Reversing Behavioral, Neuroanatomical, and Germline Influences of Intergenerational Stress

Supplemental Information

Supplemental Methods and Materials

Olfactory Treatments: **F0-Trained** – Males were trained to associate Acetophenone or Lyral presentation with mild foot-shocks. For this purpose, the Startle-Response system (SR-LAB, San Diego Instruments) was modified to deliver discrete odor stimuli as previously described (1-3). The animals were trained on 3 consecutive days with each training day consisting of 5 trials of odor presentation for 10 secs co-terminating with a 0.25 sec 0.4mA foot-shock with an average inter-trial interval of 120 secs. Both Acetophenone and Lyral (from Sigma, and IFF, respectively) were used at a 10% concentration diluted with Propylene Glycol. These odors were chosen based on prior work that demonstrated that the M71 odorant receptor is activated by Acetophenone, and that the MOR23 odorant receptor is activated by Lyral (4, 5). **F0-Exposed** – Males were treated like the F0-Trained group with the important exception that odor presentations were not accompanied by any foot-shocks. **F0-Extinguished** – Males were first treated in exactly the same manner as the F0-Trained group. The next day, these males were placed in a different context and exposed to 30 presentations of the odor that had been previously conditioned with, in the absence of any foot-shock, and this was conducted again for two more consecutive days, for a total of 3 days.

Freezing Behavior of F0 Animals: Within session freezing during conditioning, extinction and testing was determined as described in (3). Briefly, for each millisecond of a 5 second activity window during the odor presentation, voltage outputs for each animal were converted to the

average voltage output and the number of milliseconds above the mean voltage output of the empty cylinder (without a mouse present) were counted as time spent mobile. The rest of time was considered as freezing and percent freezing reported.

Odor-Potentiated Startle of Adult Offspring: We measured *baseline* behavioral sensitivity of the F1 offspring to odors using an Odor Potentiated Startle (OPS) behavioral assay that we have used previously and that measures acoustic startle response to a noise burst (1).

Animals were habituated to the startle chambers for 5-10 minutes on 3 separate days. On the day of testing, animals were first exposed to 15 Startle-alone (105 dB noise burst) trials (Leaders), before being presented with 10 Odor+Startle trials randomly intermingled with 10 Startle-alone trials. The Odor+Startle trials consisted of a 10 sec odor presentation co-terminating with a 50 msec 105 db noise burst. For each animal, an Odor-Potentiated Startle (OPS) score was computed by subtracting the startle response in the first Odor+Startle trial from the startle response in the last Startle-alone Leader. This OPS score was then divided by the last Startle-alone leader and multiplied by 100 to yield the percent OPS score (% OPS) reported in the results.

β-galactosidase Staining: Brains were rapidly dissected and placed into 4% paraformaldehyde for 10 minutes at room temperature, after which they were washed 3 times in 1X PBS for 5 minutes each time. M71-LacZ was stained for β-galactosidase, using 45 mg of X-gal (1 mg/ml) dissolved in 600 μl of DMSO and 45 ml of a solution of 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM MgCl in 1X PBS, and incubated at 37°C for 3 hours.

Measurement of Glomerular Area in the Olfactory Bulb: A microscope-mounted digital camera was used to capture high-resolution images of the β-galactosidase stained M71 glomeruli at 40X magnification. Images were converted to grayscale and equalized for background brightness. The

distribution of pixel brightness was exported in ImageJ as gray levels from 0 = black to 255 = white. X-gal labeled glomerular area was quantified as pixels, less than a set threshold gray level of 150 (optimized for axon vs background). Each glomerulus was traced using the lasso tool in Photoshop and the area was recorded from the histogram tool. This quantitation was conducted by two experimenters both blinded to the experimental groups.

Western Blotting: 10uL of protease cocktail inhibitor mix from Sigma (Cat# P8340) was mixed with 1mL and 500 uL of this mixture was added to a tube containing a fresh frozen MOE. The MOE was homogenized in this mixture using a plastic pestle and then an electric homogenizer. The tissue was then placed on a shaker for 15 min at 4˚C to ensure complete lysis. The lysate was centrifuged at 4˚C for 10 minutes at 12,000 rpm. The supernatant was transferred to a new tube on ice. Amount of protein in the lysate was determined using the Pierce BCA Protein Assay Kit. Standard SDS-PAGE was performed using 15-40 ug of protein per sample. After gel electrophoresis and transfer of the protein to Nitrocellulose membrane, the blots were probed with primary antibody (details noted below), the antibody detected by a peroxidase-coupled secondary antibody and signal detected using ECL substrate (Super Signal West Dura Extended Duration Substrate) and a BioRad Chemidoc MP-Imaging system.

Detecting LacZ:

Primary Antibody – mouse anti-LacZ (Cat # 40-1a) from Developmental studies Hybridoma Bank; 1:200 dilutions with 2.5% nonfat dry milk in 1X TBS-0.1% Tween20, overnight on a rocker at 4˚C.

Secondary Antibody – Anti mouse HRP, Cat # 7076S (Cell Signaling); 1:2000 dilution with 2.5% nonfat dry milk in 1X TBS-0.1% Tween20, 1 hour at room temperature.

Number of litters examined in each condition: F1-Exposed – 4 litters, F1-Trained – 4 litters, F1-Extinguished – 4 litters.

Detecting GFP:

Primary Antibody – Rabbit anti-GFP(ab6556) from Abcam; 1:2500 dilutions with 2.5% nonfat dry milk in 1X TBS-0.1% Tween20, overnight on a rocker at 4˚C.

Secondary Antibody – Anti rabbit HRP; Cat # 7074S (Cell Signaling) 1:2000 dilution with 2.5% nonfat dry milk in 1X TBS-0.1% tween 20, 1 hour at room temperature.

Number of litters examined in each condition: F1-Exposed – 8 litters, F1-Trained – 7 litters, F1-Extinguished – 7 litters.

Detecting β-actin as a loading control:

Primary Antibody – anti-Beta Actin (8H10D10) from Cell Signaling technology, dilution 1:5000 with 2.5% nonfat dry milk in 1X TBS-0.1% Tween20, 1 hour at room temperature.

Secondary Antibody – Secondary Antibody: Anti mouse HRP, Cat # 7076S (Cell Signaling); 1:2000 dilution with 2.5% nonfat dry milk in 1X TBS-0.1% Tween20, 1 hour at room temperature.

The amount of protein in every sample was quantified relative to β-actin by performing optical density measurements using ImageJ.

qPCR for Olfr151, Olfr160 and Olfr16 in MOE of F1 Animals:

Olfr151-F GTGACAGAGTTTATCCTCGGG

Olfr151-R TGCCCAGGTTTCCTACCATG

Olfr160-F GCTGTAGATCAGGGGGTTGA

Olfr160-R ATTCATCCTGTCCAGCATCC

Olfr16-F GACAACAGGGTTCAGCAAGG

Olfr16-R CACCTGTGCCTCTCATCTCA

OMP-F CCAAAGGTGATGAGGAAA

OMP-R CCAACCTCATGACACGCCAGCTGCT

Methyl DNA ImmunoPrecipitation (MeDIP) on DNA in F0 Sperm: Ten days after treatment, sperm was collected from F0-Exposed, F0-Trained, and F0-Extinguished males. Briefly, the cauda epidydymis was dissected into 1mL 1X PBS (Sigma), and sperm were allowed to swim into the medium for 1 hour at 37°C. Six epidydymis were used per sample, and each experimental group had 3-4 samples (1 sample = 3 animals). Mature motile sperm were then collected using density gradient centrifugation using PureSperm buffer and wash protocols (Spectrum Technologies). The sperm pellet was stored at -80°C until further use. Sperm were then lysed using RLT buffer (Qiagen), 150mM DTT, 2% SDS, 200ug/mL of Proteinase K (Qiagen) and 4ug of RNaseA (Qiagen), followed by Phenol:Chloroform:IsoamylAlcohol (pH 8) (ThermoScientific) purification and isopropanol precipitation. DNA was sonicated using a Covaris Ultrasonicator to yield fragments ranging from 100-500bp. Immunoprecipitation was carried out using an anti-5mC antibody (Active Motif) and the DNA-antibody complexes captured using magnetic DynaBead technology. Immunoprecipitated DNA was isolated by phenol-chloroform extraction and ethanol precipitation and used in quantitative PCR reactions on an ABI 7900 Real-Time PCR machine. H19-ICR, Nanog, and H1tr were used as positive controls for the MeDIP, while H1t-R and TsH2b were used as negative controls.

H19ICR-F GCCTCAGTGGTCGATATGGTTT

H19ICR-R AAAGGGACCCCCTCCAGAA

H1t-F	ACGTAGGTGCCATGGGTAAGA
H1t-R	CCCGCCTGAATCTCAAGAGA
Olfr151mC-F	ATTGTTGTCTCTAAAGTAACAATTAGTTCA
Olfr151mC-R	TAAATTGTTCATTGCCCCATTATATT
Olfr160mC-F	GTGATGAGCCTCTGGAAACTTTAA
Olfr160mC-R	CCACTATCATGATGATTCCTTTTTCTT
Olfr16mC-F	TTGTGACACGACTTTTCTGGTTCCCC
Olf16mC-R	TGAAAGTGGTGCTAATAGGAGAAGATGC

Notes on animal behavior:

1. A case could be made for us to refer to the enhanced odor potentiated startle observed in F1-Trained animals as an index of fear rather than the term “sensitivity” that we use in this manuscript and have used (1). However, we hesitate to do so. To definitively call the F1 behavior as an index of fear would require two experiments that are beyond the scope of this current study. First, we would need to condition F0 animals in an appetitive task and not observe enhanced startle of the F1 offspring to the odor but potentially a preference for this odor. Second, we would need to observe activation of some subset of fear- and anxiety-related circuitry like the amygdala and BNST in the F1 offspring after exposure to the F0 odors.

2. It must be emphasized that experiments with a particular odor were performed at the same time and independently of the other odor. Therefore, any differences in behavior observed across cohorts may be a consequence of variables ranging from the experimental (e.g. cage changes

that may have occurred for one cohort of animals and not the other, despite our attempts to minimize such occurrences) to biological (the olfactory epithelia possessing differing numbers of Acetophenone and Lyral-responsive olfactory sensory neurons thereby affecting baseline olfactory behavior to each of the odors).

3. In our previous manuscript (1), our control group comprised of F0-Home animals that had been left undisturbed in their home cages before siring F1-Home offspring. Olfaction is an extremely salient sensory modality for rodents, and previous work has demonstrated that odor exposure alone can affect olfactory acuity and behavior (5, 6), as well as olfactory receptor gene expression within a generation (5-9). To address any potential effects of odor exposure alone, in this study, we used F0- or F1-Exposed animals as controls for the stress or salience of odor exposure alone to the F0 generation that may otherwise be absent in an odor-naïve F0-Home cohort.

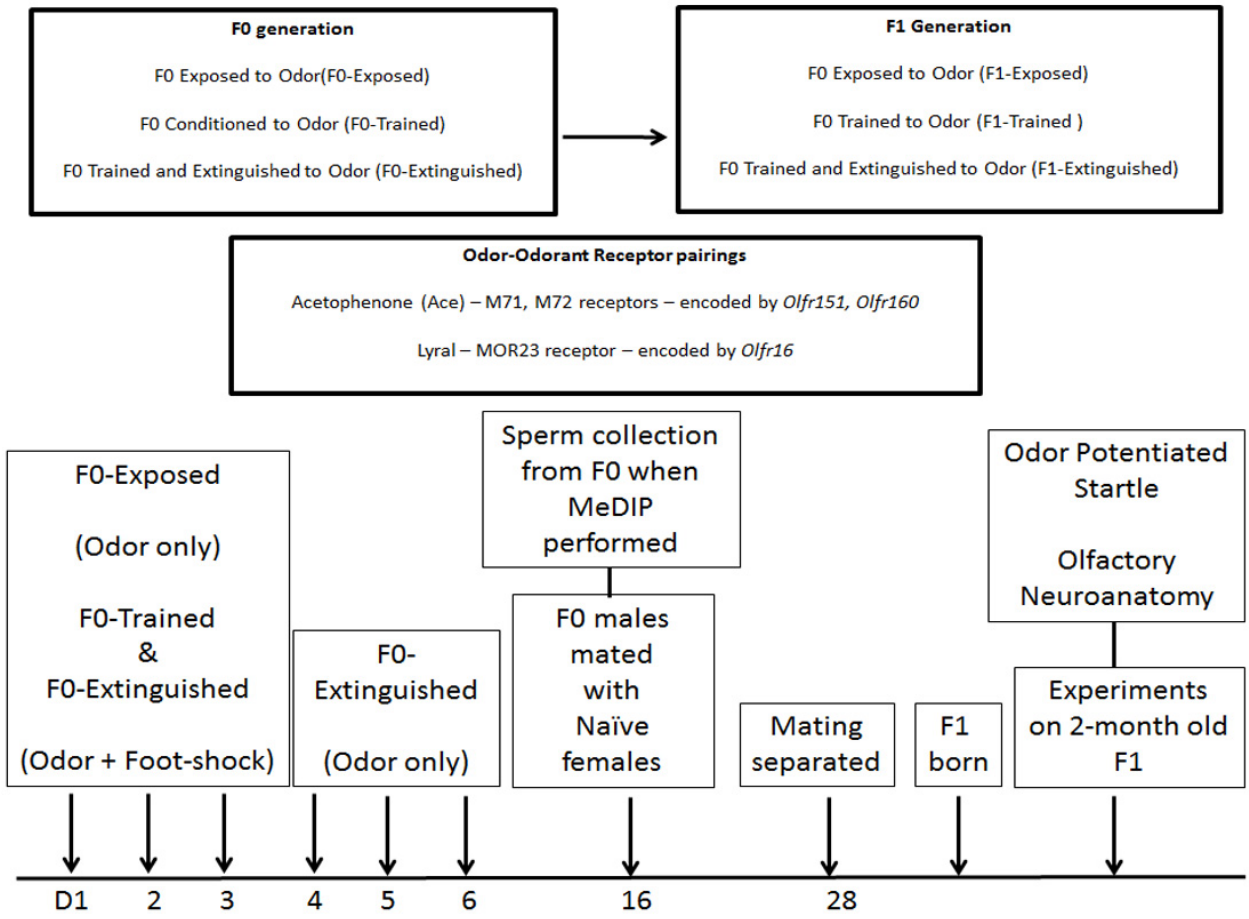
4. We did not decide if F0 animals proceeded to mate or not based on their responses to the odors during the period of treatment (F0-Exposed, F0-Trained, F0-Extinguished) (Supplementary Fig. 2) and all F0 animals were allowed to sire litters (although not all did, due to the vagaries of mating).

5. All behavior was performed in a double-blind manner and data acquired using automated computer software programs (SRLab). Animal groups were blinded to the experimenter and instead cage card numbers and tail or ear markings were used to assign identity to the animals in a cage.

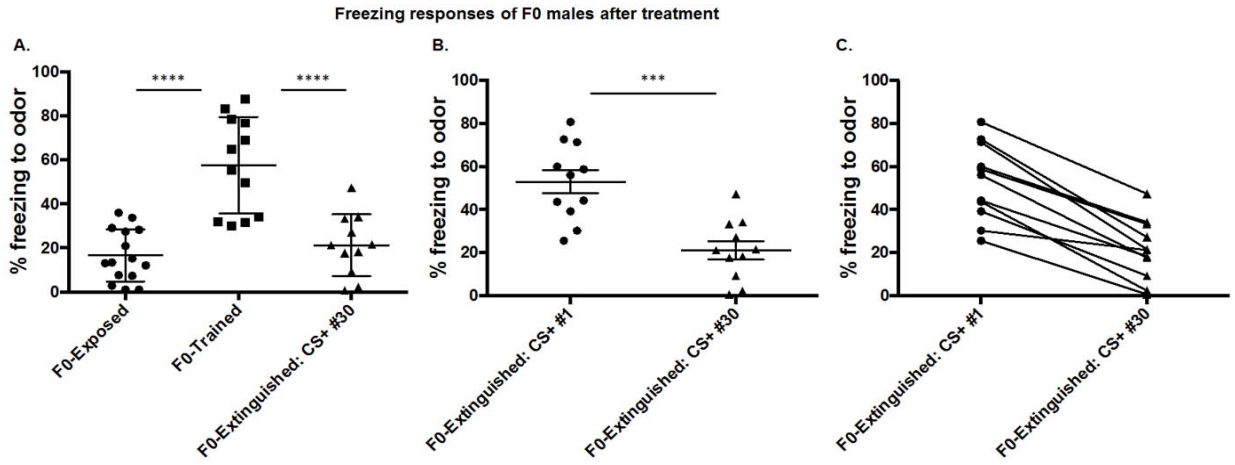
Supplemental Results

Conditioning and Extinction Training of F0 males is Efficient

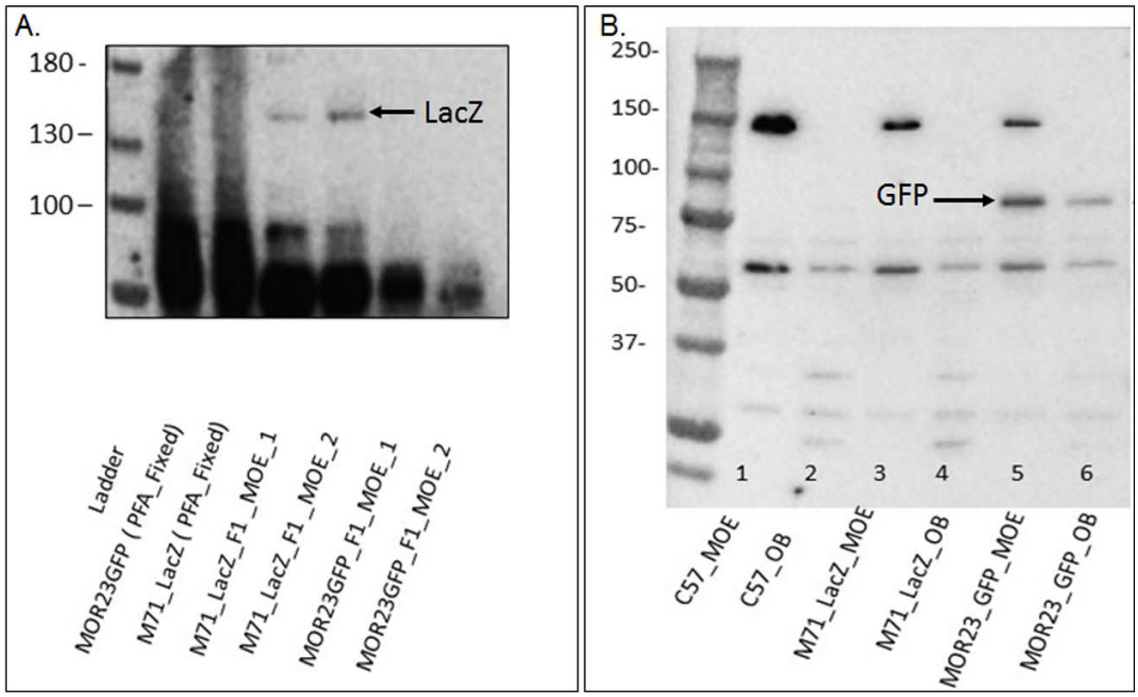
We present evidence across odors that our treatments of F0 animals were efficient (Supplemental Figure S2). F0-Trained animals did in fact show significantly higher freezing when presented with the conditioning odor compared to animals that had only been exposed to the odor (F0-Exposed) or that had been exposed to extinction training after previous conditioning (F0-Extinguished) (Supplemental Figure S2A) (F0-Exposed n=15, F0-Trained n=12, F0-Extinguished n=11) (ANOVA: $F(2,35)=24.19$, $p < 0.0001$. Post-hoc: F0-Exposed vs F0-Trained-Ace ****$p<0.0001$, F0-Trained vs F0-Extinguished ****$p<0.0001$). We further present data that our extinction protocol worked by showing high freezing levels to the conditioning odor when first presented with it compared to freezing levels at the end of the extinction training (Supplemental Figure S2B) (n = 11, t-test: $t=4.627$ $df=20$, ***$p <0.001$).



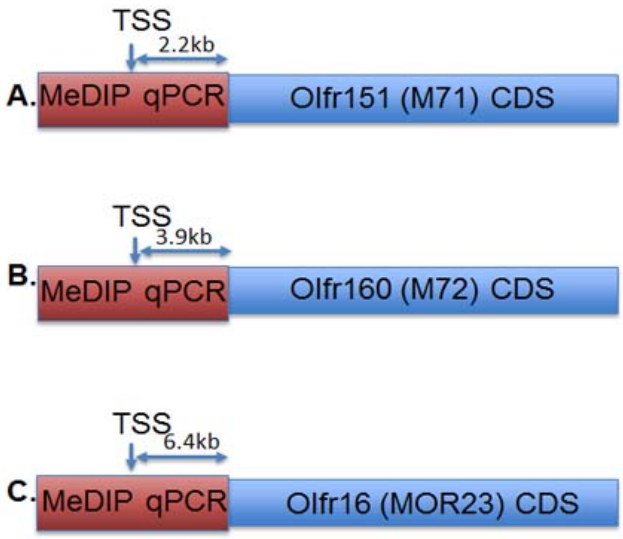
Supplemental Figure S1: Two month-old adult male mice were either exposed to Acetophenone or Lyral (F0-Exposed) or conditioned to Acetophenone or Lyral (F0-Trained). Another group of mice were conditioned to the odors and then exposed to extinction training (odor only presentations) (F0-Extinguished). Ten days after the last odor presentations to the F0-Extinguished group, males were either mated to naïve C57 females or sacrificed to harvest sperm for the MeDIP studies. Males were separated from the females after 12 days of mating and F1 offspring were born (F1-Exposed, F1-Trained, F1-Extinguished). At two-months of age the sensitivity of these F1 offspring toward the F0 conditioning odor was tested using an Odor Potentiated Startle assay and olfactory neuroanatomy measured by visualizing glomeruli in the olfactory bulb and performing Western Blotting for LacZ or GFP proteins when M71-LacZ and MOR23-GFP animals were used.



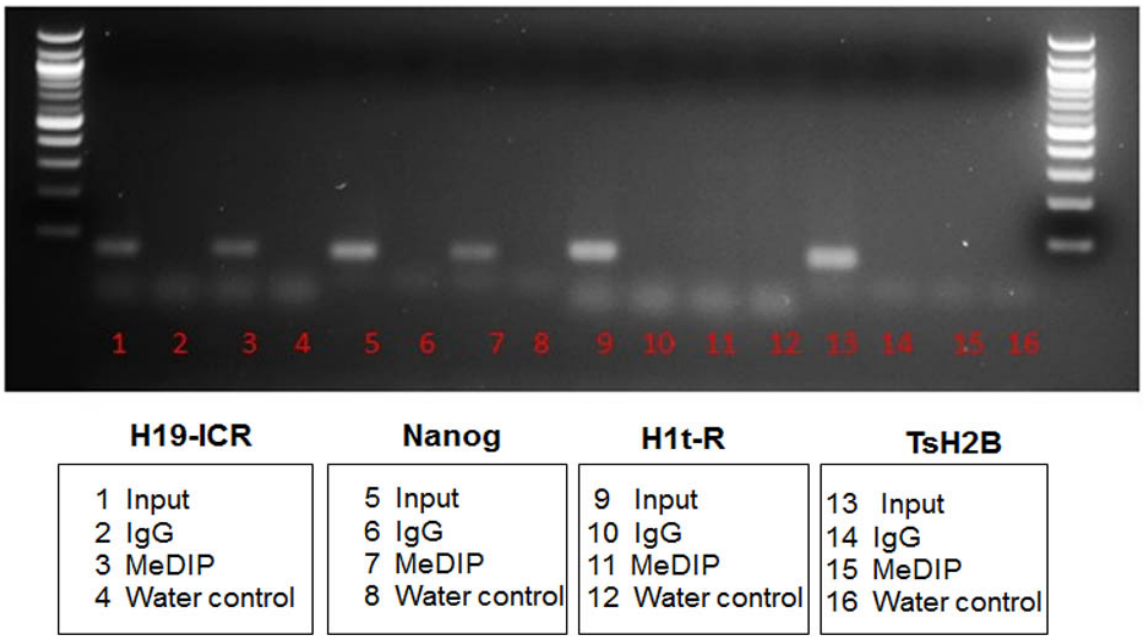
Supplemental Figure S2: Treatment of F0 males is efficient. (A) Training and extinction protocols are efficient: F0-Trained animals that have been fear conditioned with an odor show high freezing levels to the odor presentation compared to F0 animals only exposed to the odor (F0-Exposed). F0-Extinguished animals that have been subjected to extinction training after previous conditioning with the odor, show low freezing compared to F0-Trained animals and their freezing is comparable to the F0-Exposed males. **(B,C) Extinction protocol is efficient:** F0-Extinguished animals show high freezing to the first CS+ (odor presentation) after being previously trained to associate this odor with mild foot-shock and their freezing levels are significantly lower during the last CS+ (odor presentation #30) of the extinction session. Representative data shown across both odors. Data presented as Mean ± SEM. *** $p < 0.001$, **** $p < 0.0001$.



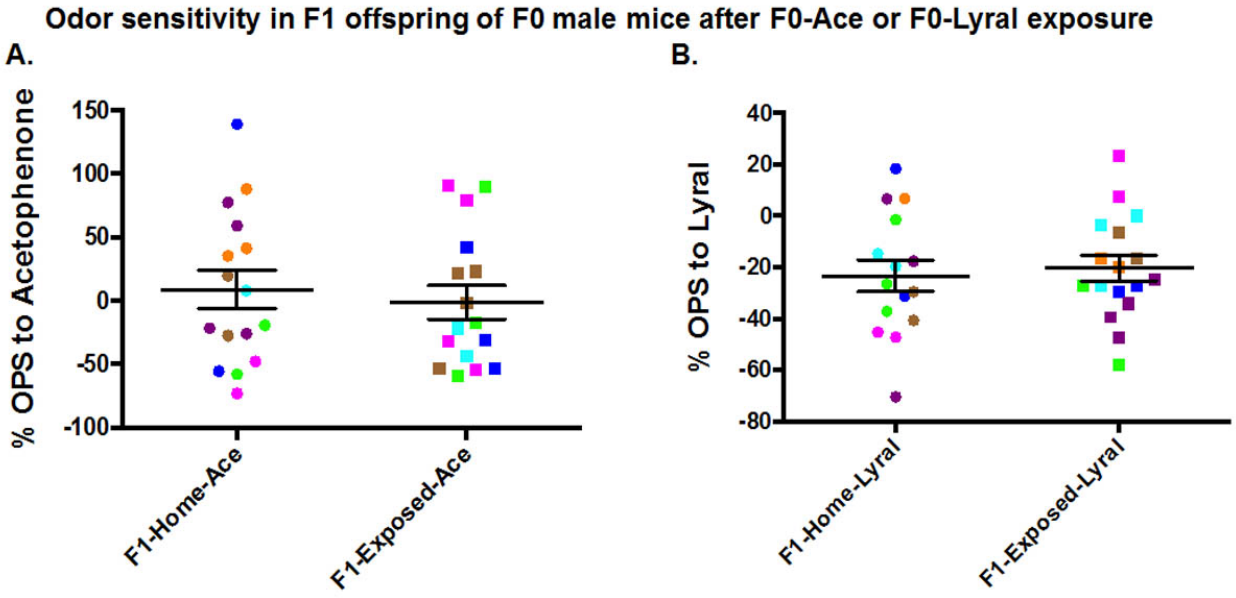
Supplemental Figure S3: Western Blots demonstrating detection of LacZ in M71-LacZ MOE and GFP in MOR23-GFP MOE. (A) We detect a band for LacZ in the MOE of M71-LacZ animals that is not detected in MOR23-GFP. **(B)** We detect a band for GFP in the MOE of MOR23-GFP animals that is not detected in M71-LacZ and C57Bl/6J animals.



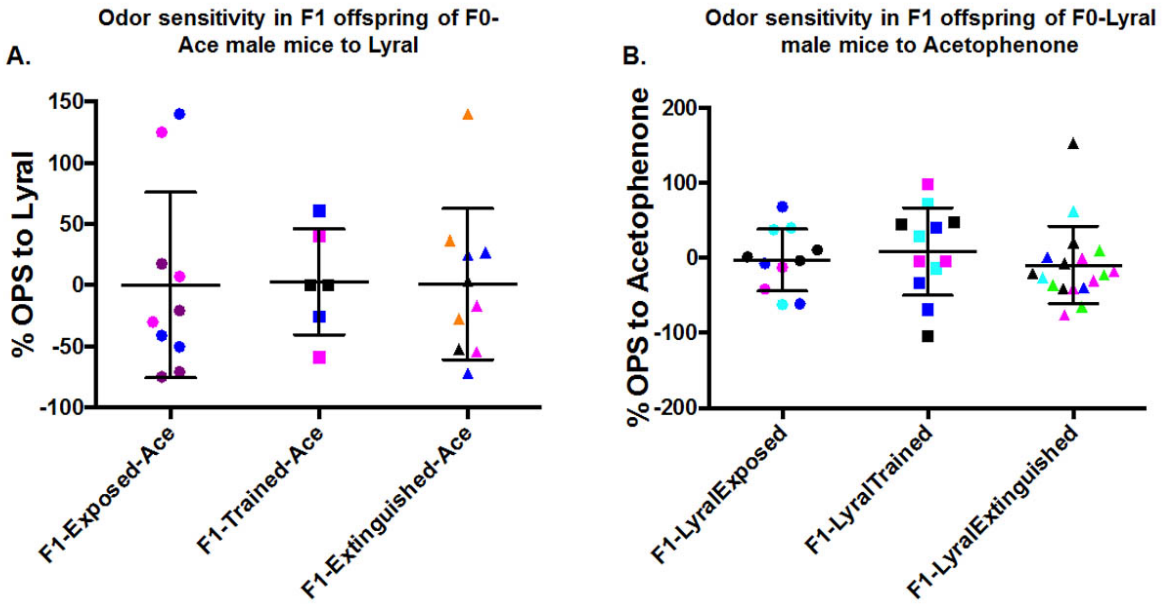
Supplemental Figure S4: Location of minimal promoters of Olfr genes within which MeDIP qPCR was performed. Studies have demonstrated the location of DNA sequences around the Transcription Start Site (TSS) needed for expression of the **(A)** M71 receptor (encoded by *Olfr 151* CDS), **(B)** M72 receptor (encoded by *Olfr 160* CDS), **(C)** MOR23 receptor (encoded by *Olfr 16* CDS). We extracted DNA from sperm of F0 males and performed MeDIP qPCR (red box) to query methylation of cytosine residues around these TSSs.



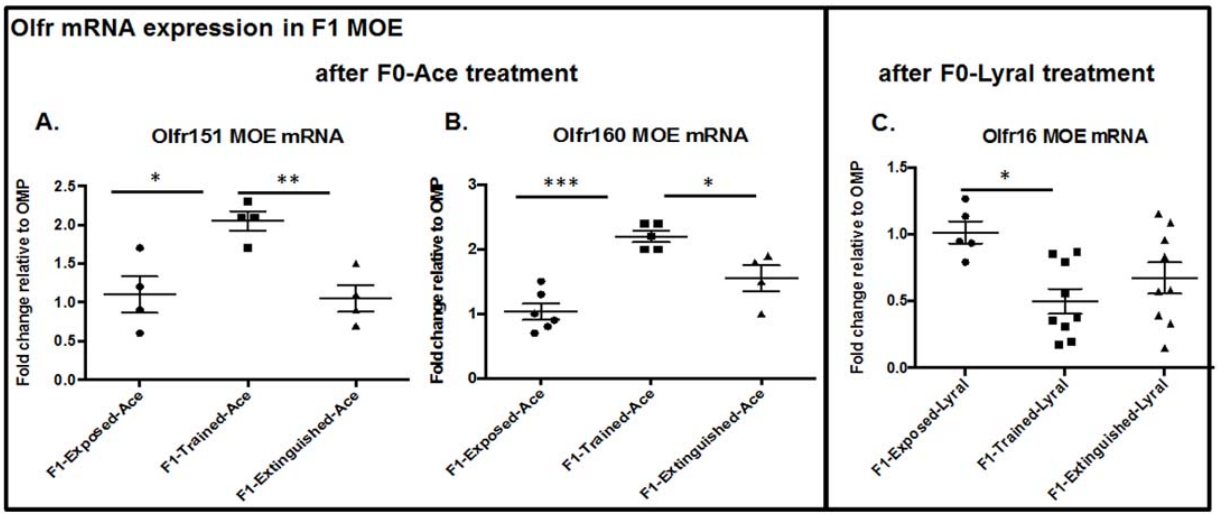
Supplemental Figure S5: Quality control for MeDIP on DNA isolated from sperm. Gel electrophoresis shows that our MeDIP protocol pulls down DNA that is typically methylated (H19-ICR and Nanog) but not DNA that typically lacks methyl groups (H1t-R and TsH2B).



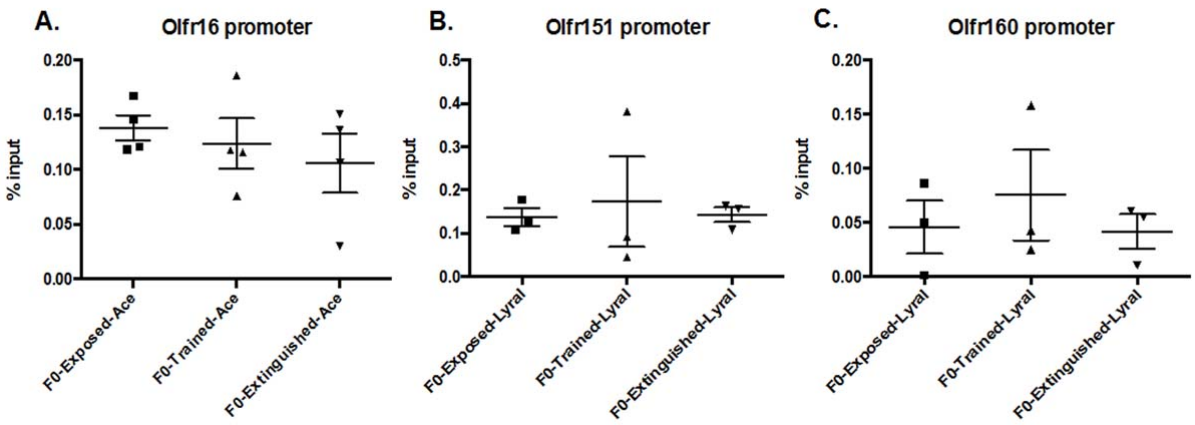
Supplemental Figure S6: Parental olfactory experience has to be salient to impact offspring. (A) F0 males treated with Acetophenone and F1 tested with Acetophenone: Exposing F0 males to Acetophenone results in F1-Exposed-Ace offspring not exhibiting behavioral sensitivity to Acetophenone when compared to F1-Home offspring sired by F0 males left undisturbed in their home cage. **(B)** F0 males treated with Lyral and F1 tested with Lyral: Exposing F0 males to Lyral results in F1-Exposed-Lyral offspring not exhibiting behavioral sensitivity to Lyral when compared to F1-Home offspring sired by F0 males left undisturbed in their home cage. Data presented as Mean ± SEM. Different colors represent individuals from the same litter.



Supplemental Figure S7: Behavioral sensitivity and reversal of behavioral sensitivity of F1 offspring does not generalize to odors that were not used to treat F0 males. (A) F0 males treated with Acetophenone and F1 tested with Lyral: F1-Exposed-Ace, F1-Trained-Ace, and F1-Extinguished-Ace animals sired by F0 males that had been treated with Acetophenone do not show any differences in sensitivity to another odorant (Lyral). **(B)** F0 males treated with Lyral and F1 tested with Acetophenone: F1-Exposed-Lyral, F1-Trained-Lyral, and F1-Extinguished-Lyral animals sired by F0 males that had been treated with Lyral do not show a higher sensitivity to another odorant (Acetophenone). Data presented as Mean ± SEM. Different colors represent individuals from the same litter.



Supplemental Figure S8: Receptor mRNA levels in the F1 MOE reflect parental olfactory experience. (A, B) The Main Olfactory Epithelium (MOE) of offspring of F0 M71-LacZ male mice that had been conditioned to Ace (F1-Trained-Ace) have higher mRNA levels of genes encoding odorant receptors *Olfr151* and *Olfr160* that respond to Acetophenone compared to offspring of F0 M71-LacZ male mice that had been exposed to Ace (F1-Exposed-Ace). Exposing F0 M71-LacZ males that had been previously fear conditioned with Acetophenone to extinction training reverses this increased mRNA expression in their F1-Extinguished-Ace offspring. **(C)** The Main Olfactory Epithelium (MOE) of offspring of F0 MOR23-GFP male mice that had been conditioned to Lyral (F1-Trained-Lyral) have lower mRNA levels of the genes encoding odorant receptor *Olfr16 that responds to* Lyral compared to offspring of F0 MOR23-GFP male mice that had been exposed to Lyral (F1-Exposed-Lyral). Exposing F0 MOR23-GFP males that had been previously fear conditioned with Lyral to extinction training does not affect *Olfr16* mRNA expression in their F1-Extinguished-Lyral offspring. Data presented as Mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.



Supplemental Figure S9. Germ-line influences reflect parental olfactory experience. (A) Methylation around the promoter of the Olfr16 gene that encodes the odorant receptor that is responsive to Lyral was not affected in the sperm of F0 males that had been subjected to treatment with Acetophenone. **(B, C)** Methylation around the promoter of the Olfr151 and Olfr160 genes that encode odorant receptors that are responsive to Acetophenone was not affected in the sperm of F0 males that had been subjected to treatment with Lyral.

Supplemental References

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