic defeat or maternal separation, result in hormonal disturbances and behavioral phenotypes in the offspring (*35–37*). Finally, toxins and addictive drugs result in an array of metabolic disturbances in the F1 population that recapitulate the paternal state (*38*). These paternal stressors are associated with metabolic and hormonal disturbances that can readily act at a distance to affect the gamete. It has been demonstrated in male gametogenesis that extracellular vesicles in the testes transmit an RNA payload as they fuse with maturing sperm (*39–42*). Such studies provide insights into a mechanism by which an olfactory sensory experience paired with fear learning could transmit receptor-specific information from one generation to the next.

Our study elaborates on a function of sensory systems in which a learned adaptation can influence future generations. Thus, the distinction between innate and learned behaviors may be fundamentally flexible — learned adaptations in the parent may have the potential to become

innate in their offspring. Understanding the mechanisms of inherited adaptation will provide insight for interventions when these changes no longer serve as adaptive to the organism.

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Author contributions:

Conceptualization: CWL, YRA, BJM

Methodology: CWL, YRA, ECBJ, HSL, BJM

Investigation: CWL, YRA, ECBJ, HSL, AK, BJM

Visualization: CWL, YRA

Funding acquisition: BJM

Project administration: CWL, BJM

Supervision: BJM

Writing – original draft: CWL, YRA, BJM

Writing – review & editing: CWL, AVA, BJM

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Supplementary Materials

Materials and Methods

Figs. S1 to S7

Movies S1 to S8

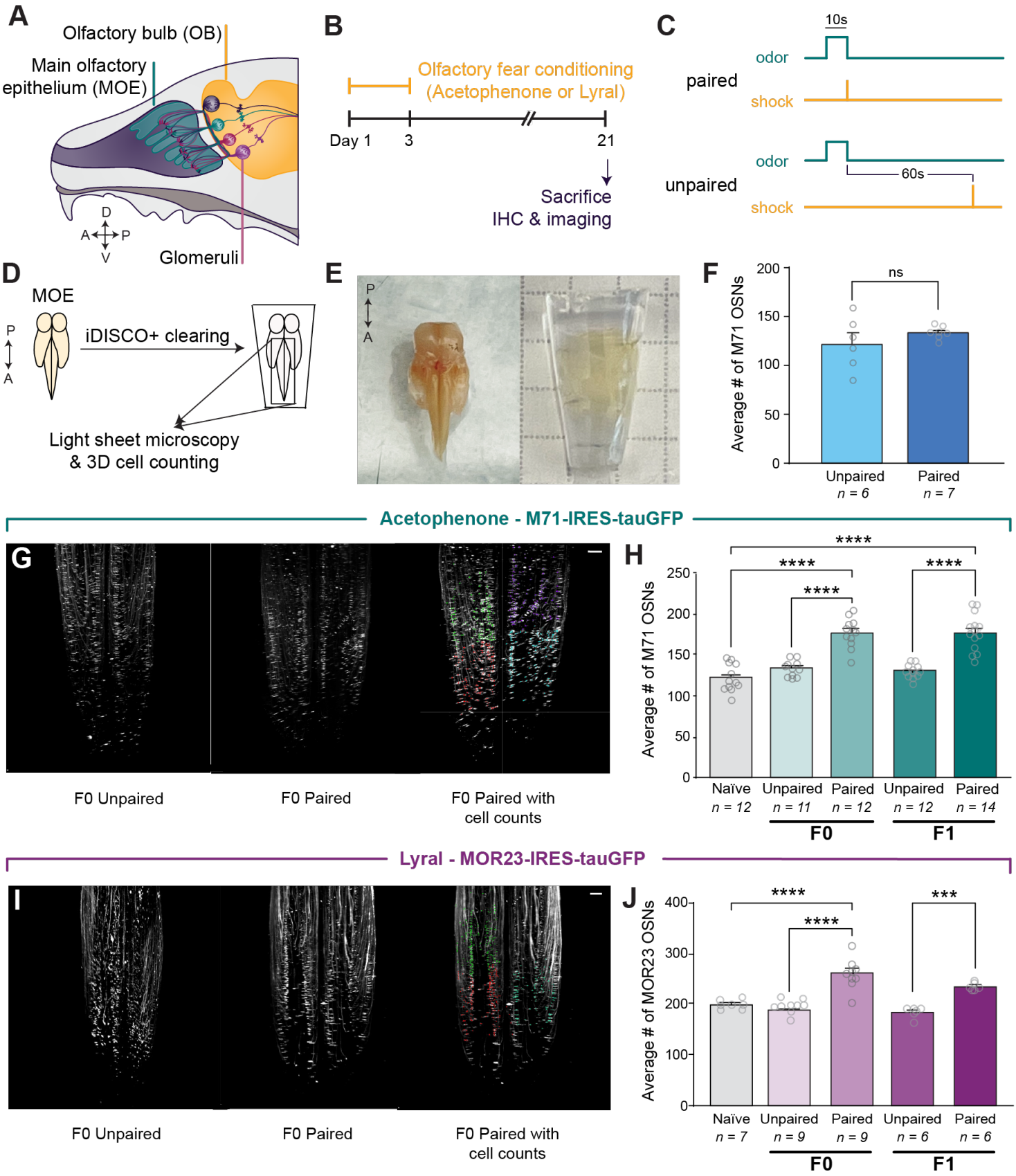


Fig. 1. Olfactory fear conditioning leads to an increase in conditioned-odor-responsive cells in parents (F0) that is heritable (F1). (A) Schematic representation of the mouse main olfactory

epithelium and olfactory bulb. MOE: main olfactory epithelium. OB: olfactory bulb. **(B)** Timeline of olfactory fear conditioning and MOE collection. **(C)** Experimental paradigms for olfactory fear conditioning groups. Mice in the paired condition received a foot shock that co-terminated with odor presentation, while mice in the unpaired condition received a foot shock 60 seconds after odor presentation. **(D)** Schematic demonstrating the process by which cells of interest in the MOE were quantified. Epithelia from both M71-IRES-tauGFP$^{+/+}$ and MOR23-IRES-tauGFP$^{+/+}$ adult mice were cleared using the iDISCO+ tissue-clearing protocol. Samples were imaged on a light sheet microscope and analyzed using Imaris spot detection software. **(E)** Images of the MOE before (left) and after (right) optical tissue clearing. **(F)** The average number of M71 olfactory sensory neurons in a 350^3 μm^3 cube of the epithelium in the propanol unpaired (light blue) and propanol paired (dark blue) conditions (Student's unpaired t-test. Unpaired vs. paired P=0.3009. n=6,7.). **(G)** Example images of M71 OSNs in zone 1 of cleared MOE from both the unpaired (left) and paired (middle) conditions. Example image of an MOE with the counted cells represented by colored dots (right). Each set of colors represents a distinct counting cube. Scale bar: 200µm. **(H)** Graph showing the differences between the average number of M71 OSNs in a 350^3 μm^3 cube of epithelium of naive (gray), acetophenone unpaired (lighter green), and acetophenone paired (darker green) conditions in F0 and F1 (One-way ANOVA. P<0.0001. Tukey's multiple comparisons. Naïve vs. F0 paired P<0.0001. F0 unpaired vs. paired P<0.0001. Naïve vs. F1 paired P<0.0001. F1 unpaired vs. paired P<0.0001. n=12,11,12,12,14.). **(I)** Example images of MOR23 OSNs in zone 1 of cleared MOE from both the unpaired (left) and paired (middle) conditions. Example image of an MOE with the counted cells represented by colored dots (right). Scale bar: 200µm. **(J)** Graph showing the differences between the average number of MOR23 OSNs in a 350^3 μm^3 cube of epithelium in naive (gray), lyral unpaired (lighter purple), and lyral paired (darker purple) conditions in F0 and F1 (One-way ANOVA. P<0.0001. Tukey's multiple comparisons. Naïve vs. F0 paired P=<0.0001. F0 unpaired vs. paired P<0.0001. F1 unpaired vs. paired P=0.0368. n=7,9,9,6,6.).

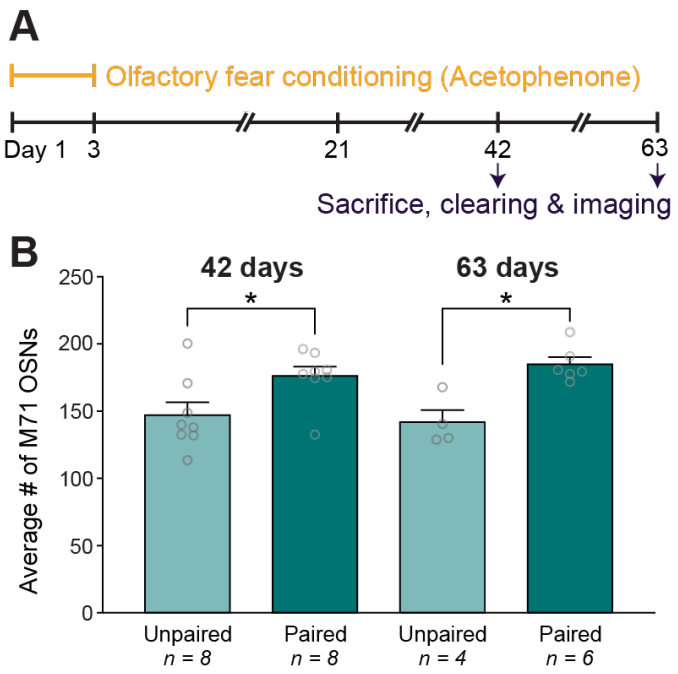


Fig. 2. Conditioned-odor-responsive cell increase is sustained through at least 9 weeks of cell turnover. (A) Timeline of olfactory fear conditioning and extended MOE collection time points. **(B)** The average number of M71 OSNs in a 350^3 μm^3 cube of epithelium of unpaired (light green) and paired (dark green) mice, 42- or 63-days post-conditioning (One-way ANOVA. P=0.0033. Tukey's multiple comparisons. 42d unpaired vs. paired P=0.0476. 63d unpaired vs. paired P=0.0011. n=8,8,4,6.).

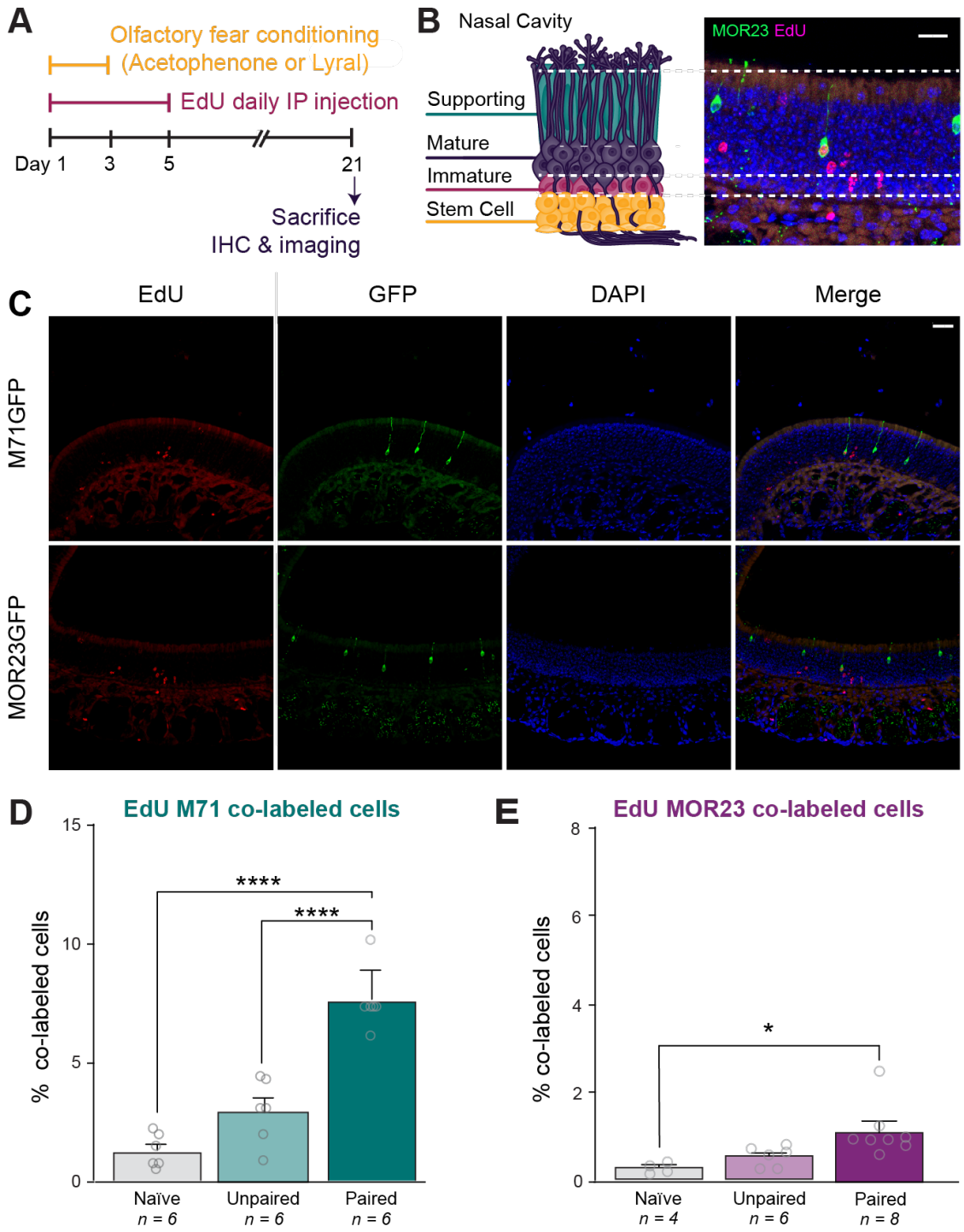
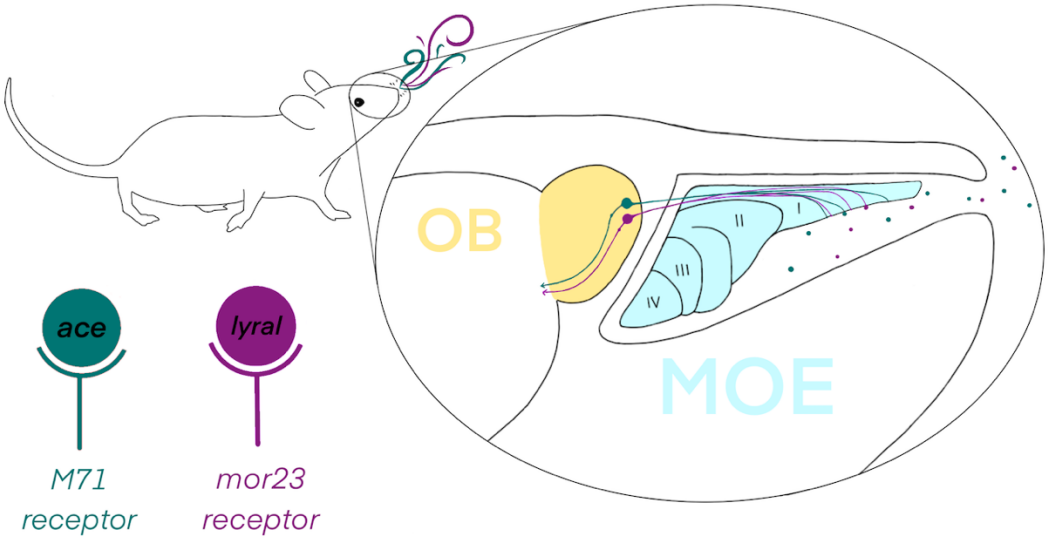


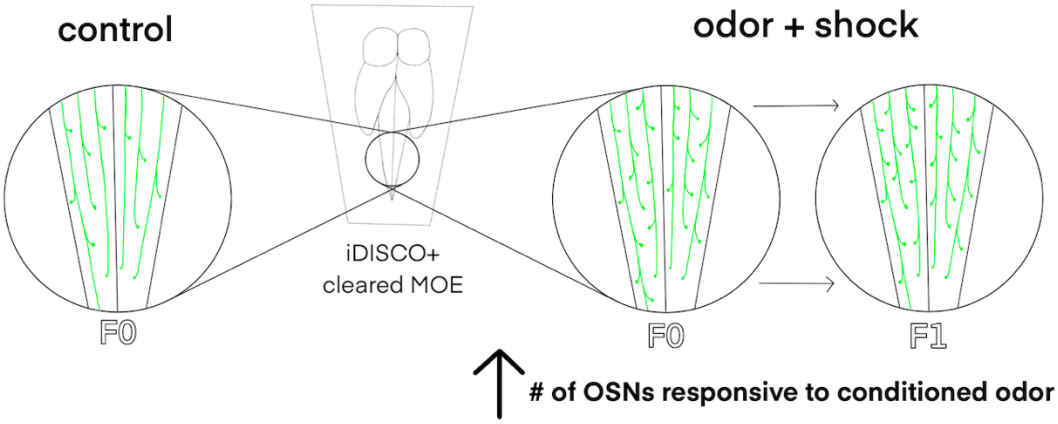
Fig. 3. Olfactory fear conditioning biases olfactory receptor choice toward conditioned-odor-responsive cell-specific identities. (A) Timeline of olfactory fear conditioning, EdU injections, and MOE collection. **(B)** Schematic representation of the distinct layers of the MOE, showing the stem cell, immature OSN, and mature OSN populations (left). Representative image of the MOE from an MOR23GFP$^{+/+}$ mouse showing EdU-positive cells (red) and a newborn (EdU+) MOR23 OSN (green). Scale bar: 20 µm. **(C)** Representative images showing staining of EdU (red, first column), endogenous GFP (green, second column), DAPI (blue, third column), and the merged

channels (fourth column) in both M71GFP$^{+/+}$ and MOR23GFP$^{+/+}$ MOE. Scale bar: 40µm. **(D)** Percentage of EdU-positive M71 OSNs in naïve, unpaired, and paired groups (One-way ANOVA. P<0.0001. Tukey's multiple comparisons. Naïve vs. paired P<0.0001. Unpaired vs. paired P<0.0001. n=6,6,6.). **(E)** Percentage of EdU-positive MOR23 OSNs in naïve, unpaired, and paired
480 groups (One-way ANOVA. P=0.0120. Tukey's multiple comparisons. Naïve vs. paired P=0.0154. Unpaired vs. paired P=0.0653. n=4,6,8.).

Graphical Abstract

One Sentence Summary
Odor-shock pairing is inherited by naïve offspring and biases neurogenesis in the nose.





Highlights:

● Olfactory fear conditioning leads to an increase in conditioned-odor-responsive cells in parents (F0) that is heritable (F1)

● Increase in conditioned-odor-responsive cells is sustained through at least 9 weeks of cell turnover in the main olfactory epithelium

● Olfactory fear conditioning in F0 biases neurogenesis specifically toward conditioned-odor responsive cell fate