Preadolescent Adversity Programs a Disrupted Maternal Stress Reactivity in Humans and Mice

Supplemental Information

Supplemental Methods and Materials

Animals

Mice were housed in a 12 h light:dark cycle. Food (Purina Rodent Chow; 28.1% protein, 59.8% carbohydrate, 12.1% fat) and water were provided *ad libitum*. HPA axis responsiveness, postpartum pup separation, and pup retrieval occurred in the 1-4 hours after lights on. Light-dark box testing occurred in the 1-4 hours after lights off.

Preadolescent Stress

Female mice underwent 14 days of chronic variable stress starting on postnatal day (PN) 21. One stressor was administered per day from the following list: 15 min restraint, 60 min fox odor exposure (1:5,000; 2,4,5-trimethylthiazole; Acros Organics, Waltham, MA), 3x cage change in one day, 36 h constant light, exposure to novel object overnight, novel 100 dB white noise overnight, and saturated bedding overnight. The order of the stressors was randomized between the first and second week of stress. Within a litter, animals were randomly assigned to the control or preadolescent stress (PAS) group. Animals in the PAS group were weaned into singly-housed cages at the beginning of stress, and were pair-housed with a same-sex, same-stress cage mate at the end of stress. Control individuals remained with the dam until they were weaned at PN28 into pair-housed cages.

HPA Axis Responsiveness

Corticosterone Response to Acute Restraint Stress

Plasma corticosterone was measured in response to a 15 min restraint stress. Mice were placed in a flat-bottomed restrainer (Braintree Scientific, Braintree, MA) during the 1-4 h after lights-on. Blood was

collected from a small nick at the end of the tail at four time points: the onset and completion of restraint (0 and 15 min, respectively), and 30 and 120 min after the onset of restraint. Samples were immediately mixed with 5 µl of 50 mM EDTA and kept on ice until centrifugation for 10 min at 5,000 rpm. Plasma was collected and stored at -80°C until analysis. Corticosterone levels were determined by ^{125}I-corticosterone radioimmunoassay (MP Biomedicals, Santa Ana, CA).

Light-Dark Box

Late pregnant females (17.5dpc, n = 6-10/group) were placed in the light compartment at the beginning of the 10 min test session. Light intensities were set at 5 lux in the dark compartment and 300 lux in the light compartment. All testing occurred 2-5 h after lights off. Total time spent in the light compartment and the number of light-to-dark transitions were analyzed with ANY-maze v4.75 software (Stoelting, Kiel, WI).

Mouse Tissue Collection and Analysis

Pregnant females were anesthetized and decapitated at 17.5dpc or 18.5dpc. The brain, pituitary gland, one adrenal gland, trunk blood, and one each of male and female placenta were collected from the dam. Brains were rapidly frozen on dry ice, while pituitary and adrenal glands were rapidly frozen on liquid nitrogen, and all were stored at -80°C. Trunk blood was collected (18.5dpc only) into a tube with 25 µl of 50 mM EDTA and kept on ice until being centrifuged for 10 min at 5,000 rpm. Plasma was collected and stored at -80°C until analysis. A placenta from one male and one female fetus was collected (18.5dpc only), hemisected, both halves rapidly frozen in separate tubes in liquid nitrogen, and stored at -80°C until analysis.

Peripheral Tissue Quantitative RT-PCR

Total RNA was isolated using TRIzol according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). cDNA was transcribed using the High-Capacity cDNA reverse transcriptase kit (Applied Biosystems, Foster City, CA). Quantitative real-time PCR (qRT-PCR) was performed using TaqMan

gene expression assays (Applied Biosystems, Foster City, CA). In the adrenal gland, gene expression of hydroxysteroid 11-beta dehydrogenase 1 (*Hsd11b1*), the reducing enzyme that can convert inactive glucocorticoid metabolites into active corticosterone, and melanocortin 2 receptor (*Mc2r*), the adrenocorticotropin hormone receptor, was measured. In the pituitary, gene expression of corticotropin releasing factor receptor 1 (*Crhr1*), the CRF receptor on pituitary corticotropes, glucocorticoid receptor (*Nr3c1*), involved in HPA negative feedback, and pro-opiomelanocortin-alpha (*Pomc*), the pro-hormone precursor to adrenocorticotropin hormone, was measured. In the placenta, gene expression of corticotropin releasing factor (*Crh*), which can enter into maternal circulation and alter maternal HPA responding, and corticotropin releasing factor binding protein (*Crhbp*), the binding protein that inactivates CRF, was measured. Each tissue was run in a separate assay. Samples were run in triplicate for the target genes and the endogenous control glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) on the same 96-well plate. Analysis was performed by the comparative Ct method, and expression levels were normalized to control subjects. Specific gene information is in Supplemental Table S1.

Paraventricular Nucleus RNA-Seq

Whole brains from 18.5dpc dams (n = 6/group) were cryosectioned at -20°C. Using a hollow 1.0 mm needle, the paraventricular nucleus (PVN) according to the atlas of Paxinos and Franklin as follows: 1.00 mm punch along the midline from two successive 300 μm slices -0.50 to -1.10 relative to bregma (atlas Figures 36-40). Punches were immediately submerged in TRIzol reagent (Invitrogen), and the tubes were placed on dry ice and stored at -80°C until RNA isolation. RNA was isolated by miRNeasy kit (Qiagen, Hilden, Germany) and suspended in RNAse-free water. mRNA-Seq libraries for RNA Sequencing (RNA-Seq) were prepared using TruSeq Library Preparation Kit v2 (Illumina, San Diego, CA) according to the manufacturers protocol. Adaptors containing six nucleotide indexes were ligated to the double-stranded cDNA. DNA was purified between enzymatic reactions and library size selection was performed with AMPure XT beads (Beckman Coulter, Sharon Hill, PA). The quantity and quality of the libraries was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Wilmington, DE) and KAPA library

quantification (KAPA Biosystems, Wilmington, MA). Single-end 75-bp sequencing was performed on libraries on the Illumina NextSeq 500 sequencer using the NextSeq High Output v2 kit.

Human Studies

Subjects

Pregnant women between the ages of 18 and 45 were recruited to an on-going study focusing on the role of maternal life stress on pregnancy and infant outcomes conducted at the Penn Center for Women's Behavioral Wellness. Women were required to be 8 to 17 weeks pregnant at the time of recruitment, to attend a 20-week ultrasound at a University of Pennsylvania Hospital System site, and to have a history of delivering a healthy full term infant. The participants were English-speaking and gave written informed consent for participation in this study, which was approved by the Perelman School of Medicine at the University of Pennsylvania Institutional Review Board.

Women were excluded from participation if they met any of the following criteria:

1. Presence of a known abnormality in the present fetus, twin pregnancy or a multiple pregnancy
2. Presence of a serious medical or neurological illness, requiring treatment during pregnancy
3. Use of an antipsychotic or antidepressant medication anytime within 2 months prior to the last menstrual period
4. Drug or alcohol abuse history within previous 2 years
5. Life-time history of psychotic disorder including, schizophrenia, schizoaffective disorder; major depression with psychotic features and bipolar disorder
6. Active psychiatric illness requiring treatment based upon the clinical judgment of the study psychiatrist
7. Hamilton Depression Rating Scale Score of > 14
8. Suicidal ideation within the previous 6 months
9. Use of steroids drugs or antihypertensives during pregnancy as they could alter physiologic arousal
10. A history of preterm birth or history of preterm labor in the active pregnancy

Assessment of Preadolescent Adverse Experience

The ACE questionnaire queries individuals regarding history of exposure to 10 different categories of adverse experiences (Supplemental Table S2). For each item that was endorsed, the responded reported the earliest age at which the event occurred. The total number of exposures is summed to create the ACE score (range: 0-10). An item was considered to be a preadolescent ACE if the experience first occurred at least 2 years prior to reported age of menarche. Participants were separated into low (0 ACE) and high (2+ ACEs) preadolescent ACE categories. None of the participants reported 1 ACE.

Cortisol Assay

Immediately after collection, samples were frozen and stored at -20°C until analysis. Frozen samples were sent either to University of Pennsylvania Translational Core laboratory or Dr. C. Kirschbaum Biopsychology laboratory (Dreseden Germany) for cortisol measurement. At the University of Pennsylvania, cortisol was measured with an antibody-coated tube by radioimmunoassay according to manufacturer protocol. There was an inter-assay coefficient of variation (CV) of 8.2% and intra-assay CV of less than 5% (Salimetrics, College Station, PA). At the Dresden laboratory, salivettes were centrifuged at 3,000 rpm for 5 min, which resulted in a clear supernatant of low viscosity. Salivary concentrations were measured using commercially available chemiluminescence immunoassay with high sensitivity (IBL International, Hamburg, Germany). The intra- and inter-assay coefficients for cortisol were below 8%. Samples sent to the two different laboratories were balanced between group. The percentage of high ACE women were compared between the two laboratories, and there was no difference (P = 0.748, Supplemental Table S4).

Statistical Analysis

An investigator blind to group conducted all data collection and analysis. Behavioral, hormonal, and gene expression measures were analyzed by t-test or by one-way analysis of variance (ANOVA) with repeated measures and Huynh-Feldt corrections where appropriate, followed by Fisher's least significant

difference post-hoc testing. Pearson correlations were calculated for postpartum separation testing measures of total corticosterone and distance travelled. For each analysis, values greater than two standard deviations away from each group mean were excluded from analysis as outliers (Supplemental Table S3). The significance level was $P < 0.05$. Statistical analyses were performed using JMP11 Pro (SAS) software. All data for these measures are reported as mean ± SEM.

Human salivary cortisol data were tested for normality and were log-transformed to improve normality. Cortisol data from B1 and B2 were averaged to obtain a baseline value for each participant, which was then subtracted from the cortisol values at each time point (T1-T4) to obtain the data used in analysis. A repeated measures mixed-model ANOVA was performed to evaluate ACE category and time as predictors of cortisol change-from-baseline, controlling for race as a confounder. A Wilcoxon rank sum test was used to analyze EPDS data. Data were not corrected for multiple comparisons.

RNA-Seq data were analyzed in the R environment for Mac using the following pipeline with the packages RSubread and DESeq: align reads to mouse genome, summarize reads, normalize read counts, annotate data, determine differentially expressed genes. To identify differentially expressed genes, the Benjamini Hochbert FDR correction was applied and an adjusted $P < 0.05$ was used. SABioscience's DECODE database was used to analyze common transcription factors.

Supplemental Results

Participants

At the time of this analysis, cortisol results had been obtained for twenty-seven mother/infant pairs who completed the infant separation protocol when the infant was 6 months of age. Of these, two women were missing results due to insufficient saliva quantity for cortisol determination for both of the first two time points and thus no baseline cortisol could be computed. These women were excluded from the analysis. The final analysis cohort consisted of 25 women, 12 of whom were classified as high preadolescent ACE. Demographic information for the 25 women are presented in Supplemental Table S4. Ages ranged from

19 to 35 with a mean of 27. Fifty-six percent were married, and 54% had a college education or higher. The racial breakdown was 8 white, 12 black, 3 Asian, and 2 were classified as other.

Determination of ACE Threshold

In the final analysis cohort of 25 women, there were no women who reported 1 preadolescent ACE. The number of preadolescent ACEs in the high group ranged from 2 to 7. To evaluate the choice of cutoff, we compared the decrease in cortisol response between women with 0 ACEs to the small group with exactly 2 ACEs and found there was a 42% reduction. We also compared women with 0 ACEs to 3+ ACEs and the reduction in cortisol was 51%.

Table S1. Peripheral tissue qRT-PCR gene information

Gene	Gene Symbol	RefSeq Reference Sequence	UniGene Cluster
Adrenal Gland			
Hydroxysteroid 11-beta dehydrogenase 1	*Hsd11b1*	NM_001044751.1	Mm.28328
Melanocortin 2 receptor	*Mc2r*	NM_001271716.1	Mm.426053
Pituitary Gland			
Corticotropin releasing factor receptor 1	*Crhr1*	NM_007762.4	Mm.1892
Glucocorticoid receptor	*Nr3c1*	NM_008173.3	Mm.129481
Pro-opiomelanocortin-alpha	*Pomc*	NM_001278581.1	Mm.277996
Placenta			
Corticotropin releasing factor	*Crh*	NM_205769.2	Mm.290689
Corticotropin releasing factor binding protein	*Crhbp*	NM_198408.3	Mm.316614
All (Control)			
Glyceraldehyde-3-phosphate dehydrogenase	*Gapdh*	NM_0080842.2	Mm99999915_g1

Table S2. Adverse childhood experiences categories

Abuse
Emotional
Physical
Sexual
Childhood neglect
Emotional
Physical
Household dysfunction
Substance abuse
Mental illness
Domestic violence against mother/stepmother
Criminal behavior
Parental separation/divorce

Table S3. Sample sizes that were included in the analysis and outliers that were excluded

Figure	Control *n*	Control Outliers	PAS *n*	PAS Outliers
1A	8	1	6	0
1B	8	0	8	0
1C	8	0	7	1
1D	3	0	4	0
1E-F	10	0	8	0
2B-E	9	1	6	1
2F	6	1	4	0
3A	5	0	6	0
3B	5	0	6	0
3C-D	11 (5M, 6F)	0	13 (6M, 7F)	0
3E	8	1	6	0
3F-G	7	0	8	0
3H-J	6	0	6	0
Figure	**Low ACE *n***	**Low ACE Outliers**	**High ACE *n***	**High ACE Outliers**
2H	13	0	12	0
S1	13	0	12	0

F = female; M = male.

Table S4. Demographic information

Demographic	**Low ACE (*n* = 13)**	**High ACE (*n* = 12)**	**p-value***
Age, mean (SD)	28.6 (4.4)	25.1 (4.4)	0.058
Race: n (%)			0.287
Caucasian	6 (46.2)	2 (16.7)	
African American	5 (38.4)	7 (58.3)	
Other	2 (15.4)	3 (25.0)	
Married/Domestic Partner	9 (69.2)	5 (41.7)	0.165
Education: n (%)			0.073
College or Graduate	9 (69.2)	4 (33.3)	
<High School	4 (30.8)	8 (66.7)	
Assay Laboratory: n (%)			0.748
Site 1	4 (30.8)	3 (25.0)	
Site 2	9 (69.2)	9 (50.0)	
History of Depressive Disorder, n (%)	3 (23.1)	4 (33.3)	0.568
Gestational Age at Delivery (weeks), mean (SD)	39.2 (1.7)	39.1 (1.2)	0.807
Gestational Age at first prenatal visit, mean (SD)	10.1 (8.7)	11.0 (9.3)	0.355
Estimated** BMI@11 weeks gestation, mean (SD)	24.04 (2.1)	30.27 (2.1)	**0.035**

*P-value from Pearson Chi-squared test of association, or Student's t-test as appropriate.
**Mean (SD) and p-value from linear regression model including gestational age at first pre-natal visit.
Low ACE = 0 preadolescent ACEs; High ACE = 2+ preadolescent ACEs.

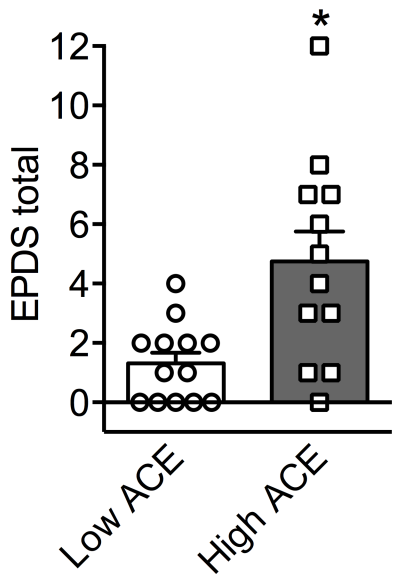

Figure S1. Preadolescent ACE exposure is associated with an increase in depression symptoms as measured by the Edinburgh Postnatal Depression Scale (EPDS) at 6 months postpartum (Z = -2.75, p = 0.006).