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Parental olfactory experience influences behavior and neural structure in subsequent generations

Brian G Dias[1,2] & Kerry J Ressler[1–3]

Using olfactory molecular specificity, we examined the inheritance of parental traumatic exposure, a phenomenon that has been frequently observed, but not understood. We subjected F0 mice to odor fear conditioning before conception and found that subsequently conceived F1 and F2 generations had an increased behavioral sensitivity to the F0-conditioned odor, but not to other odors. When an odor (acetophenone) that activates a known odorant receptor (*Olfr151*) was used to condition F0 mice, the behavioral sensitivity of the F1 and F2 generations to acetophenone was complemented by an enhanced neuroanatomical representation of the *Olfr151* pathway. Bisulfite sequencing of sperm DNA from conditioned F0 males and F1 naive offspring revealed CpG hypomethylation in the *Olfr151* gene. In addition, *in vitro* fertilization, F2 inheritance and cross-fostering revealed that these transgenerational effects are inherited via parental gametes. Our findings provide a framework for addressing how environmental information may be inherited transgenerationally at behavioral, neuroanatomical and epigenetic levels.

Responding to environmental stimuli is crucial to the survival of organisms and often manifests as alterations in the structure and function of the nervous system. When and how information from the environment results in experience-dependent alteration of nervous system structure and function are fundamental questions in behavioral neuroscience.

An important, but often ignored, factor that influences adult nervous systems is exposure of parents to salient environmental stimuli before the conception of their offspring. Such information transfer would be an efficient way for parents to 'inform' their offspring about the importance of specific environmental features that they are likely to encounter in their future environments. However, this would necessitate the transgenerational inheritance of environmental information via the germ line by offspring not even conceived at the time. Although our understanding of such non-Mendelian modes of inheritance is continually being revised in terms of the epigenetic inheritance of traits[1], empirical data to support transgenerational epigenetic inheritance of behavioral traits in mammals are beginning to accumulate at the level of morphological, behavioral and metabolic traits[2–15].

We used olfactory fear conditioning to address when and how the olfactory experience of a parent might influence their offspring. Specifically, we focused on the olfactory system, given its well-understood molecular biology and neuroanatomy[16–18], the ability to differentially target odorant-receptor pairs in the same modality for differential and well-controlled behavioral studies, and previous findings that experience-dependent alterations occur in olfactory neuroanatomy and behavior following olfactory conditioning[19].

We examined how specific features of the parental sensory environment before conception can influence sensory nervous system structure and function in a cue-specific manner in subsequently conceived F1 and F2 generations. Bisulfite sequencing of olfactory receptor genes in the sperm of the F0 and F1 generations revealed differences in methylation that may mark the specific olfactory receptor gene for enhanced transcription in the subsequent generation. Finally, using *in vitro* fertilization (IVF), F2 and cross-fostering studies, we found that the behavior and structural alterations were inherited and were not socially transmitted from the F0 generation.

RESULTS

Olfactory fear conditioning to study descendant generations

We examined whether olfactory fear conditioning of the F0 generation leads subsequently conceived adult F1, F2 and IVF-derived generations to exhibit F0-like behavioral sensitivity toward the F0 conditioned odor, and whether there were neuroanatomical changes at the level of the main olfactory epithelium (MOE) and olfactory bulb in these generations (**Supplementary Fig. 1**). The odors that we used were chosen on the basis of prior work demonstrating that the M71 odorant receptors (encoded by the *Olfr151* gene) expressed by olfactory sensory neurons (OSNs) in the MOE are activated by acetophenone[20]. The use of a chemical mixture that contained compounds very similar to propanol did not elicit any responses from M71 cells, suggesting that propanol does not activate M71 receptors. In addition, glomerular activity patterns elicited by acetophenone or propanol (http://gara.bio.uci.edu) are different and non-overlapping, suggesting that a different population of OSNs primarily responds to each odor.

In this procedure, 2-month-old sexually inexperienced and odor naive C57Bl/6J male mice or homozygous M71-LacZ transgenic male mice were left in their home cage (F0-Home) or conditioned with acetophenone (F0-Ace) or propanol (F0-Prop). Subsequently conceived adult male offspring (F1) belonged to three groups: F1-Home, F1-Ace and F1-Prop (Online Methods). It is important to note that no F0 males were excluded from the study after training and that

[1]Department of Psychiatry and Behavioral Sciences, Emory University School of Medicine, Atlanta, Georgia, USA. [2]Yerkes National Primate Research Center, Atlanta, Georgia, USA. [3]Howard Hughes Medical Institute, Chevy Chase, Maryland, USA. Correspondence should be addressed to B.G.D. (bdias@emory.edu) or K.J.R. (kressle@emory.edu).

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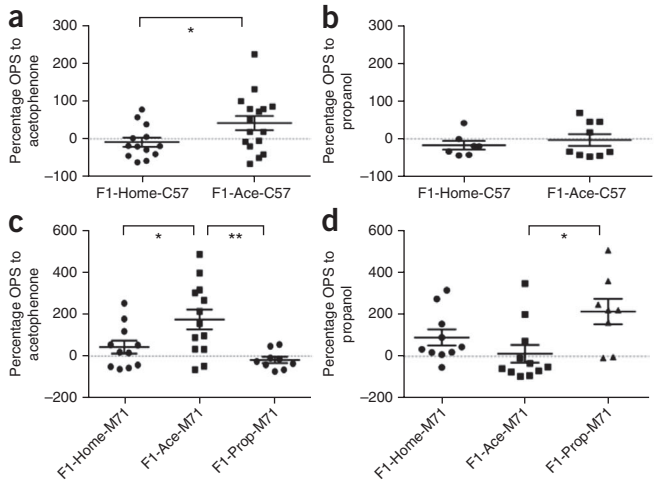

Figure 1 Behavioral sensitivity to odor is specific to the paternally conditioned odor. (**a**,**b**) Responses of individual C57Bl/6J F1 male offspring conceived after the F0 male was fear conditioned with acetophenone. F1-Ace-C57 mice had an enhanced sensitivity to acetophenone (**a**), but not to propanol (control odor, **b**) compared with F1-Home-C57 mice (F1-Ace-C57, $n = 16$; F1-Home-C57, $n = 13$; t test, $P = 0.043$, $t_{27} = 2.123$). (**c**,**d**) Responses of M71-LacZ F1 male offspring conceived after the F0 male was fear conditioned with acetophenone or propanol. F1-Ace-M71 mice had an enhanced sensitivity to acetophenone (**c**), but not to propanol (**d**), compared with F1-Home-M71, and F1-Prop-M71 mice. In contrast, F1-Prop-M71 mice had an enhanced sensitivity to propanol (**d**), but not acetophenone (**c**) (F1-Home-M71, $n = 11$; F1-Ace-M71, $n = 13$; F1-Prop-M71, $n = 9$; OPS to acetophenone: ANOVA, $P = 0.003$, $F_{2,30} = 6.874$; F1-Home-M71 versus F1-Ace-M71, $P < 0.05$; F1-Ace-M71 versus F1-Prop-M71, $P < 0.01$; OPS to propanol: ANOVA, $P = 0.020$, $F_{2,26} = 4.541$; F1-Ace-M71 versus F1-Prop-M71, $P < 0.05$). Data are presented as mean ± s.e.m. $^*P < 0.05$, $^{**}P < 0.01$.

all of them were mated with naive females. Thus, any findings that we obtained were not the results of extreme phenotype biasing or a previously existing genetic sensitivity. Both C57Bl/6J and M71-LacZ mice possess the M71 odorant receptor in their olfactory epithelium[21] and both can consequently detect acetophenone. The main difference between the strains is that the OSNs of the M71-LacZ mice produce β-galactosidase in M71-expressing neurons and can therefore be visualized[22]. This procedure allowed us to examine a seldom studied factor that might markedly influence the nervous systems of adults; namely, the experience of the F0 generation before conception.

Transgenerational olfactory sensitivity after F0 conditioning

Fear-potentiated startle (FPS) is a behavioral test to assay for fear learning[23]. FPS manifests as an augmented startle response in the presence of the aversive conditioned cue. In our case, to assay for behavioral sensitivity to an odor, we used a modified FPS protocol that consists of odor presentation before the startle stimuli. An odor-potentiated startle (OPS) score is computed, in which an enhanced OPS reflects a greater startle to the odor relative to control, when the odor is paired with the startle stimulus. Traditionally, FPS tests have been used to query the emotional state of the animal and the valence of the stimulus paired with the startle. It is important to note that we did not use this test as a measure of valence of the odor, but rather as a readout of the sensitivity toward that odor, similar to FPS tests that have been used to test the sensitivity of mice to natural odors such as fox urine[24]. Enhanced OPS to acetophenone in our experiment would be interpreted as an enhanced behavioral sensitivity to acetophenone, not necessarily an increase in fear to acetophenone. Making any statements about valence specificity and the emotional value of the odor would necessitate subjecting the F0 generation to an appetitive odor-conditioning task.

In the F0 generation, we previously reported that olfactory fear conditioning adult males to acetophenone increases FPS when the startle stimuli are paired with acetophenone presentation[19]. In the F1 generation, we found that C57Bl/6J F1-Ace mice (F1-Ace-C57) showed enhanced OPS (unconditioned) to acetophenone compared with C57Bl/6J F1-Home mice (F1-Home-C57) (**Fig. 1a**). No differences between groups were found when propanol was paired with the startle, indicating that the response was specific to acetophenone (**Fig. 1b**). Similarly, F1-Ace-M71 showed enhanced OPS to acetophenone, but not to propanol, compared with F1-Home-M71 and F1-Prop-M71 (**Fig. 1c**,**d**). In contrast, F1-Prop-M71 showed enhanced OPS to propanol, but not to acetophenone (**Fig. 1c**,**d**). These data suggest a double dissociation and specificity of the odor association, along with the inheritance of a behavioral sensitivity that is specific to the F0-conditioned odor.

To further corroborate the enhanced behavioral sensitivity to the F0-conditioned odor, we conducted an independent behavioral assay that directly probes behavioral sensitivity using an odor concentration curve and the association time of the mice with these concentrations. We found that F1-Ace males were able to detect acetophenone at lower concentrations than F1-Prop males, whereas F1-Prop males detected propanol at lower concentrations than F1-Ace males (**Fig. 2a**,**b**), further suggesting an enhanced detection sensitivity that is specific to the F0-conditioned odor. Although we make a case for both the OPS and association time assays testing for behavioral sensitivity, we used OPS in our subsequent experiments because of our ability to carefully calibrate odor presentation and removal, parameters that might influence habituation to odors and skew experimental results.

Most noteworthy for these data is the fact that the naive F1 mice had never been exposed to any of the odors with which they were tested. Taken together, these data indicate that the behavioral sensitivity to an odor in adult offspring is specific to the odor that the F0 male was conditioned to, as shown across two different odorants and two different strains of mice. Furthermore, given the fact that

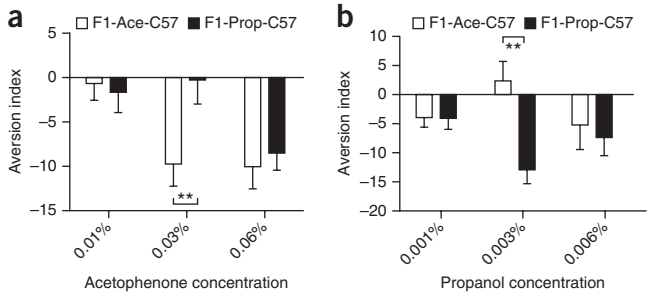

Figure 2 Sensitivity of F1 males toward F0-conditioned odor. Association time with either the concentration of odor on the x axis or an empty chamber was recorded. An aversion index was computed by subtracting the amount of time spent in the open chamber from the time spent in the odor chamber. (**a**) When tested with acetophenone, F1-Ace mice detected acetophenone at a lower concentration (0.03%) than F1-Prop mice, with both groups eventually showing equal aversion at the 0.06% concentration ($P = 0.005$ with Bonferroni correction for multiple comparisons). (**b**) When tested with propanol, F1-Prop mice detected propanol at a lower concentration (0.003%) than F1-Ace mice, with both groups eventually showing equal aversion at the 0.006% concentration ($P = 0.0005$ with Bonferroni correction for multiple comparisons) (F1-Ace-C57, $n = 16$; F1-Prop-C57, $n = 16$). Data are presented as mean ± s.e.m. ($^{**}P < 0.01$).

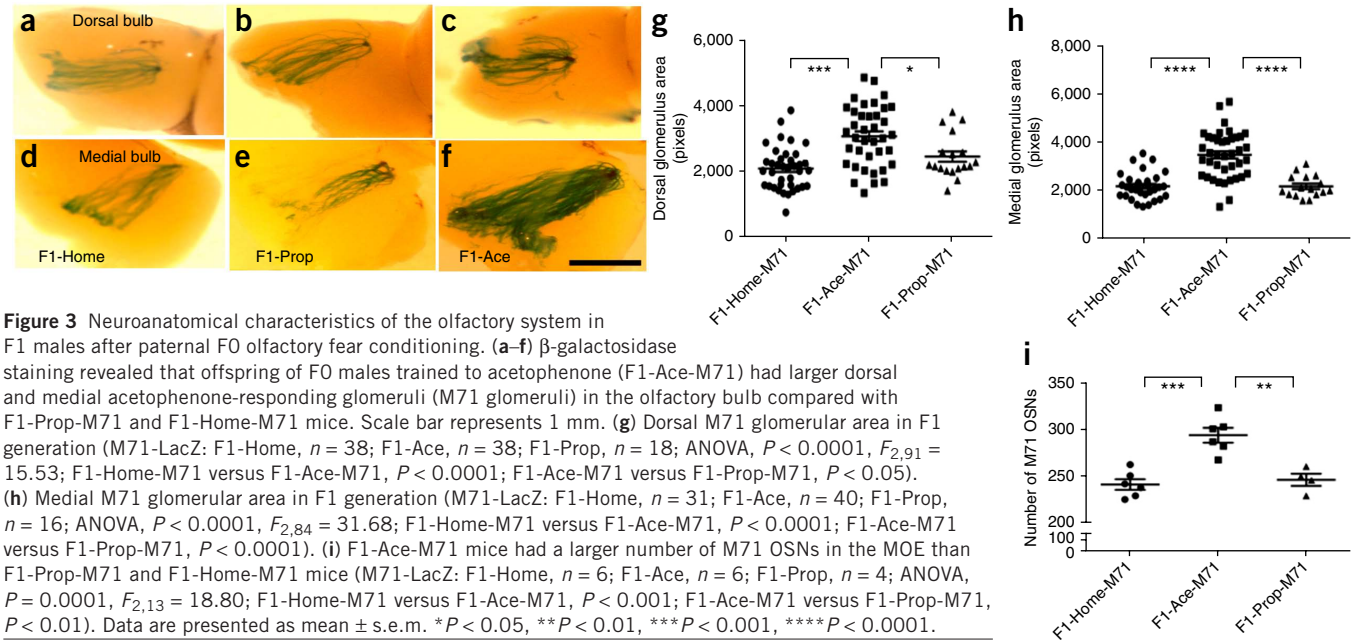

Figure 3 Neuroanatomical characteristics of the olfactory system in F1 males after paternal F0 olfactory fear conditioning. (**a**–**f**) β-galactosidase staining revealed that offspring of F0 males trained to acetophenone (F1-Ace-M71) had larger dorsal and medial acetophenone-responding glomeruli (M71 glomeruli) in the olfactory bulb compared with F1-Prop-M71 and F1-Home-M71 mice. Scale bar represents 1 mm. (**g**) Dorsal M71 glomerular area in F1 generation (M71-LacZ: F1-Home, $n = 38$; F1-Ace, $n = 38$; F1-Prop, $n = 18$; ANOVA, $P < 0.0001$, $F_{2,91} = 15.53$; F1-Home-M71 versus F1-Ace-M71, $P < 0.0001$; F1-Ace-M71 versus F1-Prop-M71, $P < 0.05$). (**h**) Medial M71 glomerular area in F1 generation (M71-LacZ: F1-Home, $n = 31$; F1-Ace, $n = 40$; F1-Prop, $n = 16$; ANOVA, $P < 0.0001$, $F_{2,84} = 31.68$; F1-Home-M71 versus F1-Ace-M71, $P < 0.0001$; F1-Ace-M71 versus F1-Prop-M71, $P < 0.0001$). (**i**) F1-Ace-M71 mice had a larger number of M71 OSNs in the MOE than F1-Prop-M71 and F1-Home-M71 mice (M71-LacZ: F1-Home, $n = 6$; F1-Ace, $n = 6$; F1-Prop, $n = 4$; ANOVA, $P = 0.0001$, $F_{2,13} = 18.80$; F1-Home-M71 versus F1-Ace-M71, $P < 0.001$; F1-Ace-M71 versus F1-Prop-M71, $P < 0.01$). Data are presented as mean ± s.e.m. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

both F0-Ace and F0-Prop mice received shocks during conditioning, these data suggest that these training-specific effects do not occur simply as a result of paternal history of the stress of shock exposure or conditioning to odors in general.

Studies that have examined the effect of parental stress after conception, either *in utero* or postnatally, have often found an anxiogenic phenotype in the offspring[25,26]. Using an elevated plus maze to assay for anxiety-like behavior, we found that prior, rather limited foot shock conditioning of the F0 generation, did not extend to generalized anxiety-like behavior in the F1 generation (**Supplementary Fig. 2a**,**b**).

To test the idea that olfactory fear conditioning of the F0 generation results in offspring that might be generally deficient in processing sensory cues and in learning and memory processes, we sought to examine whether auditory fear conditioning was affected in our experimental groups. Across all experimental groups, adult male F1 offspring subjected to auditory fear conditioning acquired, consolidated and extinguished fear similarly (**Supplementary Fig. 3a**–**c**).

F0 olfactory experience affects F1 neuroanatomy

Previously[19], we reported that the behavioral response (increased FPS to acetophenone) of F0-Ace conditioned males is complemented by an increase in the number of acetophenone–responsive M71-expressing OSNs in the MOE and M71 glomerular area in the olfactory bulbs. To examine whether alterations in the neuroanatomical representation of the conditioned odor accompanied the behavioral sensitivity reported above, we used standard β-galactosidase staining in naive M71-LacZ F1 males that had neither been behaviorally tested with, nor exposed to, any of the conditioned odors. We found that the dorsal and medial M71-specific glomeruli in the olfactory bulb of F1 offspring of acetophenone-trained F0 males (F1-Ace-M71) were significantly increased in size compared with those of the offspring of home cage or propanol-trained F0 males (F1-Home-M71 and F1-Prop-M71, respectively) (ANOVA, $P < 0.0001$ for dorsal and medial glomeruli; **Fig. 3a**–**h**). This increase in M71 glomerular area was accompanied by a significant increase in the numbers of M71 OSNs in the MOE (ANOVA, $P < 0.0001$; **Fig. 3i**).

These data suggest that the effect of paternal olfactory fear conditioning on neuroanatomy is associated with increased numbers of OSNs and increased glomerular area, both specific for the F0-conditioned odor. We posit that this increased structural representation in the main olfactory epithelium and olfactory bulb may underlie the specific enhanced olfactory sensitivity that we observed in the behavioral experiments (**Figs. 1** and **2**). We were concerned that performing behavior would make the offspring no longer odor naive, and thereby potentially confound the interpretation of the neuroanatomical results. Thus, all of the neuroanatomy data were generated using animal cohorts independent of any behavior data. Correlations between behavior and neuroanatomy within and between generations present an interesting and important future direction for research.

Inheritance of effects in the F2 and IVF-derived generations

Two mechanisms could explain how information about the F0-conditioned odor could be transferred to the subsequently conceived male offspring: inheritance via the gametes or transmission via a social route that is reminiscent of the transmission of maternal care in rodents[27]. To begin to dissociate these two possibilities, we conducted experiments with the F2 generation and with IVF-derived mice. Naive F1 males (F1-Ace, F1-Prop) were mated with naive females to generate F2 adults (F2-Ace, F2-Prop) whose F0 ancestors had been conditioned with either acetophenone or propanol. For the IVF experiment, sperm from F0 males was collected 10 d after the last conditioning day, and IVF was performed by the Transgenic Mouse Facility at Emory University in a location independent of our laboratory at Yerkes where we conducted all of the other studies reported. Subsequently conceived IVF offspring (F1-Ace-IVF and F1-Prop-IVF) were raised to adulthood and tissue was collected in this facility.

When tested in our behavioral assay, F2-Ace-C57 mice exposed to odors for the first time showed increased OPS to acetophenone compared with F2-Prop-C57 mice, whereas F2-Prop-C57 mice showed an increased OPS to propanol (**Fig. 4a**,**b**). This persistent behavioral sensitivity to the F0-conditioned odor was accompanied by corresponding increases in glomerular size in an independent set of F2 M71-LacZ mice that had no previous exposure to the odors used.

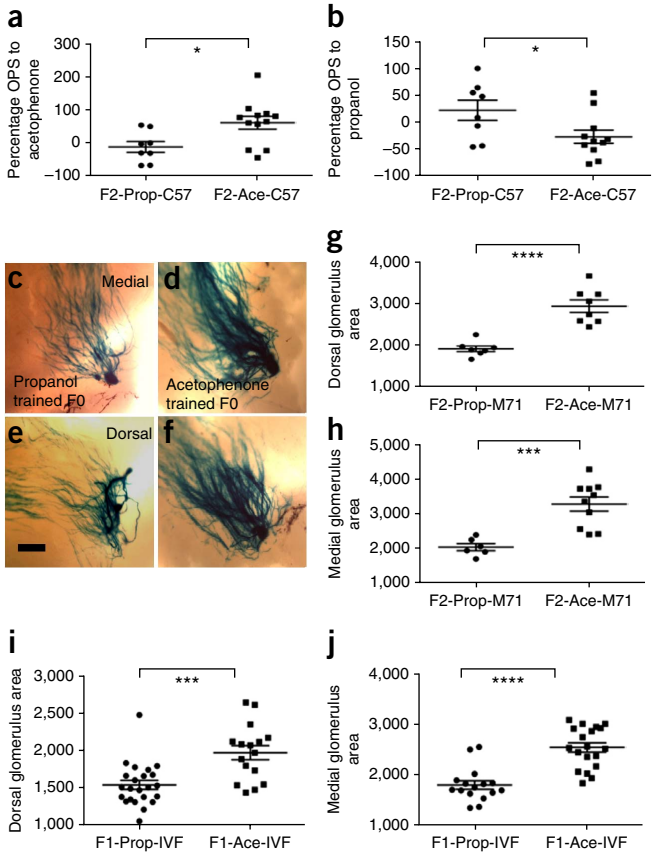

Figure 4 Behavioral sensitivity and neuroanatomical changes are inherited in F2 and IVF-derived generations. (**a**,**b**) Responses of F2-C57Bl/6J males revealed that F2-Ace-C57 mice had an enhanced sensitivity to acetophenone compared with F2-Prop-C57 mice (**a**). In contrast, F2-Prop-C57 mice had an enhanced sensitivity to propanol compared with F2-Ace-C57 mice (**b**; F2-Prop-C57, $n = 8$; F2-Ace-C57, $n = 12$; OPS to acetophenone: t test, $P = 0.0158$, $t_{18} = 2.664$; OPS to propanol: t test, $P = 0.0343$, $t_{17} = 2.302$). (**c**–**f**). F2-Ace-M71 mice whose F0 generation male had been conditioned to acetophenone had larger dorsal and medial M71 glomeruli in the olfactory bulb than F2-Prop-M71 mice whose F0 generation had been conditioned to propanol. Scale bar represents 200 µm. (**g**) Dorsal M71 glomerular area in F2 generation (M71-LacZ: F2-Prop, $n = 7$; F2-Ace, $n = 8$; t test, $P < 0.0001$, $t_{13} = 5.926$). (**h**) Medial M71 glomerular area in F2 generation (M71-LacZ: F2-Prop, $n = 6$; F2-Ace, $n = 10$; t test, $P = 0.0006$, $t_{14} = 4.44$). (**i**) Dorsal M71 glomerular area in IVF offspring (F1-Prop-IVF, $n = 23$; F1-Ace-IVF, $n = 16$; t test, $P < 0.001$, $t_{37} = 4.083$). (**j**) Medial M71 glomerular area in IVF offspring (F1-Prop-IVF, $n = 16$; F1-Ace-IVF, $n = 19$; t test, $P < 0.001$, $t_{33} = 5.880$). Data are presented as mean ± s.e.m.*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

The dorsal and medial M71-specific glomeruli in the olfactory bulbs of F2-Ace-M71 mice were significantly increased in size compared with those of F2-Prop-M71 mice (**Fig. 4c**–**h**). Similar results were obtained in our IVF study, using sperm from F0-Ace and F0-Prop males to generate offspring. We found that F1 offspring generated with sperm from acetophenone-trained F0 males (F1-Ace-IVF) had significantly larger dorsal and medial M71-specific glomeruli in the olfactory bulb, as compared with offspring generated with sperm from propanol-trained F0 males (F1-Prop-IVF) (t test, $P < 0.001$ for dorsal and medial glomeruli; **Fig. 4i**,**j**). We could not perform behavioral analyses on IVF-generated offspring because of animal quarantine issues. These data indicate that behavioral sensitivity and neuroanatomical alterations in the nervous system are specific to the F0-conditioned odor and persist until at least the F2 generation, as well as in the IVF-derived F1 generation, thereby pointing to an inheritance of these effects.

Cross-fostering supports inheritance of information

Our observations of the behavioral and structural changes specific to the F0-conditioned odor being retained in the F2 generation, and the persistence of the structural effects after IVF, argue against social transmission and make a strong case for transgenerational inheritance. Notably, our results are highly specific in the olfactory sensory modality toward the F0-conditioned odor, and both F0-Ace and F0-Prop males were subjected to the same shock training conditions that might be deemed stressful and potentially conveyed to the mother. This argues against the idea that our results might merely be the transmission of a stressful paternal experience to the mother during the time of co-habitation. To ensure that our experimental groups were balanced for both odor and shock exposure, many of our experiments utilized F0-Prop as the control group rather than F0-Home.

To further address this issue, and to address potential maternal transmission, we conducted a cross-fostering study. Sexually naive female mice were conditioned with acetophenone or left in their home cage. They were then mated with odor-naive males for 10 d, after which the male was removed. Subsequent offspring were then divided into the following groups: offspring of home cage mothers (F1-Home), offspring of acetophenone-conditioned mothers (F1-Ace), offspring of home cage mothers cross-fostered starting at postnatal day 1 by mothers conditioned to acetophenone (F1-Home(fostered)), and offspring of acetophenone conditioned mothers cross-fostered by home cage mothers (F1-Ace(fostered)) (**Supplementary Fig. 4**). Notably, the females were only exposed to the conditioning odor before mating, and never while pregnant, precluding the possibility that offspring were directly exposed to any odor-related fear and *in utero* learning. We conducted this cross-fostering study in females for two main purposes. First, we sought to examine whether these effects were specific to paternal conditioning or could also be inherited via the female germ line. Second, given the possibility that mating with the F0 conditioned male in some way altered maternal behavior toward subsequently born offspring, we wanted to account for any differences in maternal investment or information transfer about the conditioned odor that might result from our conditioning protocol.

We found that, similar to the situation in which the F0 male (father) was conditioned to acetophenone, F1-Ace mice in this maternally trained experiment had an enhanced OPS to acetophenone compared with F1-Home controls (**Fig. 5a**). If our behavioral findings were a result of a 'social transmission' mode of information transfer, we would have predicted a reversal of the above result. Instead, we found that the F1-Ace-C57(fostered) male offspring still had a higher OPS to acetophenone than F1-Home-C57(fostered) offspring (**Fig. 5b**), suggesting a biological, rather than social, mode of inheritance.

For the equivalent experiment to visualize neuroanatomy, we performed a similar cross-fostering experiment using M71-LacZ females, and used female mice conditioned to propanol as our control group (offspring labeled as F1-Prop). We found that the increased dorsal and medial glomerular area persisted in F1-Ace mice even after they were cross-fostered by mothers conditioned to propanol (F1-Ace-M71(fostered)). In contrast, F1-Prop mice cross-fostered by mothers conditioned to acetophenone (F1-Prop-M71(fostered)) did not show any increases in M71 glomerular area (**Fig. 5c**–**h**). In summary, these cross-fostering results, taken together with our IVF and F2 studies, strongly

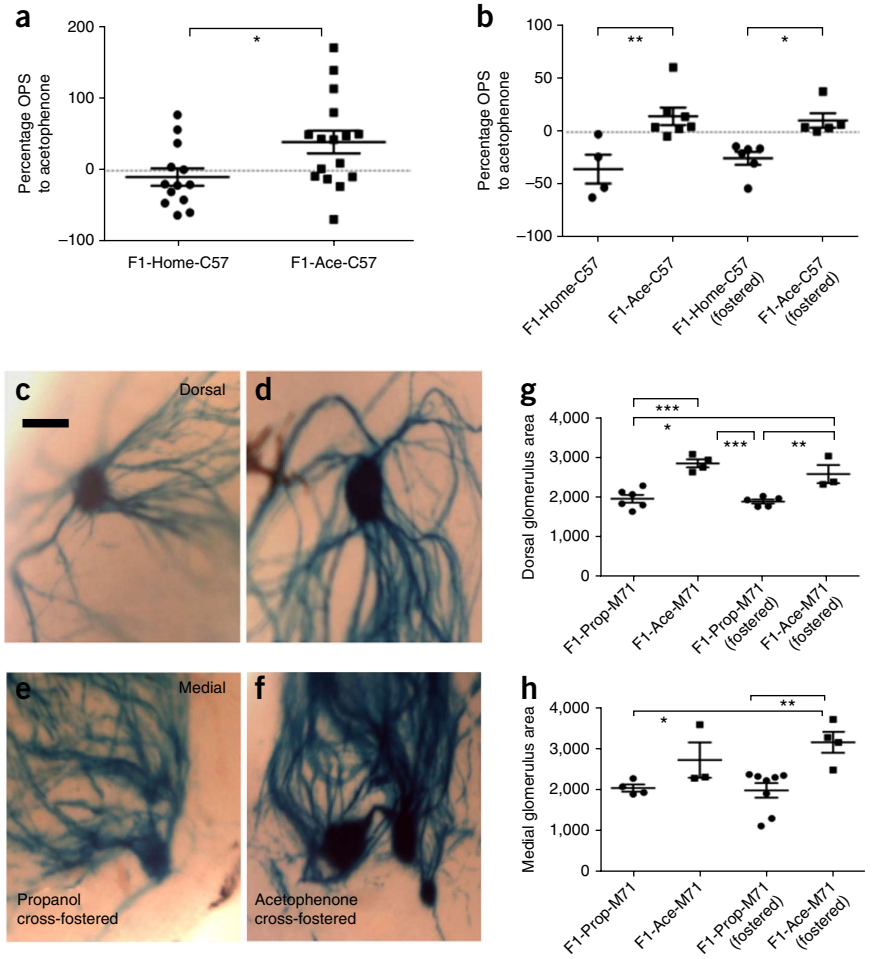

Figure 5 Behavioral sensitivity and neuroanatomical changes persist after cross-fostering. (**a**) F1 offspring of mothers that had been fear conditioned with acetophenone (F1-Ace-C57) showed enhanced sensitivity to acetophenone compared with F1-Home-C57 controls (F1-Home-C57, $n = 13$; F1-Ace-C57, $n = 16$; t test, $P = 0.0256$, $t_{27} = 2.362$). (**b**) Cross-fostering behavior. F1-Ace-C57 males had higher OPS to acetophenone than F1-Home-C57 males ($P < 0.01$). F1-Ace-C57(fostered) males still had higher OPS to acetophenone than F1-Home-C57(fostered) males ($P < 0.05$) (ANOVA, $P = 0.0011$, $F_{3,18} = 6.874$, planned *post hoc* comparisons). (**c**–**f**) Cross-fostering neuroanatomy. F1-Ace-M71 males cross-fostered by mothers conditioned to propanol (F1-Ace-M71(fostered)) continued to have larger M71 glomeruli than F1-Prop-M71 males cross-fostered by mothers conditioned to acetophenone (F1-Prop-M71(fostered)). Scale bar represents 100 µm. (**g**) Dorsal M71 glomerular area in F1 cross-fostered generation (M71-LacZ: F1-Prop, $n = 6$; F1-Ace, $n = 4$; F1-Prop(fostered), $n = 5$; F1-Ace(fostered), $n = 3$; ANOVA, $P < 0.0001$, $F_{3,14} = 17.52$; F1-Prop versus F1-Ace, $P < 0.001$; F1-Prop(fostered) versus F1-Ace(fostered), $P < 0.01$). (**h**) Medial M71 glomerular area in F1 cross-fostered generation (M71-LacZ: F1-Prop, $n = 4$; F1-Ace, $n = 3$; F1-Prop(fostered), $n = 8$; F1-Ace(fostered), $n = 4$; ANOVA, $P < 0.01$, $F_{3,15} = 5.933$; F1-Prop (fostered) versus F1-Ace(fostered), $P < 0.01$). Data are presented as mean ± s.e.m. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

suggest that our behavioral and structural data are a consequence of biological inheritance.

Altered epigenetic signature at Olfr151 (M71) locus in sperm

Given that our data suggests a biological inheritance of our behavioral and structural effects via parental gametes, we sought to examine sperm of the F0 generation males for epigenetic clues that might explain an enhanced representation for the M71 receptor (**Fig. 6**). CpG methylation is one mechanism by which a particular genetic locus can be marked for altered transcription, with less CpG di-nucleotide methylation typically being associated with more transcription. Bisulfite sequencing around the *Olfr151* (M71) locus and the non-acetophenone–responsive *Olfr6* locus (**Supplementary Fig. 5**) was conducted by Active Motif on DNA extracted from sperm of F0-Prop and F0-Ace mice. *Olfr6* converges at a glomerular space that is distinct from glomerular activity patterns elicited by acetophenone or propanol (http://gara.bio.uci.edu) and we therefore used it as a control odorant receptor for bisulfite sequencing studies. We found that the *Olfr151* ($P = 0.0323$; **Fig. 6a**), but not *Olfr6* ($P = 0.54$; **Fig. 6c**), locus was significantly less methylated in sperm from F0-Ace males compared with F0-Prop males. In addition, after correcting for multiple comparisons, one particular CpG di-nucleotide at the 3′ end of *Olfr151* was significantly less methylated in F0-Ace males than in F0-Prop males ($P = 0.003$; **Fig. 6b**,**d**).

These findings led us to hypothesize that relative hypomethylation of *Olfr151* in F0 sperm may lead to inheritance of the hypomethylated *Olfr151* in F1 MOE and F1 sperm, creating an inheritance cascade. A related idea would be that, during the stochastic odorant receptor choice process in the MOE[18,28], *Olfr151* (M71) would be more likely to be expressed in the next generation as a consequence of the epigenetic signature around that locus in the sperm. When bisulfite-converted DNA from sperm of the F1 generation was sequenced, we found that, similar to the F0 scenario, the *Olfr151* locus was hypomethylated in F1-Ace sperm compared with F1-Prop controls (**Fig. 6e**). In addition, after correcting for multiple comparisons, two particular CpG di-nucleotides in *Olfr151* were significantly less methylated in F1-Ace sperm compared with F1-Prop sperm ($P = 0.002$; **Fig. 6f**). These data suggest that inheritance of an epigenetic signature around a salient genetic locus accompanies our transgenerational effects. At the level of the MOE, we did not find any differences in the methylation at the *Olfr151* locus of either the F1 or F2 generations (**Supplementary Fig. 6**). This is perhaps unsurprising given that other modes of epigenetic modifications have been implicated in the marking of olfactory receptor loci in the MOE[29], and mandates future investigation. For example, DNA methylation and histone modifications are known to be dependent on each other[30], and changes in the methylation pattern in *Olfr151* in sperm DNA that we observe may potentially result in histone modifications around *Olfr151* in MOE DNA.

Published data also support the idea of epigenetic marking in sperm by indicating that sperm-associated histones are retained with chromatin of the paternal genome at the one-cell embryo stage[31,32]. To investigate the possibility that histone modifications mark the *Olfr151* (M71) locus, we collected sperm from F0-Ace and F0-Prop males 10 d after the last day of conditioning and performed native-chromatin immunoprecipitation (N-ChIP) on the sperm chromatin. Briefly, chromatin was extracted from sperm and immunoprecipitated with antibodies that recognize histone modifications, after which

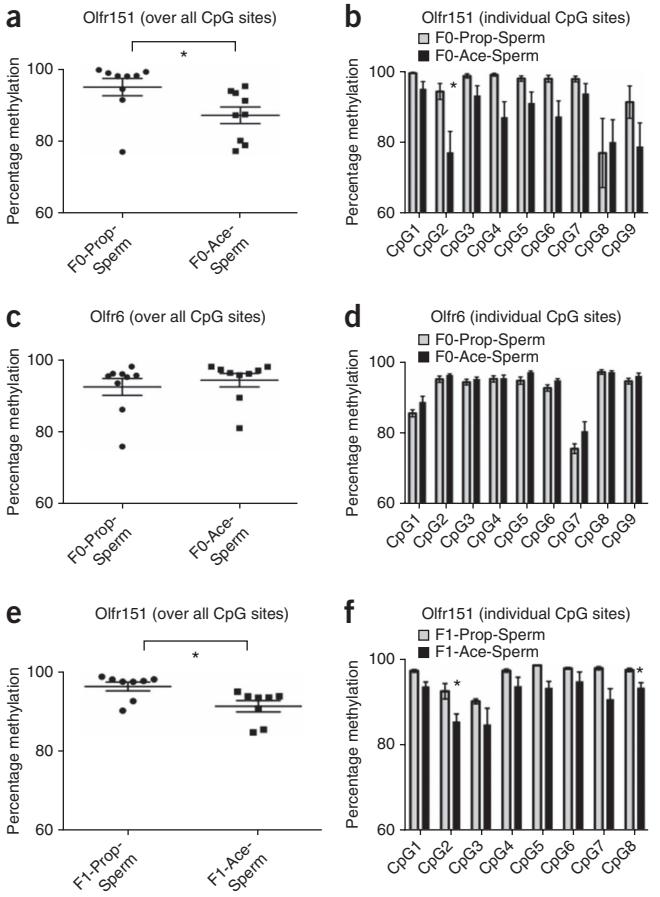

Figure 6 Methylation of odorant receptor genes in sperm DNA from conditioned F0 and odor naive F1 males. (**a**) Bisulfite sequencing of CpG di-nucleotides in the *Olfr151* (M71) gene in F0 sperm revealed that F0-Ace mouse DNA ($n = 12$) was hypomethylated compared with that of F0-Prop mice ($n = 10$) (t test, $P = 0.0323$, $t_{16} = 2.344$). (**b**) A particular CpG di-nucleotide in the *Olfr151* (M71) gene in F0 sperm was hypomethylated in F0-Ace mice ($n = 12$) compared with F0-Prop mice ($n = 10$) ($P = 0.003$, Bonferroni corrected). (**c**) We found no differences in methylation between F0-Ace ($n = 12$) and F0-Prop ($n = 10$) mice across all of the CpG di-nucleotides queried in the *Olfr6* gene in F0 sperm ($P > 0.05$). (**d**) Across specific CpG di-nucleotides in the *Olfr6* gene, we found no differences in methylation between F0-Ace ($n = 12$) and F0-Prop ($n = 10$) mice (Bonferroni corrected). (**e**) Bisulfite sequencing of the *Olfr151* (M71) gene in F1 sperm revealed that F1-Ace mouse DNA ($n = 4$) was hypomethylated compared with that of F1-Prop mice ($n = 4$) (t test, $P = 0.0153$, $t_{14} = 2.763$). (**f**) Bisulfite sequencing of CpG di-nucleotides in the *Olfr151* (M71) gene in F1 sperm revealed that two particular CpG di-nucleotides in the *Olfr151* (M71) gene were hypomethylated in F1-Ace mice ($n = 4$) compared with F1-Prop mice ($n = 4$) ($P = 0.002$, Bonferroni corrected). Data are presented as mean ± s.e.m. $^*P < 0.05$ after correction.

quantitative PCR was performed for the *Olfr151* gene. We did not observe any differences in histone-mediated epigenetic signatures around the M71 locus when chromatin was immunoprecipitated with antibodies that recognize histone modifications that either permit (acetylated H3) or repress (H3trimethyl K27) to transcription (**Supplementary Fig. 7**). The fact that the M71 locus was not epigenetically marked via histones in the F0 sperm could indicate that we did not immunoprecipitate with the relevant histone-modification antibody or that the epigenetic basis of this inheritance might not be histone based, instead relying on other mechanisms, such as DNA methylation (as reported above) or non-coding RNA, as has been demonstrated for the *Kit* locus[33].

DISCUSSION

Focusing on classical conditioning in an F0 generation before conception and using specific odors as the conditioned stimuli allowed us to tag a specific olfactory experience and follow the salience of that experience at the level of behavior and neuroanatomy through subsequent generations. We found that the F1 and F2 generations were extremely sensitive to the specific odors used to condition F0 mice. Using a transgenic mouse in which OSNs expressing a specific odorant receptor can be visualized, we found that the behavioral sensitivity was accompanied by an altered olfactory neuroanatomy for the conditioned odor. The fact that these changes persisted after IVF, cross-fostering and across two generations is indicative of biological inheritance. Finally, we observed that the sperm of the F0 and F1 generation males bear epigenetic marks that could be the basis for such inheritance.

There have been other studies that examined the transmission of stimulus-specific behavioral and structural adaptations in the nervous system from parents to their offspring, albeit with substantial differences from our experimental design. For example, *in utero* taste aversion learning affects the offspring's preference and avoidance of flavors and odors in the mother's diet during gestation[34]. In addition, quality of maternal care is transmitted across generations in rodents[27]. Furthermore, fetal origins of diseases have been proposed for a multitude of disorders as having their roots in the experience of the fetus to the parental environment while *in utero*[35]. From a chemosensory perspective, anti-predatory behavior is transmitted from gravid female crickets to their offspring when the females are exposed to a high density of a predator[36]. Finally, indirectly related to our study is a report that supplementing the mouse maternal diet with acetophenone at various stages of gestation increases M71 glomerular area and preference for acetophenone in adolescent offspring[37]. This last study exemplifies how the olfactory sensitivity and neuroanatomy of offspring can bear imprints of parental experience.

However, it is imperative to realize that all of the aforementioned manipulations of the parental condition have occurred when the pups or embryos are *in utero*, thereby assaying behavior and neuroanatomy in animals that are extremely different from those conceived after perturbation to the parent. In other words, the fetuses in the cited studies were directly exposed to the environmental perturbation. This important point about perturbation of the parental (F0) environment affecting the F1 embryo directly, as well as the F2 germ line, has been used to argue that true transgenerational inheritance should manifest itself in the F3 generation[38]. It is important to note that the F2 mice that we tested are a full and complete generation removed from the environmental perturbation of their parent; as such, our observations suggest a transgenerational phenomenon. Our IVF data complement this point further.

Most recently, several studies have factored paternal effects and transgenerational inheritance of behavior and metabolic states into their experimental design. First, paternal diet has been shown to have marked effects on the metabolic physiology of offspring conceived after the father's diet had been manipulated[7]. Second, exposure to the anti-androgenic endocrine disruptor vinclozolin during embryonic gonadal sex determination affects fertility and behavior in at least four subsequent generations, and it is associated with epigenetic changes in the sperm of descendant male offspring[2,9,39]. A recent study used a social defeat procedure in mice and found paternal transmission of depressive-like behavior in subsequently conceived adult offspring.