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Brain and placental transcriptional responses as a readout of maternal and paternal preconception stress are fetal sex specific

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Abstract

Introduction.—Despite a wealth of epidemiological evidence that cumulative parental lifetime stress experiences *prior* to conception are determinant of offspring developmental trajectories, there is a lack of insight on how these previous stress experiences are stored and communicated intergenerationally. Preconception experiences may impact offspring development through alterations in transcriptional regulation of the placenta, a major determinant of offspring growth and sex-specific developmental outcomes. We evaluated the lasting influence of maternal and paternal preconception stress (PCS) on the mid-gestation placenta and fetal brain, utilizing their transcriptomes as proximate readouts of intergenerational impact.

Methods.—To assess the combined vs. dominant influence of maternal and paternal preconception environment on sex-specific fetal development, we compared transcriptional outcomes using a breeding scheme of one stressed parent, both stressed parents, or no stressed parents as controls.

Results.—Interestingly, offspring sex affected the directionality of transcriptional changes in response to PCS, where male tissues showed a predominant downregulation, and female tissues showed an upregulation. There was also an intriguing effect of parental sex on placental programming where paternal PCS drove more effects in female placentas, while maternal PCS produced more transcriptional changes in male placentas. However, in the fetal brain, maternal PCS produced overall more changes in gene expression than paternal PCS, supporting the idea that the intrauterine environment may have a larger overall influence on the developing brain than it does on shaping the placenta.

Discussion.—Preconception experiences drive changes in the placental and the fetal brain transcriptome at a critical developmental timepoint. While not determinant, these altered transcriptional states may underlie sex-biased risk or resilience to stressful experiences later in life.

Graphical abstract

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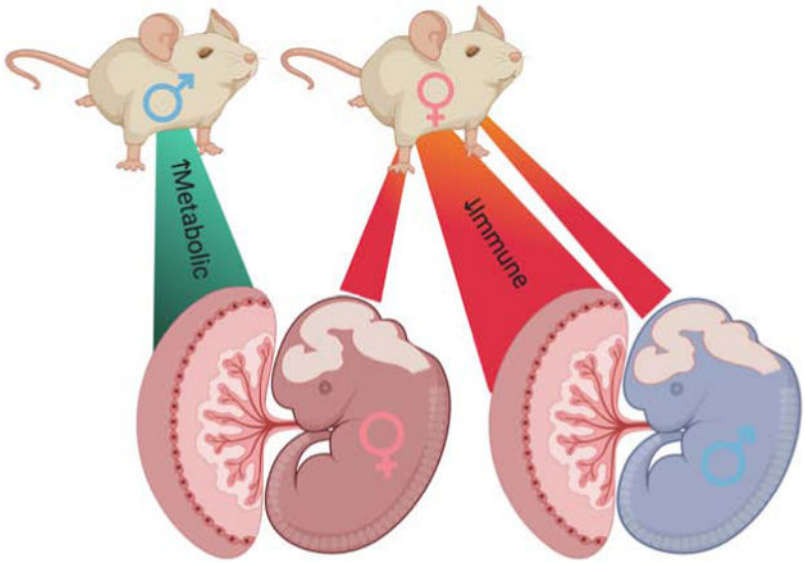

Lifetime stress and trauma, or the cumulative stressors experienced by an individual across their lifespan, is a predisposing factor for the development of chronic diseases, including neuropsychiatric disorders [1–4]. Neuropsychiatric disorders affect approximately 19.1% of adults in the United States [5]. Evidence from both rodent models and humans suggest that the physiological and psychological consequences of lifetime stress can be transmitted from parent to offspring, i.e. intergenerational transmission [5–7]. A mechanism by which maternal and paternal experiences prior to conception impact offspring development is through altering the transcriptional landscape in embryonic and extra-embryonic tissues, including the placenta [8–10]. Prenatal stress significantly alters sex-specific placental gene expression, driving sex differences in neurodevelopmental programming and offspring growth, metabolism, and behavior in adulthood [12–16]. Preconception trauma in women, and stress in female rodents, is associated with changes in offspring brain development, stress sensitivity, and neuropsychiatric outcomes [17–22]. Although studies have addressed the effects of maternal stress during gestation, little is known about the mechanisms by which maternal preconception experiences elicit such effects on offspring outcomes [17,23,24]. Recent studies have also demonstrated that paternal preconception experiences are transmitted to offspring via altered sperm content [25–28]. Specifically, paternal preconception stress programs offspring neurodevelopment and stress reactivity via sperm small-RNA content [29–33]. While maternal and paternal preconception contributions have been independently examined, an understanding of parental influences and driving forces on fetal development at the molecular level is lacking.

Conception involves a complex interplay of opposing maternal and paternal interests orchestrated at the level of the epigenome. Imprinting is the most widely recognized example of this “genetic-conflict hypothesis”, where paternally derived genes promote growth to maximize offspring fitness and maternally derived genes suppress growth to mitigate the energetic costs of gestation [34–36]. Post-conception, genetic-conflict is mediated at the placenta. As both the barrier and central integrator of signals between the

maternal and fetal compartments, the placenta must meet the metabolic demands of the fetus and maintain maternal survival [35,37,38]. In response to a dynamic maternal milieu, broad placental transcriptional responses drive physiological adaptations to maintain support in a fetal sex-specific manner [39–44]. Inappropriate or ill-timed reactivity to perturbations in the maternal environment by the placenta can result in nutrient deficits and restricted resources to highly metabolic tissues, such as the fetal brain [14,45–47]. Proper placental development and function is a key determinant of gestational growth and long-term offspring health outcomes [48–57]. Sex-specific parental experiences *prior* to conception may affect the developing fetal and placental transcriptome, potentially altering the developmental trajectory of offspring in a fetal sex-specific manner.

In this study, adult male and female mice were exposed to four weeks of chronic stress. In order to examine the enduring effects of pre-conception stress (PCS), two weeks prior to breeding mice were returned to standard housing conditions. To determine potential independent and interacting effects of maternal and paternal PCS on sex-specific offspring development, we assessed global changes in the placental and fetal brain transcriptome at embryonic day 12.5 (E12.5). This timepoint coincides with maturation of the placenta and differentiation of the hypothalamus, critical events in tissues determining offspring growth and neurodevelopment [58,59]. We therefore evaluated changes in the placenta and fetal brain transcriptome as a proxy for the potential enduring effects of PCS on offspring development. While not determinant, such changes provide a critical snapshot of altered pathways that may underlie sex-biased risk or resilience later in life.

Materials and Methods

Animals

Mice for parental preconception stress studies were generated from one breeding cohort of virgin in-house bred C57BL/6:129 hybrid strain mice. Mice were housed in a 12 hr light/dark cycle and provided *ad libitum* access to food (Purina Rodent Chow; 28.1% protein, 59.8% carbohydrate, 12.1% fat) and water. All studies were performed according to experimental protocols approved by the University of Pennsylvania Institutional Animal Care and Use Committee and were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Preconception Stress

On postnatal day (PN) 28, male (n=32) and female (n=32) mice were weaned into pair-housing and randomly assigned to a control non-stressed group or to an experimental group that underwent 28 days of preconception stress (PCS), consistent with our paternal preconception stress model [29]. Briefly, seven different stressors were administered, one per day, and order was randomized across weeks of stress. Stressors included 36 hr constant light, 15 min exposure to fox odor (1:5000 2,4,5-trimethylthiazole; Acros Organics, Geel, Belgium), novel object (marbles) overnight, 15 min restraint in a 50 mL conical tube, multiple cage changes, novel 100 dB white noise overnight, and saturated bedding overnight.

Breeding scheme

Consistent with previous experiments, following stress PCS mice were returned to standard housing for 14 days to recover from the acute effects of stress on reproductive behavior [25]. Control and PCS mice were then paired in a full factorial design resulting in the following breedings: non-stressed controls (C♂-C♀, n=8), PCS dams (C♂-S♀, n=8), PCS sires (S♂-C♀, n=8), and PCS dams and sires (S♂-S♀, n=8). Breeding pairs were housed together for a maximum of 4 nights, separated when a plug was observed, and litters were collected at embryonic day 12.5 (E12.5). All offspring were generated from one breeding cohort of PCS

Tissue Collection

Pregnant females were anesthetized and decapitated at E12.5. Placentas and fetal brains were collected from each litter, rapidly frozen in liquid nitrogen, and stored at −80°C. Tissues from one male and one female from each litter were used for further analyses.

RNA Sequencing and analysis

RNA from E12.5 placentas and fetal brains was isolated by RNeasy kit (Qiagen, Valencia, CA) and suspended in RNAse-free water. Libraries for RNA Sequencing were prepared using TruSeq Library Preparation Kit v2 (Illumina, San Diego, CA) according to the manufacturer's protocol. Quantity and quality of libraries were assessed an Agilent 4200 TapeStation (Agilent Technologies, Wilmington, DE). Individually barcoded libraries were pooled by tissue and by sex, such that 3 control (C♂-C♀), 3 maternal PCS (C♂-S♀), 2 paternal PCS (S♂-C♀), and 6 biparental PCS (S♂-S♀) samples were sequenced on each Illumina NextSeq 500 (single-end 75bp) flow cell, to control for batch effects. Fastq files containing an average of 50 million reads were processed in the R environment using Kallisto (version 0.43.1) to perform pseudoalignment and abundance quantification of reads to the mouse transcriptome (Mus musculus v. 79). Gene set enrichment analysis (GSEAv2.0.7, Broad Institute, Cambridge, MA) of normalized counts was used to interpret broad patterns of gene expression and determine greater-than-chance enrichment of biological pathways in a threshold-free manner (i.e. without consideration for differential gene expression). Collections of c2 (curated: Kegg), and c5 (gene ontology) annotated gene sets were obtained from the Molecular Signature Database (MSigDBv3.0.1, Broad Institute, Cambridge, MA) available for use with GSEA software. Gene set permutations (1000) were computed in GSEA to determine FDR, nominal p value, and normalized enrichment score (NES) of each gene set. Significance thresholds were set at FDR ≤ 0.0025, $p \leq 0.01$, and NES ≥ 1.6 to account for gene set permutation.

Results

Reproductive outcomes of parental PCS pairings

Adult male and female mice were paired in full factorial design two weeks after completion of the PCS paradigm to generate 4 pairing groups: non-stressed controls (C♂-C♀), PCS dams (C♂- S♀), PCS sires (S♂-C♀), and PCS dams and sires (S♂-S♀). Final viable litters fitting the criteria of successful mating two weeks post-stress and staged at E12.5, are described in Table 1. Despite variability in plug and pregnancy rates between groups, these

differences were not statistically significant ($\chi^2_{(3, N=20)} = 3.60$ and $\chi^2_{(3, N=14)} = 2.57$, $p > 0.05$, respectively).

Placental sex-specific transcriptomic responses to parental PCS

To examine how maternal and paternal preconception experience may be reflected at the level of the placenta by mid-gestation, we compared transcriptional profiles of male and female placentas from control (C♂-C♀), maternal PCS (C♂-S♀), paternal PCS (S♂-C♀), and bi-parental PCS (S♂-S♀) using RNA-sequencing. By GSEA analysis with enrichment criteria of FDR ≤0.0025 $p \leq 0.01$ and NES ≥1.6, there was a main effect of PCS on female placental gene expression, irrespective of parental sex (Table 2), where gene sets involved in pyruvate and folate metabolism were especially enriched in PCS female placentas relative to controls (Fig 1 **and** Supplementary Table 1). In analyzing parent-specific contributions of PCS, we found that cell metabolism and cell cycling gene sets were increased in placentas of maternal PCS, but paternal PCS female placentas increased gene sets for carbohydrate, lipid, and amino acid metabolism. Bi-parental PCS female placentas were positively enriched for steroid biosynthesis and cell metabolism gene sets, specifically the TCA cycle and glycolysis/gluconeogenesis. Paternal effects were most similar to bi-parental PCS in female placentas where both increased expression of gene sets involved in PPAR signaling and complement and coagulation.

In male placental tissue, gene expression was substantially altered by PCS regardless of parental sex (Table 2), where specifically there was a reduction in expression of gene sets involved in immune function and allograft rejection relative to control males (Fig 1 **and** Supplementary Table 2). In analyzing parent-specific contributions of PCS, we found that maternal PCS reduced expression of gene sets for complement and coagulation and the extracellular matrix, while increasing expression of cell metabolic and mRNA surveillance gene sets relative to control male placentas. Gene sets involved in MAPK signaling were reduced in expression, and gene sets regulating lipid, amino acid and carbohydrate metabolism increased expression in bi-parental PCS offspring placentas relative to control male placentas. Maternal effects were most similar to bi-parental PCS in male placentas where both reduced expression of gene sets involved in additional immune pathways, and increased expression of DNA repair gene sets relative to control male placentas.

Fetal brain sex-specific transcriptomic responses to parental PCS

To examine PCS parent-of-origin effects reflected in the developing brain, transcriptional profiles of E12.5 brains were also determined by RNA-sequencing. GSEA analysis of the E12.5 brain transcriptome revealed a main effect of PCS, regardless of parental sex, on both male and female offspring fetal brain gene expression compared to offspring brains from non-stressed parents (Table 3). Specific effects of maternal PCS showed that female offspring brains increased gene expression of pathways related to DNA replication and repair, and nucleotide metabolism, but neuron and synapse development gene sets, such as calcium signaling, axon guidance, and long term potentiation were decreased in expression (Fig 2 **and** Supplementary Table 3). Bi-parental PCS female brains increased expression of complement and coagulation, PPAR signaling, and cellular metabolism gene sets, while decreasing expression of TGF-B, MAPK, and cell-cell signaling gene sets relative to control

female brains. Relative to control offspring, no gene sets were significantly altered by paternal PCS in female brains. Bi-parental and maternal PCS female brains similarly decreased calcium signaling gene sets and increased steroid biosynthesis gene set expression.

In male brains, PCS increased expression of genes involved in generating the spliceosome, and decreased genes involved in complement and coagulation, CYP450 metabolism, and histidine metabolism relative to control males (Fig 2 **and** Supplementary Table 4). Maternal PCS increased cell cycle, DNA repair, and transcriptional machinery gene set expression, but decreased gene sets involved in humoral immunity, nutrient transport, and fatty acid metabolism. Paternal PCS reduced gene expression related to immune signaling, calcium signaling, and nitrogen metabolism. Bi-parental PCS increased expression of oxidative phosphorylation and proteolysis genes, while gene sets regulating steroid hormone biosynthesis were reduced in expression relative to controls. Bi-parental and maternal PCS both increased expression of DNA replication and transcription gene sets, and relatively reduced expression of complement and coagulation, extracellular matrix, PPAR signaling, and metabolism of amino acids, carbohydrates, and retinol gene sets compared to controls.

Discussion

Despite evidence that parental adverse life experiences prior to conception are a strong determinant of offspring neurodevelopmental outcomes, there is a lack of understanding as to how maternal or paternal lifetime stress interact to influence offspring development. Developmental trajectories are modulated in an offspring sex-specific manner through changes in the epigenetic and transcriptional patterns of placental tissues contributing to sex differences in fetal development [13,15,60,61]. In the current study, we probed for parent- and offspring-sex-specific changes in the placental and fetal brain transcriptome as a snapshot of mid-gestation, a key period of placental maturation and brain development, in response to MPS.

The placenta expresses the fetal genetic sex, which facilitates differences in male and female placental transcription, largely promoted by the X and Y chromosomes [10,50,62,63]. These divergent gene expression patterns alter placental function and responses to the maternal environment, ultimately driving sex differences in offspring developmental trajectories [12,16,64–66]. Consistent with previous studies, we found that offspring exhibited sex-specific changes to the transcriptional landscape of the mid-gestation placenta in response to parental preconception stress (PCS). Indeed, increased expression of immune-related genes was also observed in the male placenta of dams exposed to early preconception stress [67]. Although preconception paternal derived programming of female offspring outcomes [68–70] and preconception maternal derived effects on adult male offspring [19,71,72] have been documented, little is known about the mechanisms driving this parent- and offspring- sex specificity. Importantly, our data also demonstrated *parental* sex-specific effects on placental transcriptional changes, such that maternal and paternal PCS offspring differed in the magnitude, directionality, and functional categories of gene sets affected. The predominant effect of paternal preconception stress on the placenta was an upregulation in expression of 19 gene sets involved in metabolic signaling, specifically in the female placenta. When

examined together with maternal effects, bi-parental PCS female placentas still shared a significant enrichment of gene sets involved in cellular and energy metabolism with paternal PCS placentas, suggesting that paternal life experiences dominate over maternal for his female offspring placenta. Paternal PCS shaping of female placental nutrient synthesis and metabolism at this dynamic time point in gestation aligns with the paternal interests in "genetic conflict theory", altering placental nutrient supply, and ultimately determining intrauterine growth and metabolism of female offspring [34,73–75]. It is unclear why this effect is only apparent in the female placenta, but as we are studying a single timepoint in development, we may be observing a sex difference dependent on the rate or this particular stage of development.

In stark contrast to female placental outcomes, male offspring, regardless of parental sex, robustly *reduced* placental gene expression when compared to same sex controls. Specifically, maternal PCS had a much greater influence on the male placental transcriptome than paternal PCS, robustly altering the expression of 45 gene sets, relative to 11 with paternal PCS, where a majority of the gene sets were involved in immunity and immune signaling, suggesting a potential shift in placental immune environment at this mid-gestation time point in the male placenta. Bi-parental PCS males shared 21 of 37 altered placental gene sets with maternal PCS, further supporting the dominant role of maternal PCS on the male placental transcriptome. Changes in the complex and precise regulation of immunity at the maternal-fetal interface may underlie susceptibility or resilience to immune challenges during gestation, specifically in the male placenta, and have long term effects on the developmental trajectory of male offspring [76–80].

The preconception environment of the parent is a strong determinant of the offspring neurodevelopmental outcomes [19,53,72,81–86]. Transcriptional adaptations of the placenta drive sex-specific changes in communication and transport at the maternal:fetal interface that impact fetal brain development [10,50,62,66]. Given the parent and offspring sex-specific changes in placental gene expression, we also examined the E12.5 brain transcriptome. Maternal PCS drove transcriptional alterations in both male and female fetal brains relative to paternal PCS. There were no significantly enriched gene sets in paternal PCS females, suggesting that at this mid-gestational time point there is: (1) minimal transcriptional influence of paternal PCS the female fetal brain, or (2) transmission of a heterogenous signal that does not fall into distinct gene sets. However, maternal PCS only shared ~25% of enriched gene sets with bi-parental PCS, which suggests some influence of paternal PCS. Similar to the placenta, there was a significant reduction in gene expression of male PCS fetal brains, excepting gene sets involved in cell cycling. Maternal PCS male brains were enriched for 23 gene sets relative to 16 in paternal PCS, which suggests some influence of paternal PCS. Similar to the placenta, there was a significant reduction in gene expression of male PCS fetal brains, excepting gene sets involved in cell cycling. Maternal PCS male brains were enriched for 23 gene sets relative to 16 in paternal PCS. When accounting for 6 gene sets enriched in all PCS groups, there was a 50% overlap of gene sets altered in bi-parental and maternal PCS relative to only 6% in paternal PCS, supporting the dominant role of maternal PCS on gene expression changes in the fetal brain for both sexes.

In summary, these findings demonstrate that parental PCS experience may shape offspring development in a parent- and offspring- sex specific manner. At mid-gestation, the fetal brain transcriptome appeared markedly altered by maternal PCS in both males and females. However, we saw far greater parental and offspring sex-specific outcomes in the placental transcriptome. Additionally, these data outline the sex-difference in transcriptional response to parental PCS; with male offspring suppressing and female offspring enriching immune and metabolic gene sets in both the placenta and the brain. Given the tight regulation of immunity and metabolism during development, these transcriptional adaptations, while not determinant, may suggest sex-specific shifts in utilization of energy resources and protection from the external environment. As these data are limited to transcriptional readouts, further studies are needed to understand the ultimate effects of these developmental events. This study supports the growing appreciation for the impact of parental lifetime stress and preconception adverse experiences on offspring development, possibly underlying sex differences in long-term health risk and resilience.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Highlights

- Preconception stress (PCS) alters offspring development in a parental and fetal sex-specific manner.
- Maternal PCS markedly decreased immune-related genes in the male placenta.
- Paternal PCS altered metabolic-related genes in the female placenta.

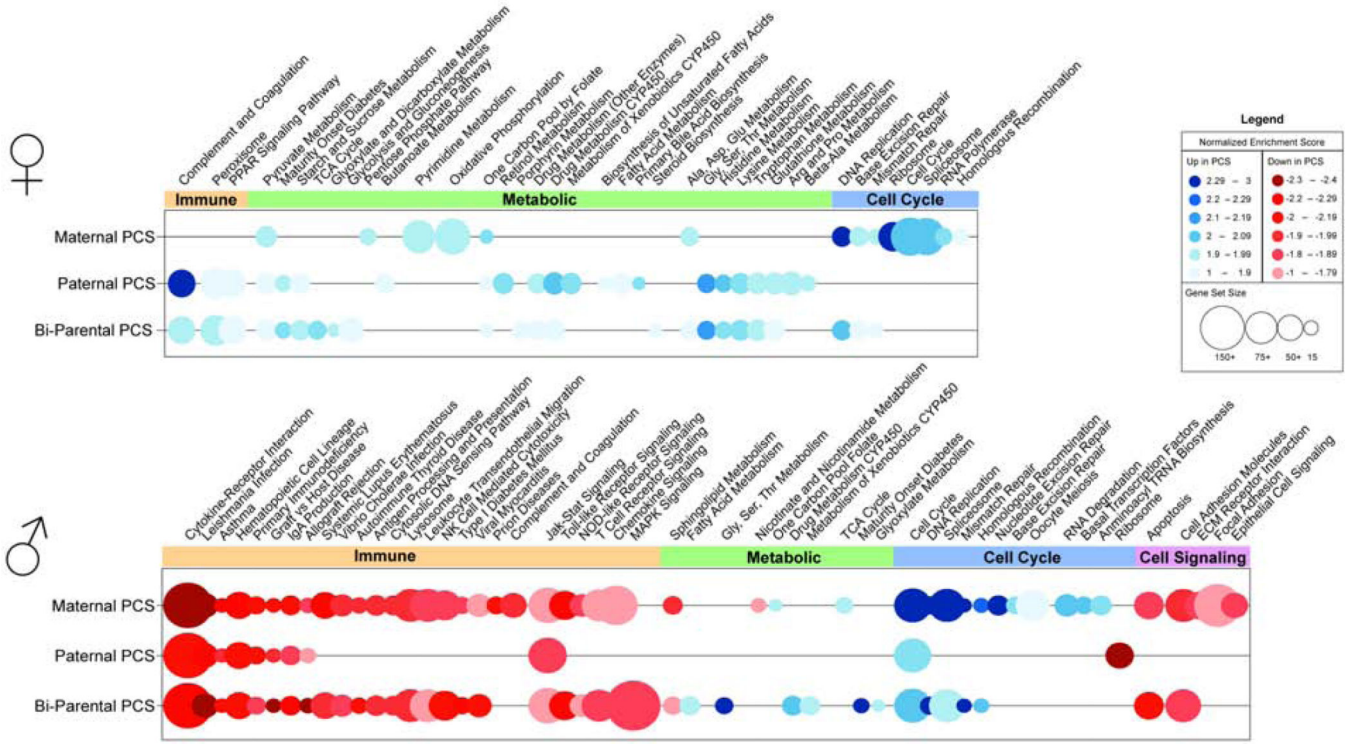

Figure 1.

Parental preconception stress dramatically reduces male offspring placental transcription. Bubble plots of gene sets identified by gene set enrichment analysis (GSEA) as significantly altered in female (top) and male (bottom) placentas of maternal (n=3), paternal (n=2), and bi-parental (n=6) PCS offspring relative to controls (n=3). Bubbles represent the normalized enrichment score (NES) of a particular gene set, where color indicates increased (blue) or decreased (red) enrichment, color intensity indicates magnitude of change, and bubble diameter represents gene set size. Differentially enriched gene sets, meeting criteria of FDR ≤ 0.0025 $p \leq 0.01$, and NES ≥ 1.6, were grouped into functional categories spanning immunity (red), metabolism (green), cell cycling (blue), and cell signaling (purple). Gene set enrichment analysis (GSEA) of placentas at embryonic day 12.5 (E12.5) indicated a marked decrease in gene expression in placentas of male PCS offspring relative to controls, specifically in genes mediating immune processes. Female PCS offspring increased placental expression of gene sets mediating metabolic processes. A full list of enriched gene sets and statistics are detailed in Supplementary Tables 1 **and** 2.

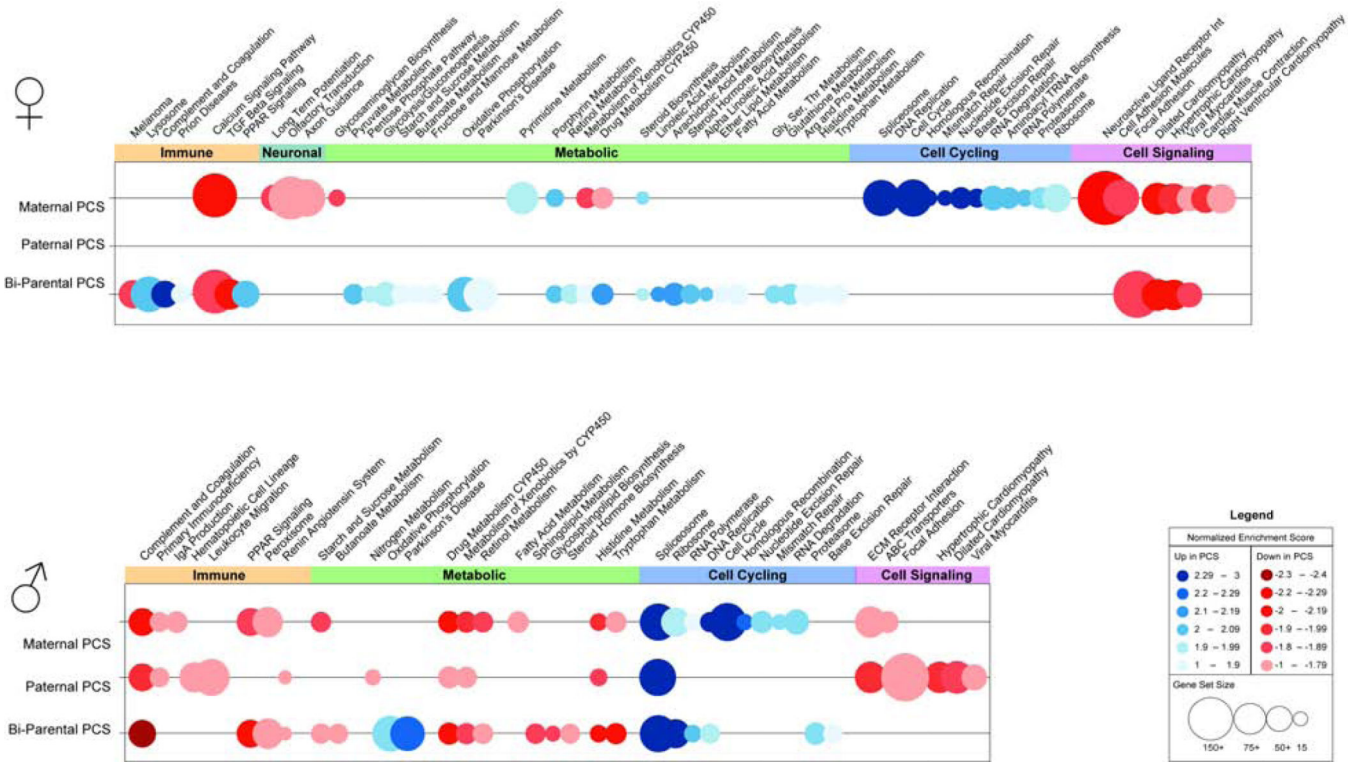

Figure 2.

Maternal preconception stress shapes the offspring fetal brain transcriptome. Bubble plots of gene sets identified by gene set enrichment analysis (GSEA) as significantly altered in fetal brains of female (top) and male (bottom) offspring of maternal (n=3), paternal (n=2), and bi-parental (n=6) PCS relative to controls (n=3). Bubbles represent the normalized enrichment score (NES) of a particular gene set, where color indicates increased (blue) or decreased (red) enrichment, color intensity indicates magnitude of change, and bubble diameter represents gene set size. Differentially enriched gene sets, meeting criteria of FDR ≤ 0.0025 $p \leq 0.01$, and NES ≥ 1.6, were grouped into functional categories spanning immunity (red), metabolism (green), cell cycling (blue), and cell signaling (purple). Gene set enrichment analysis (GSEA) of placentas at embryonic day 12.5 (E12.5) indicated a marked decrease in gene expression in placentas of male PCS offspring relative to controls, specifically in genes mediating immune Supplementary Tables 3 **and** 4.

Table 1.

Summary of breeding outcomes.

	Pairings				
	C♂-C♀ (n=8)	**C♂-S♀ (n=8)**	**S♂-C♀ (n=8)**	**S♂-S♀ (n=8)**	***P value*[b]**
Vaginal Plug Observed, *n* (%)	3/8 (37.5)	6/8 (75)	3/8 (37.5)	8/8 (100)	0.31
Pregnant/Plugged, *n* (%)	3/3 (100)	3/6 (50)	2/3 (67)	6/8 (75)	0.46
Litter Size[a]	10.33 ± 1.53	12 ± 1.00	10.5 ± 0.71	9.67 ± 3.98	0.96
Males per Litter[a]	7.3 ± 2.08	4.3 ± 1.15	5 ± 1.41	5.3 ± 2.73	0.79
Females per Litter[a]	3 ± 1.00	7.7 ± 1.53	5.5 ± 0.71	4.3 ± 2.07	0.51

[a]Values represent litter characteristics at E12.5 expressed as mean ± SD

[b]Pearson's $\square^2$

Table 2.

Summary of placental gene sets altered in parental PCS offspring.

	Female			Male		
	Maternal	**Paternal**	**Bi-parental**	**Maternal**	**Paternal**	**Bi-parental**
KEGG	15	22	23	45	11	37
BP	25	41	14	286	4	95

Total gene sets with significantly increased or decreased expression in male and female PCS offspring placentas relative to controls. GSEA enrichment criteria were FDR ≤ 0.0025 $p \leq 0.01$, and NES ≥ 1.6.

Table 3.

Summary of fetal brain gene sets altered in parental PCS offspring.

	Female			Male		
	Maternal	**Paternal**	**Bi-parental**	**Maternal**	**Paternal**	**Bi-parental**
KEGG	17	0	33	23	16	22
BP	235	0	22	204	22	66

Total gene sets with significantly increased or decreased expression in male and female fetal PCS offspring brains relative to controls. GSEA enrichment criteria were FDR ≤ 0.0025 $p \leq 0.01$, and NES ≥ 1.6.