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Parental olfactory experience influences behavior and neural structure in subsequent generations

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Associated Data

▾ Supplementary Materials

Abstract Go to ↓

Olfactory attractive molecules, 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 and subsequently bred them to naive females to produce F1 offspring. Male and female F1 offspring exposed to the F0-conditioned odor, but not to other odors, When we subjected F1 offspring to behavioral assays, we found that F1 offspring exhibited a learned avoidance of the F0-conditioned odor, but not other odors. We also observed a hypersensitivity to the F0-conditioned odor, but not to other odors.

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. The OPS score was then the percentage of time spent freezing to the odor leader and multiplied by 100 to yield the percent OPS score (% OPS) reported in the results. Mice were exposed to the acetophenone-potentiated startle (acetophenone + startle) and propanol-potentiated startle (propanol + startle) procedures on independent days in a counter-balanced fashion.

Auditory fear conditioning

Mice were pre-exposed to sound attenuated conditioning chambers (San Diego Instruments) for three consecutive days before training. On the day of auditory fear conditioning, mice received five conditioned-unconditioned stimulus pairings (conditioned stimulus: 30-s, 6-kHz, 75-dB tone; unconditioned stimulus: 500-ms, 0.6-mA foot shock) with a 5-min inter-trial interval. The percentage of time spent freezing to the tones was measured by SR-LAB software (San Diego Instruments). The consolidation of fear memory was tested 24 h after conditioning in a novel context (modified chamber, Med Associates) where mice were exposed to 15 conditioned stimulus tones with a 1.5-min inter-trial interval. Freezing during the tone presentations was measured with FreezeView software (Cushman Instruments). The extinction retention test occurred 24 h after extinction training and consisted of 30 conditioned stimulus-tone presentations to the mice.

Odor sensitivity

Mice were placed in a three-chambered box and allowed to explore between all three chambers for 10 min. A particular concentration of odor (10_{-2}, either acetophenone or propanol) contained in a 1.5-ml Eppendorf tube was placed in one of the chambers with the middle chamber empty and an empty Eppendorf tube in the farthest chamber. Association time with either the odor or the empty chamber was recorded. An association index was computed by subtracting the amount of time spent in the odor chamber from the time spent in the odor chamber. Pilot experiments on independent mice revealed an increasing aversion for either acetophenone or propanol as the concentration increased. Independent mice were used in the acetophenone and propanol experiments (F1-Ace-C57, n = 16; F1-Prop-C57, n = 16).

IVF

IVF was carried out by the Emory Transgenic Mouse Facility (TMF) located in a different building from our colony (http://med.emory.edu/research/core_labs/transgenic_mouse/) across the Emory Campus. Briefly, F0-M/T males were first conditioned either to acetophenone or propanol as outlined above. 10 d after fear conditioning, sperm was collected from the caudal epididymis and vas deferens of these males in our facility, and then transported to the TMF wherein IVF was conducted by TMF personnel blinded to the experimental conditions of the sperm samples according to protocols followed by Jackson Laboratory[21]. *In vitro* fertilization culture medium, Mouse Vitro Fert (MVF, Cook Medical), was used for sperm isolation, IVF and zygote culture. Superovulated C57BL/6 female mice were used as oocyte donors. Sperm were co-incubated with oocytes in MVF for 4 h in a 5% CO_2 incubator, the presumptive zygotes were washed, and were cultured overnight in a 150-μl MVF drop in the incubator. In the second morning, two-cell embryos were washed and placed in MVF drops (pseudopregnant CD-1 female mice of 0–13 weeks of age were used as embryo recipients. 15–20 embryos were transferred into one oviduct of each female. Pups were born after 19 d and weaned from their foster moms at 3.5 weeks of age, they were reared to 2 months of age and then tissue was collected in the TMF facility for β-galactosidase staining on the MOE and olfactory bulb.

β-galactosidase staining, quantitation of MOE OSN number and glomerular area in bulb

The MOE and olfactory bulbs of 2-month-old M71-LacZ mice were processed for β-galactosidase staining, and then M71 OSN number and glomerular area were quantitated using previously published protocols[22]. Briefly, lateral whole-mount MOE and brains were rapidly dissected and placed into 4% paraformaldehyde (wt/vol) for 10 min at +2-8 °C, after which they were washed three times in 1x phosphate-buffered saline (PBS) for 5 min. M71-LacZ was stained for β-galactosidase using 45 mg of X-gal (1 mg ml^{-1}) 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 h.

Quantitation of MOE M71-positive OSN number

The lateral whole mount MOE was imaged using a microscope-mounted digital camera. β-galactosidase-stained blue OSNs were manually counted by an experimenter blinded to the experimental groups.

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 versus 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.

N-ChIP on sperm

N-ChIP was conducted on sperm chromatin using previously described procedures[22]. Briefly, the cauda epidydymis was dissected into 1 ml of M2 medium (Sigma), and sperm were allowed to swim into the medium for 1 h at 37 °C. Five epidydymis were used per sample, and each experimental group had three samples. At least 3 x 10^6 sperm were used for each ChIP. Sperm were then collected by centrifugation at 4 °C for 10 min at 500g, and resuspended in 1x PBS, 1 mM PMSF. Sperm were then lysed on ice for 10 min in 1x PBS, 1 mM PMSF, 0.5% Triton X-100 (vol/vol). Nuclei were pelleted by centrifugation at 4 °C for 10 min at 371g. The pellet was then suspended in 1x PBS, 1 mM PMSF, 10 mM DTT, and incubated at 37 °C for 30 min, before the addition of 0.6 mM $CaCl_2$ and MNase (Sigma) to yield mono-, di- and tri-nucleosomal chromatin. Immunoprecipitation was then carried out as described for the MOE and followed the previously described[22]. The antibodies were used at 1:1,000 and were specific to H3 trimethyl lysine-27 (07-449) and acetyl histone H3 (06-599) from Upstate. 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. 5 mM sodium butyrate was added to all buffers and wash solutions to inhibit histone deacetylases. Primers for the control genes were the same as those used in ref. [22]. ChIP on sperm was conducted on two independent sets of samples with similar results.

NGS analysis of bisulfite PCR amplicons from sperm and MOE DNA

F0-C57 males were fear conditioned to either acetophenone (F0-Ace-C57, n = 12) or propanol (F0-Prop-Sperm, n = 10) as outlined above. 10 d after fear conditioning, sperm was collected from these males in our facility. As outlined above, a separate group of F0-Ace-C57 and F0-Prop-C57 males sired F1 offspring. At 2 months of age, sperm and MOE were collected from F1-Ace-C57 and F1-Prop-C57 mice (n = 4 each). As yet another independent experiment, F1-Ace or F1-Prop mice sired F2-Ace-C57 and F2-Prop-C57 mice, respectively. MOE from this F2 generation (F2-Ace-C57, and F2-Prop-C57) were collected at 2 months of age. All samples were shipped to Active Motif (http://www.activemotif.com) for genomic DNA extraction, bisulfite conversion, PCR-based library generation and sequencing. We coded the samples, and personnel at Active Motif were blinded to this code.

PCR primers to the target regions were designed with the MethPrimer software (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi) (Supplementary Fig. 2). For the F1 sperm, F1 MOE and F2 MOE samples, a shorter amplicon was generated, and we queried eight CpG sites in this analysis, compared with nine sites in the F0 generation. The queried CpG sites 1–8 were the same in the F0 and F1 generations. Genomic DNA was isolated from the sperm samples using Quick-Gene mini prep kit (Zymo Research), and bisulfite-converted using MethylMiner (Active Motif). PCR reactions (40–55 cycles) were performed using Invitrogen's Platinum PCR supermix.

DNA samples containing approximately the same amounts of two (or four) bisulfite PCR products (~300 ng DNA total) were treated with T4 DNA polymerase, Klenow large fragment and T4 polynucleotide kinase to generate 5′-phosphorylated blunt ends. After concatemerization with T4 DNA ligase, the sample was sonicated to an average fragment length of 150–300 bp using a Misonix cuphorn sonicator 3000. Libraries were generated from these sonicated DNA samples using the standard Illumina protocol. The 8 (16) samples were indexed with 6-bp barcodes (independent Illumina index read). Sequencing on Hi-Seq generated ~5–10 million reads per sample. Reads were aligned to chr7 and chr9 reference sequences (mm9 assembly) using the Bismark software (version 0.7.3)[23]. Alignment and methylation information was captured in BAM files, and percentage methylation and read coverage at each CpG site was determined by running the appropriate Bismark scripts. Alignments to the strands and genomic locations not expected to be present in the PCR products were filtered out using a combination of SAMtools[24] and standard UNIX commands.

Supplementary Material

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Supplementary Material

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Acknowledgments

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Footnotes

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Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

AUTHOR CONTRIBUTIONS

B.G.D. conceived of the project, designed and performed experiments, analyzed the data, and wrote the paper. K.J.R. obtained funds, designed experiments, analyzed the data, wrote the paper and supervised the project.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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