Male Germline Transmits Fetal Alcohol Adverse Effect on Hypothalamic Proopiomelanocortin Gene Across Generations

Supplemental Information

Supplemental Methods and Materials

Animals and Treatments

Sprague-Dawley rats were bred in our animal facility. During the breeding period, 1-2 female rats were kept with 1 male in each cage. Once sperm was detected in the vagina of the female rat, it was moved to a single cage, and that day was defined as gestational day (GD) 0. On GD 7-21, pregnant rats were fed rat chow *ad libitum* (AD), a liquid diet containing ethanol (BioServe Inc., Frenchtown, NJ) (AF), or pair-fed an isocaloric liquid control diet (with the ethanol calories replaced by maltose-dextrin) (PF). The concentration of ethanol varied (1.7-5.0% v/v) in the diet for the first 4 days to habituate the animals with the alcohol diet. After this habituation period, animals were fed the liquid diet containing ethanol at a concentration of 6.7% v/v. Age-matched normal females were also bred at the same time as the experimental animals, fed with rat chow throughout the time, and were used as foster dams. Cross fostering was used to avoid the behavior or physiological changes of the original mom on pups during alcohol removal after birth. Pups from all treatment groups were fostered by an untreated dam to account for the possible impact of cross fostering. Only one pup from each litter was used in an experiment in order to prevent gene homogeneity. Rats were sacrificed between 60 and 80 days after birth.

In order to investigate whether the effect of alcohol on *POMC* gene is transmitted through AF males or females to the offspring, we produced two different lines of breeding: male germline (AFM) and female germline (AFF). For producing AFM, male AF rats were bred with normal female rats to produce the F2 generation males and females, and the F2 generation males were then bred with normal females to produce the F3 generation males and females. Similarly

for the female germline (AFF), female AF rats were bred with normal males to produce F2 generation males and females, and the F2 generation females were then bred with normal males to produce F3 generation males and females. For PF and AD animals, F1 males and females were bred to produce F2 generation, and F2 males and females were bred to produce F3 generation. F1-F3 generations of rats were sacrificed by decapitation when they were 2 months old.

Stress responses were evaluated by measuring plasma levels of stress hormones following intraperitoneal injection of 100 μg/kg lipopolysaccharide (LPS; Sigma, St. Louis, MO) or saline at various time periods. Animal surgery and care were performed in accordance with institutional guidelines and complied with the National Institutes of Health policy.

Digestion with Methylation-Sensitive Enzymes Followed by Polymerase Chain Reaction (PCR)

DNA was isolated from the homogenized hypothalamic tissue using Qiagen DNA Kit (Qiagen, Valencia, CA). One microgram of the isolated DNA was digested either with the methylation-sensitive restriction enzyme *HpaII* (Promega Inc, Madison, WI) or with the methylation-insensitive isoschizomer *MspI* (Promega) at 37ºC for 4 h. The DNA incubated only with the enzyme's buffer served as a negative control. After the incubation, the digested as well as negative control samples were subjected to real-time PCR (RT-PCR) with the primers chosen from the regions flanking the restriction sites of the *POMC* gene (GenBank: X03171.1). The degree of methylation was calculated as follows: (1- (Ct *HpaII* digest – Ct *HpaII* buffer))/(Ct *MspI* digest – Ct *MspI* buffer), where the difference (Ct *MspI* digest – Ct *MspI* buffer) served as a measure of the DNA template accessibility.

SYBR Green Methylation-Specific (MSP) Real-Time PCR

The bisulfite conversion of DNA was performed with the help of EZ DNA Methylation-Direct Kit (Zymo Research, Orange, CA). The PCR primers sequences of the oligos are presented in Table 1 and manufactured by Sigma. Primers were designed to be "methylation-specific" or "unmethylation-specific" with respect to the particular cytosine nucleotide in the CpG pair under analysis. The ratios of the methylation-specific to unmethylation-specific responses were quantified by the ΔCt method. All runs were performed in duplicate.

TaqMan MSP Real-Time PCR for *POMC and Dnmt3a*

The PCR primers and fluorogenic probes were designed to be "methylation-specific" or "unmethylation-specific" with respect to the particular cytosine nucleotides in the CpG sites of the -70 to -153 region of the rat *POMC* promoter. The sequences of the oligos are presented in Table 1. Amounts of methylated and unmethylated amplicons were quantified using the standard curve method. The methylated DNA standard for the methylation-specific assay was prepared by methylation of hypothalamic rat DNA with *SssI* CpG methylase (Zymo Research, Inc). The unmethylated DNA standard for the unmethylation-specific assay was prepared by PCR amplification of the segment -64 to -220 of the rat *POMC* promoter region. PCR was performed with the help of ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA) using TaqMan Universal PCR Master Mix (Applied Biosystems). The PCR primers for the *Dnmt3a* gene were designed using the MethPrimer program (http://www.urogene.org/methprimer/index1.html) and manufactured by Sigma. Rat high methylated and rat low methylated DNA controls (EpigenDx, Worcester, MA) were also

bisulfite converted and used for the preparation of the standard curve of diluted complementary DNA which was utilized in each run. All runs were performed in duplicates.

Bisulphite Quantitative Pyrosequencing Methylation Assay

The bisulfite conversion of DNA was performed with the help of EZ DNA Methylation-Direct Kit (Zymo). Quantitative DNA methylation analysis of the bisulphite-treated DNA was performed by pyrosequencing using PSQ 96MA system (Qiagen) with the PyroGold SQA reagent kit, according to the manufacturer's instructions. Regions of interest were amplified from bisulphite-treated genomic DNA, with forward and reverse primers, one of which was biotinylated (Table S1). Standard reaction conditions were Platinum® Taq buffer (Life Technologies, Carlsbad, CA). Purification of the PCR product with streptavidin Sepharose HP beads and hybridization of the biotinylated PCR products and the sequencing primer were conducted as described in the PSQ96 sample preparation guide, using a vacuum filtration sample transfer device (Qiagen). Sequencing was performed on a PSQ 96MA system with the PyroGold SQA reagent kit, and the results were analyzed according to the manufacturer's instructions. In the pyrosequencing study, we have sequenced only one strand of DNA. Our cut-off point of a pyrosequenced DNA value was 10% above the mean ratio of methylation and unmethylation DNA values.

Measurement of mRNA Levels

Total RNA was extracted from tissue sample using a Micro to Midi Kit with Trizol (Invitrogen, Grand Island, NY). The RT-PCR and PCR conditions were as previously described (1, 2). All runs were performed in duplicate. We used GAPDH, b-actin and 18S as housekeeping

genes. The ratio of mean quantity of gene of interest to the mean quantity of GAPDH was compared between different groups. All primers were designed by Applied Biosystems. RT-PCR was performed using the ABI prism 7500HT sequence detection system (Applied Biosystems).

Immunocytochemical Detection of Histone Modifier and Methyltransferase Proteins in β-endorphin Neurons

Hypothalamic tissues of AF, PF, and AD rats were sectioned at 20 μm thickness from plate 19 to plate 23 in the stereotaxic atlas to cover the arcuate nucleus (ARC) area (3) and placed on the same glass slide. Brain sections were double-immunostained with a polyclonal antibody against rat β-endorphin (BEP) (Peninsula Laboratories Inc., San Carlos, CA, 1:200) or corticotrophin-releasing hormone (CRH) (Bachem, Torrance, CA, 1:500) and monoclonal antibody against di and trimethylated histone H3 at lysine 4 (H3K4me2,3), dimethylated histone H3 at lysine 9 (H3K9me2) or acetylated H3 at lysine 9 (AcH3K9) (all from Abcam, Cambridge, MA, and in 1:500 dilution), Dnmt1 (Santa Cruz Biotechnology, Santa Cruz, CA; 1:100), or 5-hmC (Diagenode, Denville, NJ, 1:500). Alexafluor 488 donkey anti-mouse or donkey anti-goat (Invitrogen, 1:1000) or AlexaFluor594 donkey anti-rabbit IgG (Invitrogen, 1:500) were used as secondary antibodies. Pictures were taken using a confocal microscope. The total number of BEP cells from each slide as well as total number of BEP cells that stained positive for either histone modifier proteins or Dnmt and 5-hmC in each brain were calculated. Five sections representatives of plates 19-23 (P19-P23 covering the whole ARC area) from each brain were double-stained for BEP and the target protein H3K4me2,3, H3K9me2, AceH3K9, Dnmt1, or 5-hmC. We counted cells that are located on the left and right sides of the third ventricle from 5 sections per brain. The total number of BEP cells (201-438 cells/ARC) and the total number of

BEP cells positive for the target protein were counted (102-321 cells/ARC). The percentage of BEP cells positive for each histone mark or for Dnmt1 was also calculated and presented in the histograms. The percentage of CRH cells (135-195 cells/PVN) positive for 5hmC (85-141 cells/PVN) was also counted in the paraventricular nucleus area of the hypothalamus (4 sections per brain) in a similar manner and presented in the histogram.

BEP, Adrenocorticotropic Hormone (ACTH) and Corticosterone Detections

The tissue level of BEP was measured using the EIA kit (Bachem, Torrance, CA), plasma corticosterone level was measured by ELISA assays (Diagnostic System Laboratories, Webster, TX) and ACTH was measured by ELISA assay (Phoenix Pharmaceuticals, Burlingame, CA).

Statistics

Data are presented as mean ± SEM. The number of animals was indicated within brackets under or above each histogram. The significant differences between different treatment groups were assessed with one-way analysis of variance (ANOVA) with post hoc analysis using the Newman Keuls post-test or by Kruskal-Wallis ANOVA followed by Dunn's posttest (methylation and unmethylation ratio data). The interaction between treatments effects and feeding effects were determined by two-way ANOVA with post hoc analysis using the Bonferroni post-test. $P < 0.05$ was considered significant.

Table S1. Forward and reverse primers.

Primers for Digestion with Methylation-Sensitive Enzymes followed by PCR
5' GGA GAT CCA ACA GCA TCC TTA ATT A 3' Forward
5' CAA CGC AAC AAG CGA ATC C 3' Reversed
5' CTG TGC TAA CGC CAG CCT CCG C Probe

Primers for SYBR Green Methylation-specific (MSP) Real-Time PCR
5' GTT TTA GCG GGT TTG TGT TAA C 3' Forward **3** M
5' TAA ACG TAA CCT TCC TAA CAA CG 3' Reverse **3** M
5' GTT TTA GTG GGT TTG TGT TAA TGT 3' Forward **3** U
5' TAA ACA TAA CCT TCC TAA CAA CAC T 3' Reverse **3** U

5' TTT TAG GTA TAT TTG TTG TGC GC 3' Forward **1** M
5' TCC TAA CAA CGC TTC TAC AAC G 3' reverse **1** M
5' ATT TTT TAG GTA TAT TTG TTG TGT GT 3' Forward **1** U
5' CCT TCC TAA CAA CAC TTC TAC AAC A 3' Reverse **1** U

5' ATT TGT TGT GCG CGT AGT TTC 3' Forward **2** M
5'TCC TAA CAA CGC TTC TAC AAC G 3' Reverse **2** M
5'TAT ATT TGT TGT GTG TGT AGT TTT Forward **2** U
5' CCT TCC TAA CAA CAC TTC TAC AAC A Reverse **2** U

5' GTT TTA GCG GGT TTG TGT TAA C 3' Forward **5** M
5' TTC TAC AAC GCA ACA AAC G 3' Reverse **5** M
5' GTT TTA GTG GGT TTG TGT TAA TGT 3' Forward **5** U
5' CTT CTA CAA CAC AAC AAA CA 3' Reverse **5** U

5' GTT AGG TGT GCG TTT TAG C 3' Forward **7** M
5' CTA ACA ACG CTT CTA CAA CG 3' Reverse **7** M
5' GGG TTA GGT GTG TGT TTT AGT 3' Forward **7** U
5' CCT AAC AAC ACT TCT ACA ACA CA 3' Reverse 7 U

5' CGG GTT TGT GTT AAC GTT AGT TTT C 3' Forward **8** M
5' GAA AAA AAA AAC TTC CCG ATC GAA ACT ACG 3' Reverse **8** M
5' TGT TTT AGT GGG TTT GTG TTA ATG TTA GTT TTT G 3' Forward **8** U
5' CAC AAA AAA AAA AAC TTC CCA ATC AAA ACT ACA 3' Reverse **8** U

Primers for Taqman MSP Real-Time PCR
Methylated
5' CGTTTTAGCGGGTTTGTGTTAAC 3' Forward
5' CTACAACGCAACAAACGAATCC 3' Reverse
5' CGATCGGGAAGTT 3' Probe
Unmethylated
5' GTGTTTTAGTGGGTTTGTGTTAATGTTAG 3' Forward
5' ACTTCTACAACACAACAAACAAATCCC 3' Reverse
5' GTTTTTGTATTTTTTAGGTATATTTG3' Probe

Pyrosequencing primers
Distal primer set
AATGCCAGGAAGGCAGATG Forward
TCCCTATCACTCTTCTCTCTTCTT Reverse
ATTAAGTTTTTTTTGATTAT Sequencing
Proximal primer set
CAGGTAATTCCACTCCATTCTGTA Forward
CCCTATCACTCTTCTCTCTTCTTT Reverse
GTTGTTAGGAAGGTTA Sequencing

M, methylated; U, unmethylated; PCR, polymerase chain reaction.

Table S2. Statistical values of ANOVA test for the data in Figure 1

Panel	Test	*F*, *df* and *P* values
A	One-way ANOVA	*F* values = 4.38, *df* = 2, *P* < 0.04
B	One-way ANOVA	*F* values = 4.44, *df* = 2, *P* < 0.02
C	One-way ANOVA	*F* values = 4.7, *df* = 2, *P* < 0.01
D	One-way ANOVA	*F* values = 4.3, *df* = 2, *P* < 0.05
E	One-way ANOVA	*F* values = 1.86, *df* = 2, *P* = 0.186
F	One-way ANOVA	*F* values = 1.19, *df* = 2, *P* = 0.33
G	One-way ANOVA	*F* = 0.07, *df* = 2, *P* = 0.93
H	One-way ANOVA	*F* values = 3.02, *df* = 2, *P* = 0.09
I	Two-way ANOVA	Interaction (*P* < 0.0001); feeding-*F* = 225, *df* = 5, *P* < 0.001; Time-*F* = 147.8, *df* = 5, *P* < 0.001
J	Two-way ANOVA	Interaction (*P* > 0.05); feeding-*F* = 23.89, *df* = 2, *P* < 0.001; LPS-*F* = 51.05, *df* = 1, *P* < 0.001
K	Two-way ANOVA	Interaction (*P* > 0.05); feeding-*F* = 22.68, *df* = 2, *P* < 0.001; LPS-*F* = 64.01, *df* = 1, *P* < 0.001
L	Two-way ANOVA	Interaction (*P* < 0.0001); feeding-*F* = 178, *df* = 5, *P* < 0.001; Time-*F* = 71.9, *df* = 5, *P* < 0.001
M	Two-way ANOVA	Interaction (*P* > 0.05); feeding-*F* = 20.52, *df* = 2, *P* < 0.001; LPS-*F* = 49.51, *df* = 1, *P* < 0.001
N	Two-way ANOVA	Interaction (*P* > 0.05); feeding-*F* = 5.77, *df* = 2, *P* < 0.01; LPS-*F* = 44.92, *df* = 5, *P* < 0.001

ANOVA, analysis of variance; *df*, degrees of freedom; LPS, lipopolysaccharide.

Table S3. Statistical values of ANOVA test for the data in Figure 2

Panel	**Test**	***F*, KWS, *df* and *P* values**
B	One-way ANOVA	$F = 6.91$, $df = 2$, $P < 0.01$
C	Two-way ANOVA	Interaction-$F = 76.91$, $df = 10$, $P < 0.001$; treatment-$F = 93.15$, $df = 2$, $P < 0.001$; primer-$F = 109.2$, $df = 5$, $P < 0.001$
D	Kruskal-Wallis ANOVA	KWS = 7.91, $df = 3$, $P = 0.019$
E	Kruskal-Wallis ANOVA	KWS = 9.2, $df = 3$, $P = 0.01$
F-238	One-way ANOVA	$F = 2.37$, $df = 2$, $P = 0.3$
F-224		$F = 2.098$, $df = 2$, $P = 0.35$
F-216		$F = 8.78$, $df = 2$, $P < 0.02$
F-62		$F = 16.19$, $df = 2$, $P < 0.005$
G-238	One-way ANOVA	$F = 1.06$, $df = 2$, $P = 0.586$
G-224		$F = 2.715$, $df = 2$, $P = 0.257$
G-216		$F = 10.68$, $df = 2$, $P < 0.005$
G-62		$F = 10.86$, $df = 2$, $P < 0.005$
H	One-way ANOVA	$F = .49$, $df = 2$, $P = 0.78$
I	One-way ANOVA	$F = .43$, $df = 2$, $P = 0.8$
K	Kruskal-Wallis ANOVA	H = 1.45, $df = 3$, $P = 0.48$
L	One-way ANOVA	$F = .24$, $df = 2$, $P = 0.88$

ANOVA, analysis of variance; *df*, degrees of freedom; KWS, Kruskal-Wallis statistic.

Table S4. Statistical values of ANOVA test for the data in Figure 3

Panel	**Test**	***F*, *df* and *P* values**
B	One-way ANOVA	$F = 5.298$, $df = 2$, $P < 0.05$
D	One-way ANOVA	$F = 5.34$, $df = 2$, $P < 0.05$
F	One-way ANOVA	$F = 14.27$, $df = 2$, $P < 0.001$
G	One-way ANOVA	$F = 8.983$, $df = 2$, $P < 0.01$
H	One-way ANOVA	$F = 6.718$, $df = 2$, $P < 0.005$
I	One-way ANOVA	$F = 5.921$, $df = 2$, $P < 0.01$
J	One-way ANOVA	$F = 3.853$, $df = 2$, $P < 0.05$
K	One-way ANOVA	$F = 5.133$, $df = 2$, $P < 0.01$
M	One-way ANOVA	$F = 22.56$, $df = 2$, $P < 0.0005$
N	One-way ANOVA	$F = 5.773$, $df = 2$, $P < 0.01$
P	One-way ANOVA	$F = 6.1$, $df = 2$, $P < 0.03$
R	One-way ANOVA	$F = 8.46$, $df = 2$, $P < 0.01$

ANOVA, analysis of variance; *df*, degrees of freedom.

Table S5. Statistical values of ANOVA test for the data in Figure 4

Panel	**Test**	***F*, KWS, *df* and *P* values**
A	Kruskal-Wallis ANOVA	KWS = 12.01, *df* = 6, *P* < 0.033
B	Kruskal-Wallis ANOVA	KWS = 11.54, *df* = 6, *P* < 0.041
C	One-way ANOVA	*F* = 3.47, *df* = 2, *P* < 0.05
D	One-way ANOVA	*F* = 4.795, *df* = 2, *P* < 0.01
E	Two-way ANOVA	Interaction-*F* = 5.552, *df* = 5, *P* < 0.005; treatment-*F* = 13.07, *df* = 5, *P* < 0.001; LPS-*F* = 725.7, *df* = 1, *P* < 0.001
F	Two-way ANOVA	Interaction-*F* = 5.289, *df* = 5, *P* < 0.005; treatment-*F* = 7.413, *df* = 5, *P* < 0.001; LPS-*F* = 343.0, *df* = 1, *P* < 0.001

ANOVA, analysis of variance; *df*, degrees of freedom; KWS, Kruskal-Wallis statistic; LPS, lipopolysaccharide.

Table S6. Statistical values of ANOVA test for the data in Figure 5

Panel	**Test**	***F*, KWS, *df* and *P* values**
B	Kruskal-Wallis ANOVA	KWS = 7.906, $df = 4$, $P < 0.0192$
C	Kruskal-Wallis ANOVA	KWS = 9.118, $df = 4$, $P < 0.0105$
D	One-way ANOVA	$F = 3.47$, $df = 2$, $P < 0.05$
E	One-way ANOVA	$F = 4.43$, $df = 2$, $P < 0.05$
F	Kruskal-Wallis ANOVA	KWS = 10.68, $df = 4$, $P < 0.0136$
G	Kruskal-Wallis ANOVA	KWS = 0.57, $df = 4$, $P > 0.91$
H	One-way ANOVA	$F = 9.082$, $df = 3$, $P < 0.001$
I	One-way ANOVA	$F = 3.28$, $df = 3$, $P < 0.01$
J	Kruskal-Wallis ANOVA	KWS = 14.98, $df = 4$, $P < 0.002$
K	Kruskal-Wallis ANOVA	KWS = 3.04, $df = 4$, $P = 0.384$
L	One-way ANOVA	$F = 4.054$, $df = 3$, $P < 0.05$
M	One-way ANOVA	$F = .9726$, $df = 3$, $P > 0.05$

ANOVA, analysis of variance; *df*, degrees of freedom; KWS, Kruskal-Wallis statistic.

Table S7. Statistical values of ANOVA test for the data in Figure 6

Panel	Test	*F*, *df* and *P* values
A	Two-way ANOVA	Interaction ($P > 0.05$); LPS-$F = 51.05$; $df = 1$, $P < 0.001$; feeding-$F = 23.89$, $df = 2$, $P < 0.001$
B	Two-way ANOVA	Interaction ($P > 0.05$); LPS-$F = 64.01$; $df = 1$, $P < 0.001$; feeding-$F = 22.68$, $df = 2$, $P < 0.001$
C	Two-way ANOVA	Interaction ($P > 0.05$); LPS-$F = 49.52$, $df = 1$, $P < 0.001$; feeding-$F = 20.52$, $df = 2$, $P < 0.001$
D	Two-way ANOVA	Interaction ($P > 0.05$); LPS-$F = 44.92$; $df = 1$, $P < 0.001$; feeding-$F = 5.74$, $df = 2$, $P < 0.01$
E	Two-way ANOVA	Interaction ($P > 0.05$); LPS-$F = 28.91$ $df = 1$, $P < 0.001$; feeding-$F = 13.74$, $df = 2$, $P < 0.001$
F	Two-way ANOVA	Interaction ($P > 0.05$); LPS-$F = 59.05$, $df = 1$, $P < 0.0001$; feeding ($P > 0.05$)
G	Two-way ANOVA	Interaction-$F = 3.7$, $df = 4$, $P < 0.05$; LPS-$F = 224.5$, $df = 1$, $P < 0.0001$; feeding-$F = 15.38$, $df = 3$, $P < 0.001$
H	Two-way ANOVA	Interaction ($P > 0.05$); LPS-$F = 84.3$, $df = 1$, $P < 0.0001$; feeding ($P > 0.05$)
I	Two-way ANOVA	Interaction ($P > 0.05$); $df = 4$, $P < 0.05$; LPS-$F = 106.1$, $df = 1$, $P < 0.0001$; feeding-$F = 23.46$, $df = 3$, $P < 0.001$
J	Two-way ANOVA	Interaction ($P > 0.05$); LPS-$F = 81.4$, $df = 1$, $P < 0.0001$; feeding ($P > 0.05$)
K	Two-way ANOVA	Interaction ($P > 0.05$); $df = 4$, $P < 0.05$; LPS-$F = 73.99$, $df = 1$, $P < 0.0001$; feeding-$F = 20.21$, $df = 3$, $P < 0.001$
L	Two-way ANOVA	Interaction ($P > 0.05$); LPS-$F = 53.4$, $df = 1$, $P < 0.0001$; feeding ($P > 0.05$)

ANOVA, analysis of variance; *df*, degrees of freedom; LPS, lipopolysaccharide.

Table S8. Statistical values of ANOVA test for the data in Figure 7

Panel	**Test**	***F*, KWS, *df* and *P* values**
A	Kruskal-Wallis ANOVA	KWS = 7.99; $df = 3$, $P < 0.018$
B	Kruskal-Wallis ANOVA	KWS = 7.17; $df = 3$, $P < 0.028$
C	Kruskal-Wallis ANOVA	KWS = 7.39; $df = 3$, $P < 0.024$

ANOVA, analysis of variance; *df*, degrees of freedom; KWS, Kruskal-Wallis statistic.

Table S9. Statistical values of ANOVA test for the data in Figure S1

Panel	**Test**	***F*, *df* and *P* values**
A	One-way ANOVA	$F = 4.607$, $df = 9$, $P < 0.01$
B	Two-way ANOVA	Interaction ($P > 0.05$); time = $F = 16.42$; $df = 2$, $P < 0.001$
C	One-way ANOVA	$F = 0.63$; $df = 2$, $P > 0.05$
D	Two-way ANOVA	Interaction ($P > 0.05$); time = $F = 1711$; $df = 5$, $P < 0.001$
E	Two-way ANOVA	Interaction ($P > 0.05$); time = $F = 560.1$; $df = 5$, $P < 0.001$

ANOVA, analysis of variance; *df*, degrees of freedom.

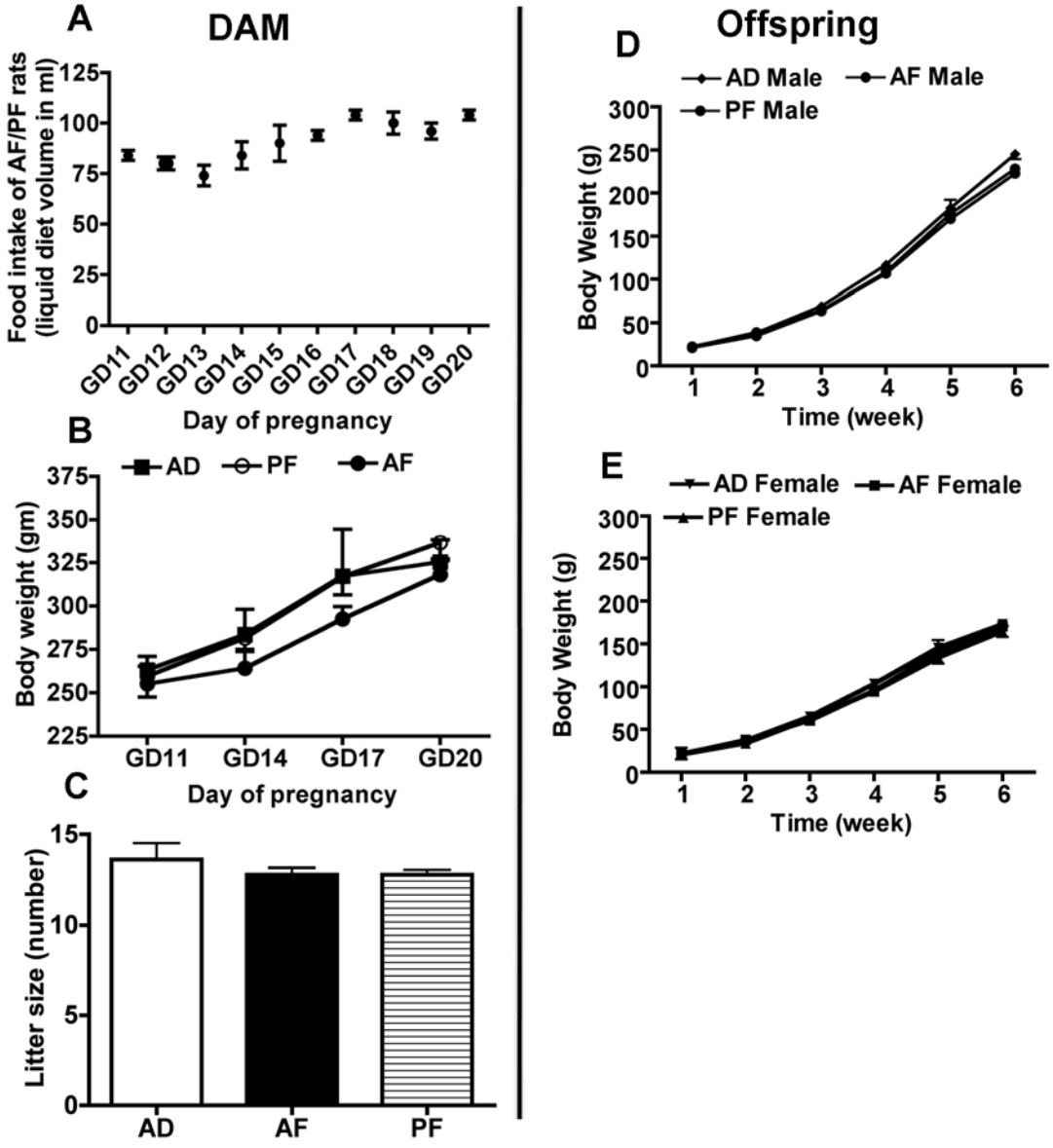

Figure S1. Characterization of the prenatal model of alcohol feeding**.** The mean daily liquid diet intake (**A**), body weight changes (**B**) and litter size (**C**) of alcohol-fed (AF), pair-fed (PF) and *ad libitum*-fed (AD) dams, and the changes of body growth of their male (**D**) and female offspring (**E**). Data are mean ± SEM values. n = 5-12. One-way analysis of variance (ANOVA) analysis identified time-dependent food-intake changes in dams at the $P < 0.01$ level. Two-way ANOVA analysis identified only time affecting results at $P < 0.001$ for the body weight changes in dams, and male and female offspring (see also Table S9).

Supplemental References

1. Boyadjieva NI, Ortigüela M, Arjona A, Cheng X, Sarkar DK (2009): Beta-endorphin neuronal cell transplant reduces corticotropin releasing hormone hyperresponse to lipopolysaccharide and eliminates natural killer cell functional deficiencies in fetal alcohol exposed rats. *Alcohol Clin Exp Res* 33: 931-937.

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