Supplementary Information for

Bisphenol A and Bisphenol S Disruptions of the Mouse Placenta and Potential Effects on the Placenta-Brain Axis

Jiude Mao[1,2], Ashish Jain[3,4], Nancy D. Denslow[5], Mohammad- Zaman Nouri[5], Sixue Chen[6,7], Tingting Wang[7], Ning Zhu[7], Jin Koh[7], Saurav J. Sarma[1,8], Barbara W. Sumner[1,8], Zhentian Lei[1,8,9], Lloyd W. Sumner[1,8.9], Nathan J. Bivens[10], R. Michael Roberts[1,9,11,*], Geetu Tuteja[3,4,*], Cheryl S. Rosenfeld[1,2,12,13*]

Author Affiliations:
[1]Bond Life Sciences Center, University of Missouri, Columbia, MO 65211, USA: [2]Biomedical Sciences, University of Missouri, Columbia, MO 65211, USA: [3]Bioinformatics and Computational Biology, Iowa State University, Ames, IA 50011, USA: [4]Genetics, Development and Cell Biology, Iowa State University, Ames, IA 50011, USA: [5]Physiological Sciences and Center for Environmental and Human Toxicology, University of Florida, Gainesville, FL 32611, USA: [6]Department of Biology, Genetics Institute, University of Florida, Gainesville, FL32610, USA: [7]Proteomics and Mass Spectrometry Facility, Interdisciplinary Center for Biotechnology Research, University of Florida, Gainesville, FL 32610, USA: [8]MU Metabolomics Center, University of Missouri, Columbia, MO 65211, USA: [9]Biochemistry, University of Missouri, Columbia, MO 65211, USA: [10]DNA Core Facility, University of Missouri, Columbia, MO 65211, USA: [11]Animal Sciences, University of Missouri, Columbia, MO 65211, USA: [12]Thompson Center for Autism and Neurobehavioral Disorders, University of Missouri, Columbia, MO 65211, USA: [13]MU Informatics Institute (MUII), University of Missouri, Columbia, MO 65211, USA.

Corresponding Authors: robertsrm@missouri.edu; geetu@iastate.edu; rosenfeldc@missouri.edu

This PDF file includes:

www.pnas.org/cgi/doi/10.1073/pnas.1919563117

Supplementary Methods

Chemicals and reagents. Bisphenol A (BPA, Chemical CAS #80-05-7, purity >99%,) and bishenol S (BPS, CAS # 80-09-1, Purity > 98%) was purchased from Sigma-Aldrich (Saint Louis, MO). Paraformaldehyde EM grade powder was purchased from PolySciences Catalogue #00380 (Warrington, PA). Methanol, methyl tert-butyl ether (MTBE) and dansyl chloride were purchased from Fisher Scientific (Optima, MA). Ammonium fluoride, corticosterone, E2, estrone and progesterone were purchased from Sigma-Aldrich and testosterone was obtained from Steraloids, Inc. (RI). Deuterium-labelled or ^{13}C steroid hormones were used as internal standards, including cortisol-d7 (Santa Cruz biotechnology, MN), 17β-E2-d5 (CDN isotopes, Quebec, Canada), progesterone-d9 (TRC, Toronto, Canada) and 17OH-Progesterone-$^{13}C_3$ (Sigma-Aldrich).

Fetal PCR Sexing and Placental RNA isolation. To determine the sex of each conceptus, DNA was isolated with the DNeasy Blood & Tissue Kit (Catalogue #69504; Qiagen, Gaithersburg, MD) from fetal tissue. Polymerase Chain Reaction (PCR) amplification was then performed for *Sry* (Y-chromosome specific gene, forward primer: 5'TCATGAGACTGCCAACCACAG3'; reverse primer: 5'CATGACCACCACCACCACCAA3') and myogenin (*Myog*- autosomal control gene; forward primer: 5'TTACGTCCATCGTGGACAGC3'; reverse primer: 5'TGGGCTGGGTGTTAGTCTTA3'), as detailed in (1). Once the sex of the conceptuses was established, one male and one female placental pair from each litter were selected for further analyses.

RNA was isolated from each of the selected placental samples with the Qiagen AllPrep DNA/RNA/miRNA Universal Kit (Catalogue #80224; Qiagen). The quantity and quality of the RNA was determined with a Nanodrop ND1000 spectrophotometer (Nanodrop Products, Wilmington, DE). The results were further confirmed by analyzing the RNA on the Fragment Analyzer (Advanced Analytical Technologies, Ankeny, IA). Only those samples that had a RNA integrity number (RIN) score above 7 were selected for RNA sequencing (RNAseq) and quantitative PCR (qPCR) analyses.

Illumina TruSeq RNA Library Preparation and Sequencing. Libraries were constructed per the manufacturer's protocol with reagents supplied in Illumina's TruSeq mRNA Stranded Library Preparation Kit. Briefly, the poly-A containing mRNA was purified from total RNA, mRNA was fragmented, double-stranded cDNA generated from fragmented RNA, and the index containing adapters were ligated to the ends.

The final construct of each purified library was determined by using the Fragment Analyzer (Advanced Analytical Technologies, Ankeny, IA) automated electrophoresis system, quantified with the Qubit flourometer by means of the quant-iT HS dsDNA reagent kit (Invitrogen), and diluted according to Illumina's standard sequencing protocol for sequencing on the NextSeq 500. Libraries were sequenced at the University of Missouri DNA Core Facility to obtain 75 base pair, single end reads. Samples were sequenced a sufficient number of times to provide approximately 110 million reads per sample. The actual number of reads obtained for each sample is listed in **SI Appendix, Table S3**.

RNAseq data processing. The reads were trimmed for Illumina adapters, for ambiguous nucleotides (N's), and for artificial poly-G (2) for reads whose 3' ends overlap with the adapter for a minimum of 3 bases with 90% identity. After trimming, reads with fewer than ten bases were discarded. The filtered trimmed reads were aligned to the reference mouse genome (mm9) by HISAT2 to achieve a high overall alignment (~97%) (3). The aligned reads were further filtered to remove reads that mapped to the mitochondrial genome. The number of reads that aligned to each protein-coding gene were counted with the htseq-count tool from HTseq software suite (4). The raw read counts were normalized for each biological replicate by converting them into log transformed transcripts per million (TPM) values (5).

Differential Gene Expression Analysis (DGEA): DESeq2. The raw read counts were used to carry out differential gene expression analysis (DGEA) by means of DESeq2 to study the effects of the treatments (BPA or BPS) (6). The genes with an average of less than 5 read counts in at least one group were filtered out before carrying out DGEA. Genes were considered upregulated if they had an absolute fold-change ≥ 1.5 and adjusted p-value ≤ 0.05. We used two DGEA models to identify differentially expressed genes between the treatment and control samples. In model 1 (~ Gender + Treatment + Batch + Gender:Treatment), the variations due to sex (Male versus Female), treatment (BPA, BPS, or Control), known batch effects (Batch 1: 30 samples, Batch 2: 10 samples), and interaction between the sex and treatment were modeled. The interaction gives information about genes that behave differently in a particular sex treated with a particular treatment. In model 2 (~ Treatment + Batch) the variations due to known batch effects (Batch 1: 30 samples, Batch 2: 10 samples), and treatment (BPA, BPS, or Control) were modeled.

Tissue-Specific Gene Enrichment Analysis. Tissue-specific gene enrichment analysis was carried out by means of TissueEnrich (7). We used the mouse ENCODE (8) dataset to carry out the enrichment analysis with default settings. Enrichments were considered significant if P was $\leq$ 0.01 and fold-change ≥ 2.

Functional Enrichment Analysis. Protein-protein interactions (PPI) for proteins encoded by DEG were determined with the STRING Database (9). Functional enrichment analysis was determined with WEB-based Gene SeT AnaLysis Toolkit (WebGestalt) (10), which is based on over-enrichment pathway analysis for mice, and the DEG were compared against all protein encoded genes in the mouse database.

qPCR. Total RNA, which had been treated with DNase to remove any genomic DNA contamination, was reverse transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Catalogue #205310, Qiagen). The qPCR procedure was performed on the Applied Biosystems 7500 Real-Time PCR System (Carlsbad, CA) using the QuantiTect SYBR Green PCR Kit (Catalogue #204143; Qiagen). Primers were designed by using NCBI Primer-Blast online (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer sequences for the genes examined are listed in **SI Appendix, Table S8**, and primers were purchased from IDT (Coralville, IA). The average efficiency for all primers was 100.3 ± 2.2 %. The qPCR conditions employed were 1) 15 minutes at 95 ºC for polymerase activation 2) 40 cycles of: denaturation 15 seconds at 95 °C, annealing 30 seconds at 55 ºC, and extension 72 ºC for 60 seconds 3) Dissociation melt curve analysis from 60 ºC to 90 ºC. Ubiquitin C (*Ubc)*, RNA Polymerase II Subunit A (*Polr2a*), and ribosomal protein L7 (*Rpl7*) were used as internal controls. With high

expression stability of *Ubc* and *Polr2a* in control placenta, these genes are considered an appropriate reference control for the mouse placenta (11). These three internal control gens were further evaluated by NormFinder software (v 0.953; https://moma.dk/normfinder-software) and all had high stability of expression with low intra and intergroup variations.

Nontargeted Metabolomics of Fetal Placental Tissue. Nontargeted Metabolomics was performed at the University of Missouri Metabolomics Center (http://metabolomics.missouri.edu/). There were significant variations in the placental sample weights. Thus, samples were extracted with a normalized volume of solvent that was proportional to adding 250 µl of H_2O, containing 25 µg/ml ribitol (internal standard), to 1.0 mg of placental tissue. For example, a 5.0 mg placental tissue sample was extracted with 1.25 ml of H_2O containing 25 µg/ml ribitol. The samples were vortexed for 20 seconds, sonicated for 15 minutes then incubated at 50 ºC for 1 h. Next, sample tubes were centrifuged at 13000 x g for 15 minutes. Equal amounts of supernatant from each sample tube were collected into an auto-sampler glass vial, dried under a gaseous nitrogen stream, methoximated with 25 µl of pyridine containing 15 mg/ml methoxyamine hydrochloride, and then trimethylsilylated with 25 µl MSTFA (N-methyl-N-(trimethyl-silyl)trifluoroacetamide) + 1% TMCS (chlorotrimethylsilane) reagent. The derivatized extracts were analyzed by non-targeted metabolic profiling by using an Agilent 6890 GC coupled to a 5973N MSD mass spectrometer with a scan range from m/z 50 to 650 (Agilent Technologies, Inc., Santa Clara, CA). Separations were achieved with a temperature program of 80 ºC for 2 minutes, then ramped at 5 ºC /minute to 315 ºC and held at 315 ºC for 12 minutes by using a 60 m DB-5MS column (J&W Scientific, 0.25 mm ID, 0.25 um film thickness) and a constant flow of 1.0 ml/minute of helium gas. A standard alkane mix was

used for Gas Chromatography Mass Spectrometry (GC/MS) quality control and retention index calculations. The data were deconvoluted by means of the program AMDIS (12) and annotated through mass spectral and retention index matching to an in-house constructed EI GC/MS spectra library of authentic standards. The remaining unidentified components were then searched and tentatively identified by using spectral matching to a commercial NIST17 mass spectral library. The combined identifications were saved as an .ELU file, and the abundance of the ions were extracted by means of custom MET-IDEA software (13, 14). The abundances were then normalized to the internal standard, ribitol, which we have previously shown to be a good negative control for mouse placental samples. The normalized values were used for statistical analyses such as partial least squares discriminant analysis (PLS-DA) and volcano plot after log transformation and Pareto scaling with Metaboanalyst software (https://www.metaboanalyst.ca/).

Quantitative Analysis of Neurotransmitters in Fetal Placental Tissue. A TSQ Altis QQQ (Thermo Scientific) MS/MS system with an ion funnel connected to a Vanquish Horizon ultra-high-performance liquid chromatography (UHPLC) was employed to analyze neurotransmitters. Prior to analysis of placental samples, multiple reaction monitoring (MRM) parameters for each standard compound were optimized (precursor m/z, fragment m/z, radio frequency (RF) lens, and collision energy) on the TSQ Altis QQQ MS/MS by direct infusion of the authentic standards at a concentration of 1 µg/ml each in 50 % (v/v) acetonitrile with 0.1 % (v/v) formic acid. Concentrations of GABA, DA, 5-HT, and its primary metabolite, 5-HIAA were measured on a Hypersil Gold column, 2.1× 50 mm, 1.9 µm (Thermo Scientific). A binary gradient of 0.1% (v/v) formic acid in water and 0.1% formic acid (v/v) in acetonitrile was used as mobile phases A and B, respectively. The gradient profile was: 0 to 25 % B from 0-3 minutes, 25 to 90 % B from

3-3.5 minutes, 90 % B from 3.5-4 minutes, and 0 % B from 4.01-6 minutes. The analytes were eluted at a constant flow rate of 0.4 ml/minute. The mass spectrometer conditions included the spray voltage applied at 3000 V in the positive mode and sheath, aux and sweep gases set to 50, 10, and 0, respectively. Temperatures of the ion transfer tube and the vaporizer were set at 200 ºC and 40 ºC, respectively. For MRM monitoring, both Q1 and Q3 resolutions were set at 0.7 m/z full width at half-maximum (FWHM) with the CID gas set at 1.5 mTorr, and the scan cycle time was at 0.8 seconds. A 14-point standard curve was established for each metabolite.

Placental samples were subsequently weighed in EZ micro test tubes (Bio-Rad). Immediately after weighing, samples were extracted in 50 mM phosphate-citrate buffer (pH 5.6) at 3:1 volume (µl): tissue (mg). To improve extraction, the solid material was pulverized with a disposable pestle (Fisher Scientific). Samples were sonicated and centrifuged, and supernatants transferred to a new test tube. Samples (25 µl each) were injected for liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis. Targeted analyses with MRM were performed for the analytes of interest as indicated in **SI Appendix, Table S9**. All chromatograms were inspected manually to determine peak shape and number of points in the peak. Blank analyses and washes were added between the samples to mediate carryover. The LC-MS/MS instrument was operated via Thermo Scientific Xcalibur Foundation Software, and acquired data processed for generation of calibration curves and quantitation of neurotransmitters by means of their Quan Browser software. A weighting factor of 1/x was applied to the data (15), linearity of the standard curves were verified by least square linear regression analysis (16, 17), and limits of detection and quantitation calculated (18).

Quantitative Analysis of Placental Estradiol, Estrone, Corticosterone, Testosterone, and Progesterone Concentrations. Pure steroid hormones, including corticosterone (200 ng/ml), progesterone (20 ng/ml), testosterone (20 ng/ml), E2 (400 ng/ml), and estrone (400 ng/ml) were mixed to prepare a calibration working solution in de-lipidized human serum, double charcoal stripped (Golden West Diagnostics, CA). The working solution was serially diluted with the serum to obtain an 8-point standard curve for each hormone. Quality control solutions for the high-, medium-, and low- concentrations were prepared to match the 4, 20, and 200 times dilution of the calibration working solution, respectively. Internal heavy isotope standards were mixed and diluted by using methanol to make a final concentration of 1 pg/ml cortisol-d7, 5 pg/ml 17β-E2, 0.2 pg/ml progesterone-d9 and 0.5 pg/ml 17OH-progesterone-$^{13}C_3$ spiking solution.

Hormones were measured by LC-MS/MS on the AB SCIEX QTRAP 6500 available in the University of Florida Analytical Toxicology Core Laboratory. Placental samples were weighed and spiked with 5 µl of mixed heavy isotope internal standards. Samples were homogenized in 245 µl methanol 10 % (v/v) in water with a Tissue Tearor Homogenizer (IKA T10, USA) and hormones were extracted with 500 µl MTBE. The extracts were dried under N_2 gas and dissolved in 50 µl methanol prior to injection of 8 µl into the LC MS/MS instrument.
To improve ionization efficiency and detection limit of E2, an additional derivatization step was performed, following the method described by Nelson and et al (19). Briefly, hormone extracts were dried and re-dissolved in 50 µl sodium bicarbonate buffer (100 mM, pH 10.5) and an equal volume of 1 mg/ml dansyl chloride in acetone was added. Each sample was vortex-mixed for 1 minute and incubated in a heating block at 60 ºC for 10 minutes. Samples were cooled on ice,

dried and reconstituted with 25 µl methanol. Samples were centrifuged for a short time to remove particulates and the supernatant was transferred to a new autosampler vial and injected into the LC-MS/MS.

Chromatographic separation was performed by means of an Eclipse plus C18 column, 2.1× 100 mm, 3.5 µm (Agilent, CA). A binary gradient, consisting of 0.2 mM ammonium fluoride in water (mobile phase A) and methanol (mobile phase B), was applied. The linear gradient started at 35 % solvent B and increased to 98 % within 12.5 minutes and remained at the level for 30 seconds and then was reduced to 10 % with a flow rate of 0.3 mL.minutes^{-1} for 15 minutes total running time.

The instrument was run under scheduled MRM method with Analyst software version 1.6.2 (ABSciex). Corticosterone, progesterone, testosterone and derivatized E2 were monitored in positive ion electrospray ionization mode, while estrone was detected in negative ion electrospray ionization mode. Curtain gas and collision gas were set at 35 kPa and 12 kPa, respectively. Ion spray voltages in positive and negative modes were 5250 V and 4400 V, respectively. Ion source temperature values in positive and negative modes were set at 550 ºC and 500 ºC, respectively. MRM transitions and other optimized parameters for de-clustering potential, collision energy and collision cell exit potential are listed in **SI Appendix, Table S10**.

Each steroid hormone was quantified based on a standard curve specific for that steroid. Derivatized E2 was quantified according to the standard curve of derivatized calibration solution. Duplicate quality control samples and methanol spiked with internal standards were used to

verify the precision and accuracy of detection (20). Blank samples made up of methanol were interspersed between samples to wash the instrument and to avoid carryover. Acquired data were further processed to generate calibration curves and quantify hormones by means of MultiQuant software ver. 3.0.1 (ABSciex). A weighting factor of 1/x was applied to the data (15) and linearity of the standard curves was verified by least square linear regression analysis (16, 17).

Integrative Correlation Analyses. We used the mixOmics R package (21) to correlate the gene expression, nontargeted metabolomics, neurotransmitters, steroid hormones, placenta histology, and 5-HT-ir and dopamine-immunoreactivity in trophoblast giant cells (GCs). We conducted sparse discriminant analysis with partial least square regression with function 'block.splsda'. The circos plot was generated by using the 'circosPlot' function with correlations calculated by the method described by González, et al. (22). A correlation coefficient ≥ 0.80 was used as the cutoff.

Supplementary Figures

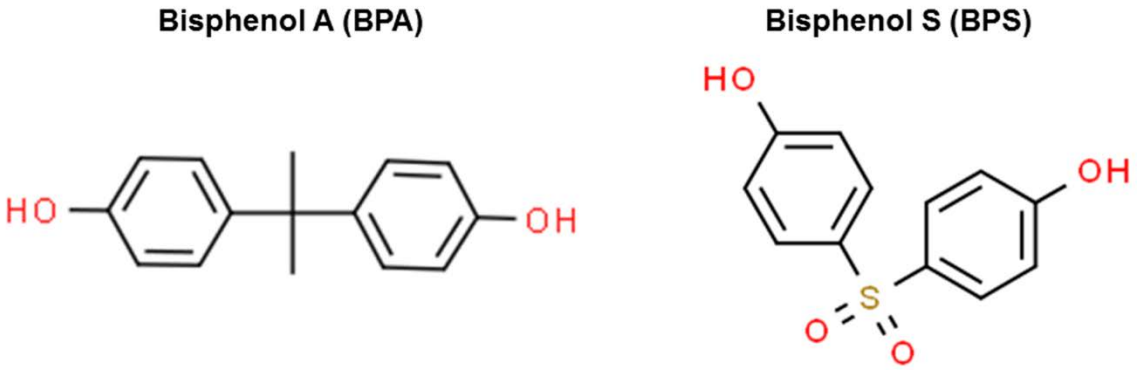

Fig. S1. Chemical structures for BPA and BPS. These figures were generated according to reference (23).

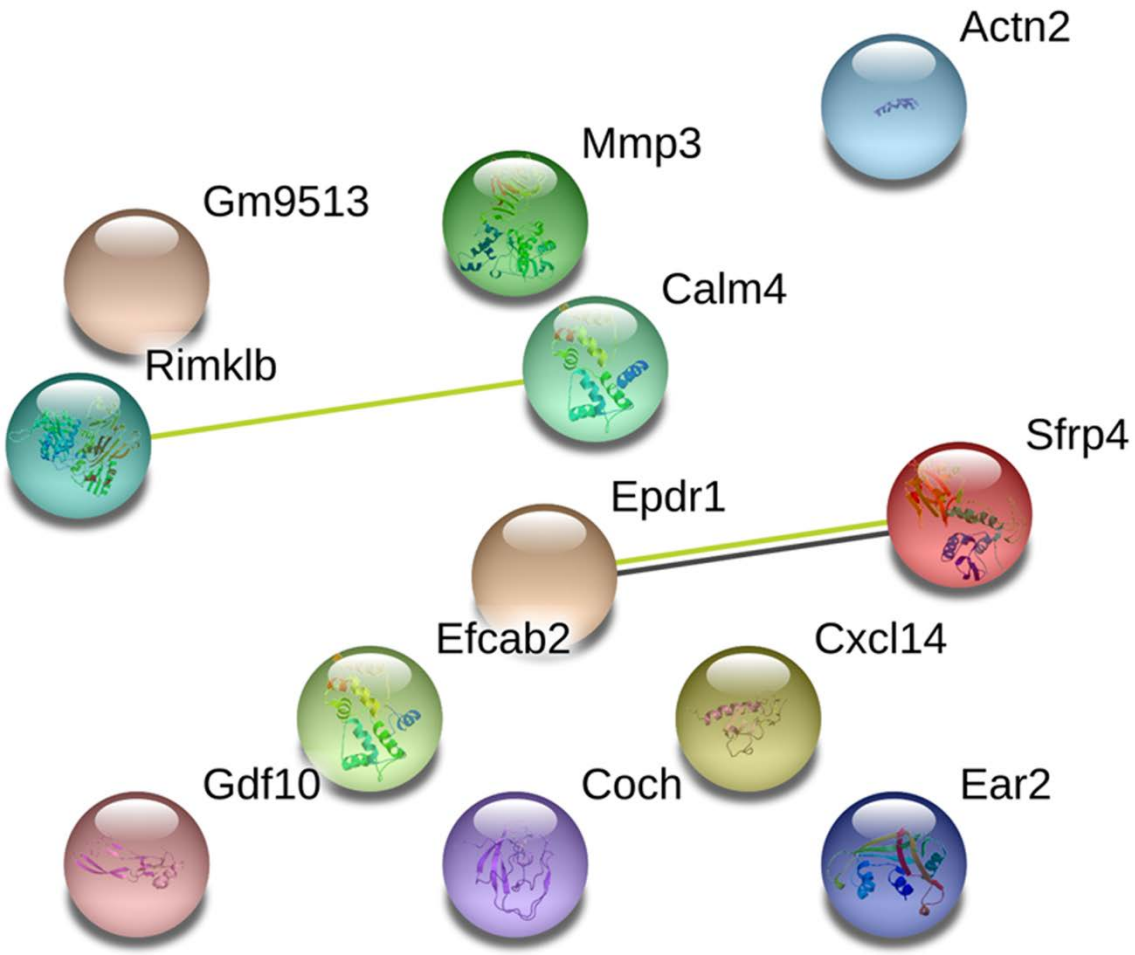

Fig. S2. STRING PPI interaction for differentially expressed (DE) genes in BPA/BPS vs. control placenta. Two PPI interactions were identified for DE genes in BPS vs control placenta. These included *Rimk1b* to *Calm4* (Co-mentioned in PubMed Abstracts, combined score= 0.49) and *Epdr* to *Sfrp4* (Co-expressed and co-mentioned in PubMed Abstracts, combined score = 0.5).

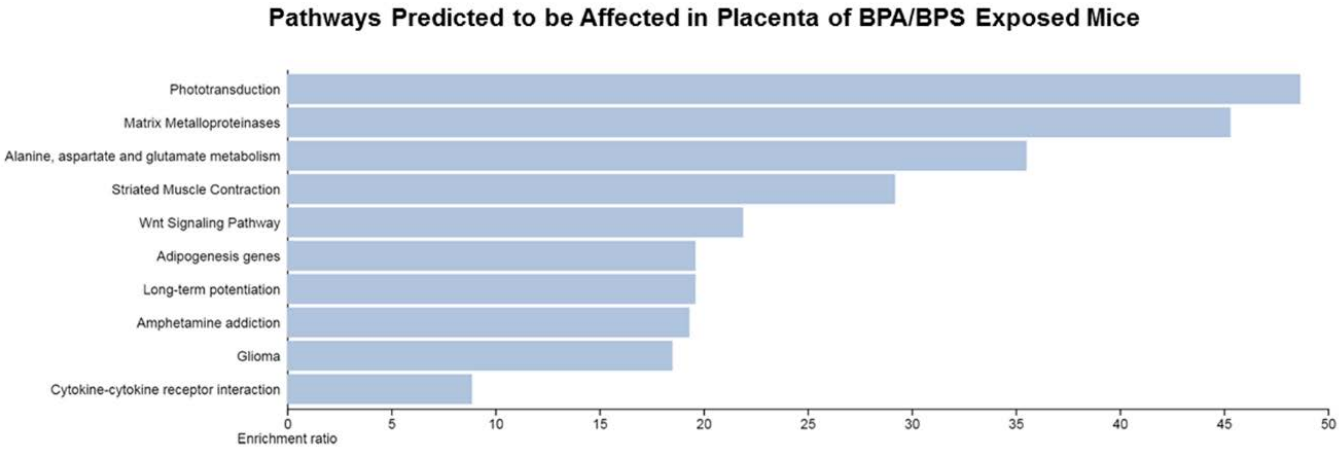

Fig. S3. Pathways predicted to be affected in placenta of BPA/BPS exposed mice. Primary pathways predicted to be affected in BPA/BPS exposed placenta include matrix metalloproteinases, Wingless Int-1 (Wnt) signaling pathway, cytokine-cytokine receptor interaction.

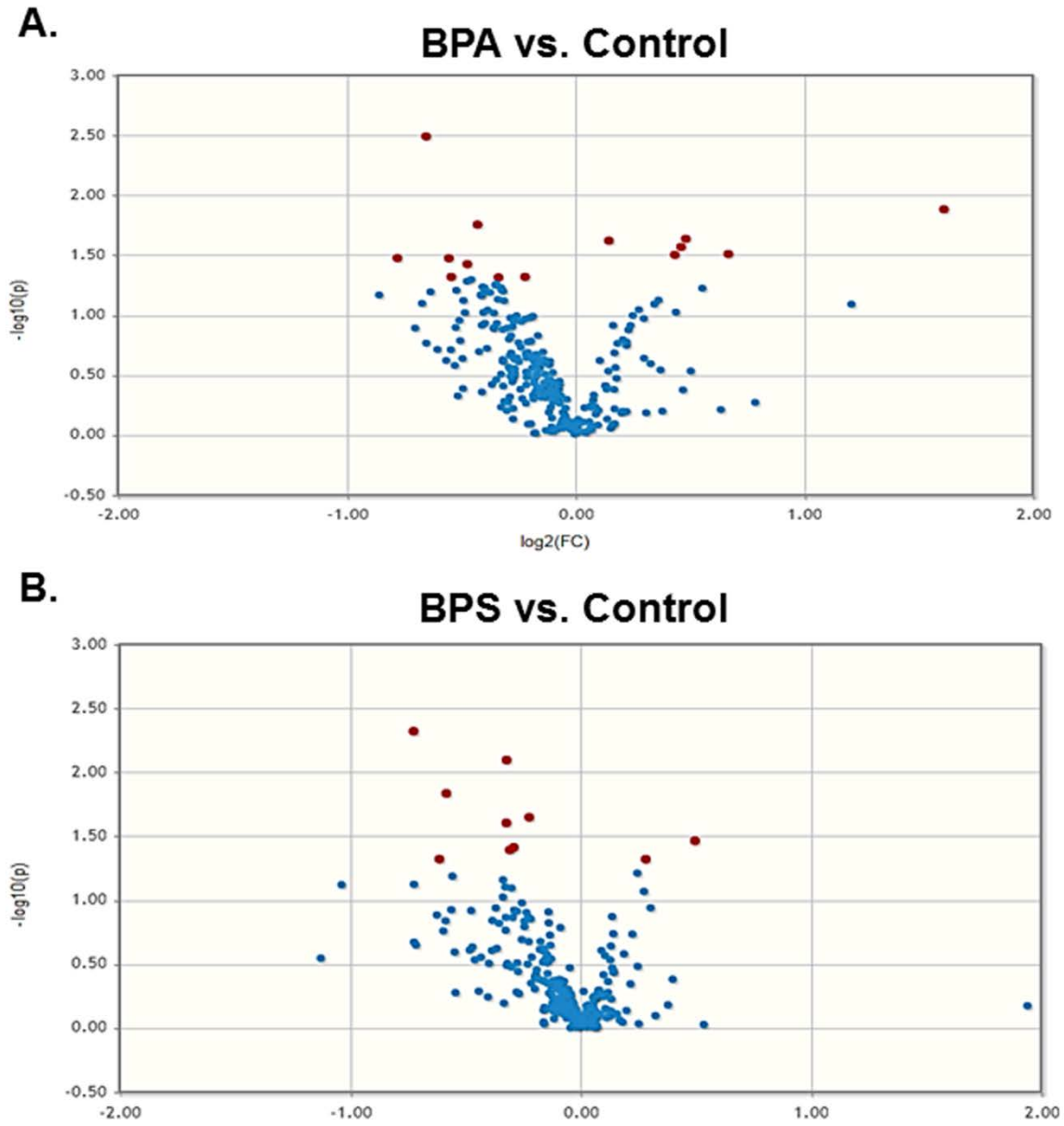

Fig. S4. Volcano plot analyses for BPA/BPS exposed vs. control placenta. A) BPA vs. control placenta. B) BPS vs. control placenta. Blue dots indicate the metabolites that are not significant for the group comparison. Red dots indicate the metabolites that are significant in BPA or BPS vs. control placenta ($P \leq 0.05$).

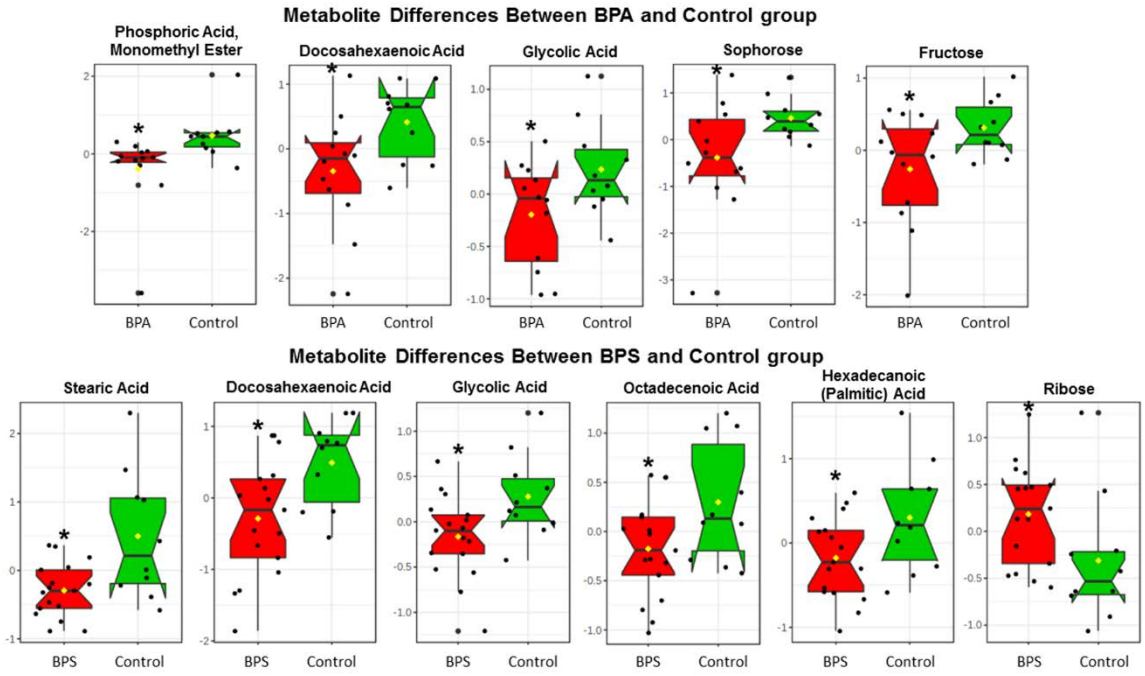

Fig. S5. Example of metabolites that are altered with most being reduced in the placenta of BPA/BPS females compared to control females. Several fatty acids, amino acid, and cholesterol were reduced in BPS females compared to controls, thereby resulting in perturbations of two major pathways affected in this group, including biosynthesis of unsaturated fatty acids and fatty acid biosynthesis. N = 5-8 biological replicates/group. *$P \leq 0.05$.

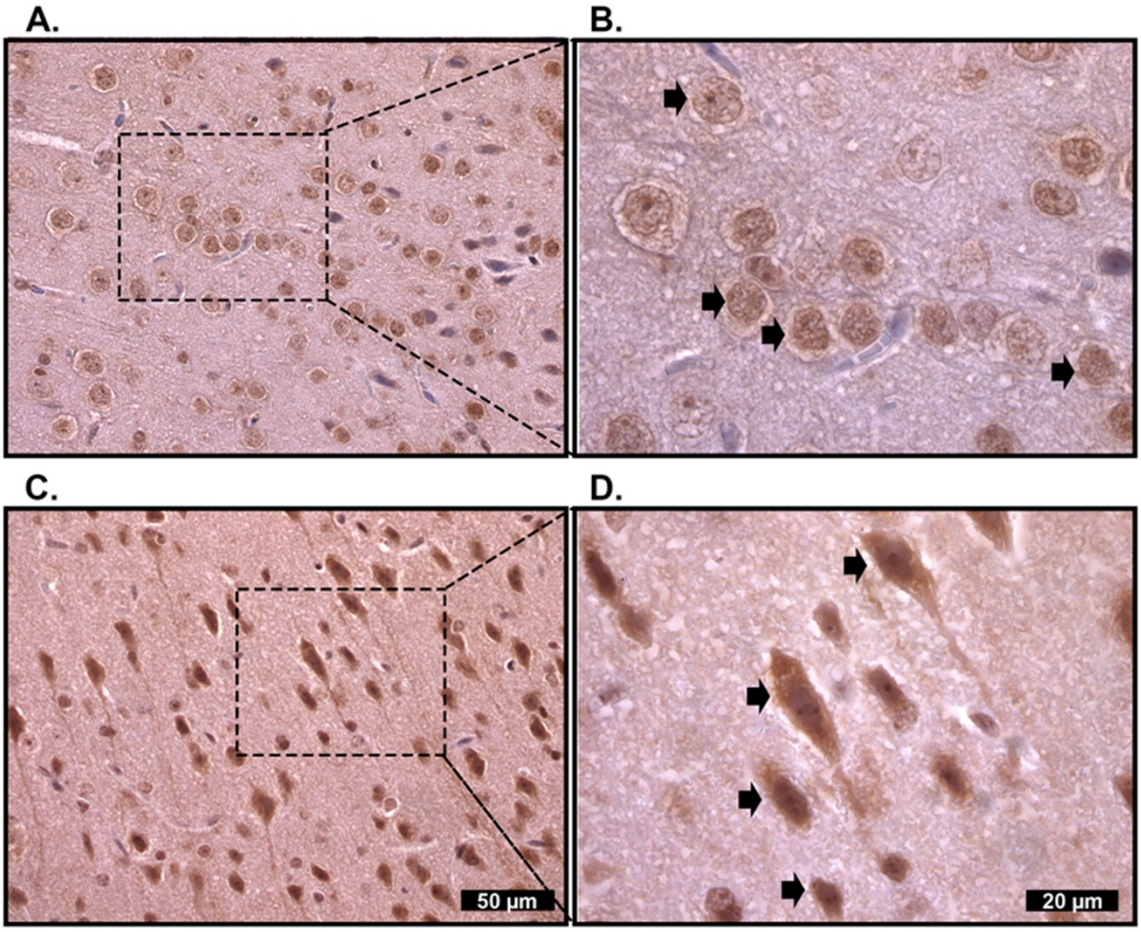

Fig. S6. Serotonin (5-HT)-immunoreactivity and dopamine-immunoreactivity in fetal mouse brain. As shown in panels A and B, fetal neurons are immunoreactive for 5-HT. Likewise, neurons also stain for dopamine (panels C and D).

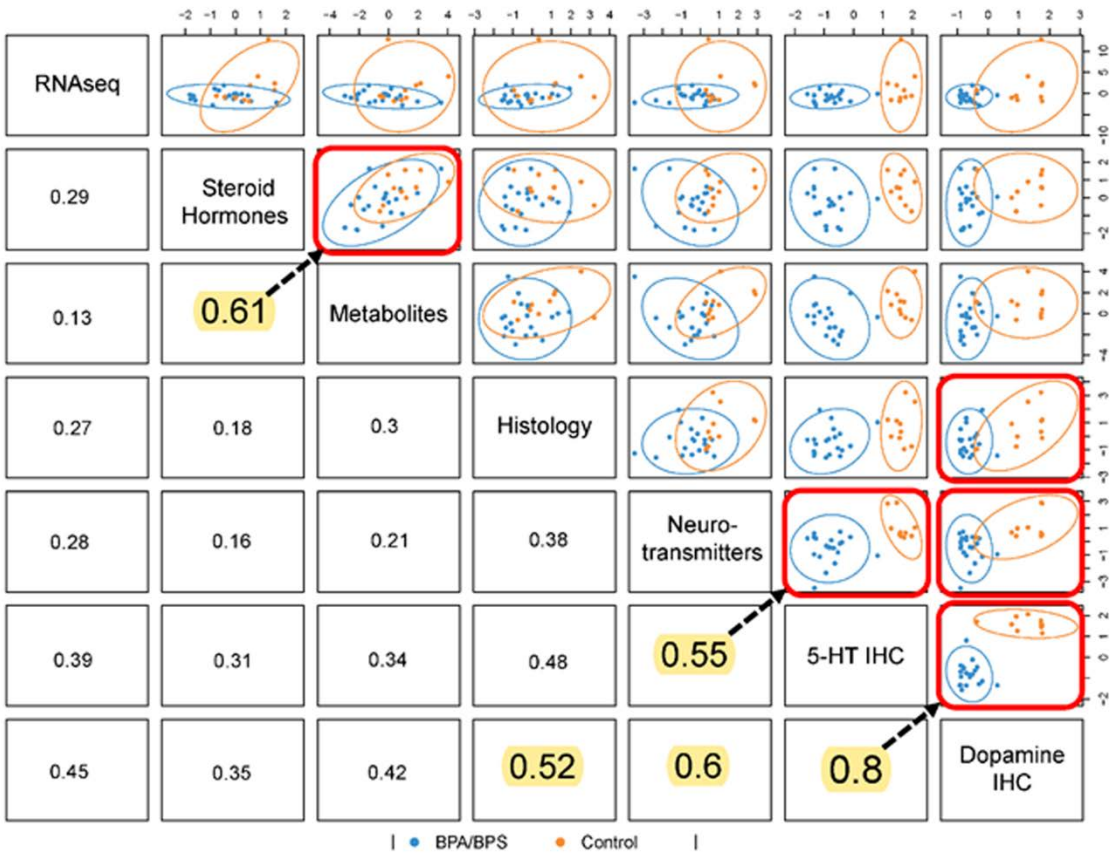

Fig. S7. R plot correlations and principal component analysis (PCA) diagrams for BPA vs. control placenta across RNAseq, steroid hormone, metabolite, placenta histological proportions, neurotransmitters, 5-HT IHC, and dopamine IHC. BPA/BPS and control values are designated in blue and orange, respectively. As shown, overall steroid hormones and non-targeted metabolites show a strong correlation, r = 0.61. Neurotransmitters and 5-HT IHC are significantly correlated, r = 0.55. Neurotransmitters and dopamine IHC are also correlated, r = 0.6. 5-HT IHC and dopamine IHC are strongly correlated, r = 0.8. Placental histological and dopamine IHC results show some correlation as well, r = 0.52.

Supplementary Tables

Table S1. Total number of litters and conceptuses for each group

Treatment	Total # of litters	Total # of conceptuses
Control	5	47
BPA	7	64
BPS	8	69

Table S2. Comparison of pregnancy success, maternal gestational weight gain from embryonic age (e)0.5 to e12.5, number of implantation sites, number of fetuses, and % male conceptuses in AIN controls, BPA, and BPS treatment groups.

	Treatment		
Parameter	**AIN**	**BPA**	**BPS**
Pregnancy success	71.4%	70.0%	80.0%
• *P value relative to AIN*		0.95	0.68
Average maternal gestational weight gain (g)	8.0 ± 0.7	8.1 ± 0.3	8.6 ± 0.5
• *P value relative to AIN*		0.97	0.5
Average # of implantation sites (total and resorbed)	9.4 ± 0.5	9.1 ± 0.6	8.6 ± 0.7
• *P value relative to AIN*		0.56	0.8
Average # of fetuses	9.0 ± 0.3	8.9 ± 0.5	8.1 ± 0.6
• *P value relative to AIN*		0.89	0.3
% male conceptuses	42.9 ± 7.9	56.9 ± 4.7	50.8 ± 7.7
• *P value relative to AIN*		0.06	0.28
• *P value relative to expected 1:1 sex ratio*	0.53	0.37	0.93

Table S3. Alignment details for each RNAseq dataset.

Sample Number	Treatment	Sex	#Total Reads	% Alignment	#Total Mapped Reads	#Total Filtered Reads
1	BPS	Female	94262947	96.27%	90746939	89596347
2	BPS	Male	112882172	96.85%	109326384	107062042
4	BPS	Female	131865896	97.31%	128318703	126109508
5	BPA	Female	122009090	97.28%	118690443	115848534
6	BPA	Male	112121913	96.97%	108724619	107116958
8	BPA	Female	127276281	96.67%	123037980	120201771
9	CTL	Male	115446270	97.06%	112052150	109939713
10	CTL	Female	128354269	96.99%	124490806	121628413
12	CTL	Female	115380537	97.21%	112161420	108860209
13	BPA	Male	130991249	97.14%	127244899	122926719
14	BPA	Female	116458911	97.38%	113407687	110719119
15	BPA	Female	128455162	97.16%	124807035	122091740
16	BPA	Male	128751353	97.00%	124888812	122563589
17	CTL	Female	132488327	97.31%	128924391	126166581
18	CTL	Male	120367483	97.17%	116961083	114565962
19	BPS	Male	136293826	96.81%	131946053	128578922
20	BPS	Female	127285031	97.21%	123733778	120548394
21	BPS	Male	123050622	97.07%	119445239	116747205
22	BPS	Female	142716383	96.93%	138334990	134286433
23	BPS	Female	130763476	97.21%	127115175	124321528
24	BPS	Male	118953934	96.58%	114885709	111878757
25	CTL	Female	104835125	97.18%	101878774	99585339
26	CTL	Male	110774799	97.37%	107861421	105302499
27	CTL	Male	126397870	97.29%	122972487	119480788
28	CTL	Female	112364669	97.55%	109611735	106451572
29	BPA	Male	124399281	96.96%	120617543	117233326
30	BPA	Female	131539633	97.00%	127593444	124626516
32	BPA	Male	131816416	96.82%	127624654	124085088
33	CTL	Male	125307152	96.62%	121071770	117285009
34	BPS	Male	145799171	96.99%	141410616	137861230
2001L2	BPS	Male	152052018	97.09%	147629917	143847411
2001L3	BPS	Female	136973004	97.29%	133266311	129602072
3002R2	BPS	Male	148887513	97.28%	144841873	140659470
3002R3	BPS	Female	133800315	96.81%	129529330	126276611
3004L2	BPS	Male	135063162	96.75%	130667812	127084333
3004L3	BPS	Female	124849981	97.27%	121435373	118552238
3015L3	BPA	Female	135063162	97.12%	134820506	131531114
3015L4	BPA	Male	132187735	97.43%	128789087	125881733
3018R3	BPA	Male	137529158	96.86%	133208647	129507993
3018R4	BPA	Female	141162189	97.24%	137272758	133228543
Average			**127174437.1**	**97.06%**	**123533708.8**	**120496033.2**

Table S4a. Fold change of placental gene expression based on BPA treatment X sex effects.

	Female				Male			
Gene Symbol	**P value***	**FDR**	**Log2 Fold Change**	**Fold-Change**	**P value**	**FDR**	**Log2 Fold Change**	**Fold-Change**
Actn2	4.76E-01	1.00E+00	-0.19	-1.15	1.43E-02	8.61E-01	0.69	1.62
Calm4	1.03E-01	1.00E+00	-1.47	-2.77	4.83E-03	8.61E-01	-3.13	-8.73
Coch	4.94E-02	1.00E+00	-1.61	-3.06	1.22E-02	8.61E-01	-2.34	-5.07
Cxcl14	5.44E-01	1.00E+00	-0.47	-1.39	2.49E-05	3.55E-01	-3.64	-12.44
Ear2/ NR2F6	2.61E-01	1.00E+00	-1.21	-2.32	NA	NA	-3.90	-14.89
Efcab2	8.20E-01	1.00E+00	-0.03	-1.02	6.61E-05	3.55E-01	0.61	1.53
Epdr1	9.21E-01	1.00E+00	0.04	1.03	1.00E-04	4.04E-01	-2.00	-3.99
Gdf10	1.23E-01	1.00E+00	-0.61	-1.53	NA	NA	-2.31	-4.96
Gm9513/ PATE1	NA**	NA	-2.87	-7.32	4.61E-01	8.80E-01	-0.75	-1.68
Guca2a	2.72E-01	1.00E+00	-1.26	-2.4	NA	NA	-3.61	-12.21
Mmp3	1.60E-01	1.00E+00	-1.03	-2.04	2.84E-03	8.61E-01	-2.54	-5.8
Rimklb	2.46E-01	1.00E+00	-0.53	-1.45	8.01E-03	8.61E-01	-1.80	-3.48
Sfrp4	1.45E-01	1.00E+00	-1.41	-2.65	NA	NA	-5.41	-42.62

*While some genes appear to be different based on maternal BPA exposure X sex interactions, none of them are different based on a false discovery rate (FDR).

**Not applicable (NA) values are reported by DESeq2 when an extreme count outlier is detected.

Table S4b. Fold change of placental gene expression based on BPS treatment X sex effects.

	Female				Male			
Gene Symbol	**P value***	**FDR**	**Log2 Fold Change**	**Fold-Change**	**P value**	**FDR**	**Log2 Fold Change**	**Fold-Change**
Actn2	9.37E-01	9.95E-01	-0.02	-1.02	5.84E-05	4.71E-01	1.12	2.17
Calm4	2.47E-01	9.61E-01	-1.04	-2.07	1.80E-04	6.04E-01	-4.16	-17.87
Coch	1.70E-01	9.61E-01	-1.12	-2.18	2.94E-04	6.04E-01	-3.38	-10.42
Cxcl14	7.78E-01	9.78E-01	-0.22	-1.17	7.57E-04	8.48E-01	-2.90	-7.49
Ear2/ NR2F6	9.37E-01	9.95E-01	-0.08	-1.06	NA	NA	-4.14	-17.65
Efcab2	6.27E-01	9.66E-01	0.06	1.05	1.06E-01	8.48E-01	0.25	1.19
Epdr1	5.99E-01	9.62E-01	-0.21	-1.17	2.02E-04	6.04E-01	-1.91	-3.75
Gdf10	5.96E-01	9.62E-01	-0.21	-1.16	NA	NA	-2.52	-5.75
Gm9513/ PATE1	NA**	NA	-1.54	-2.91	4.10E-03	8.48E-01	-2.91	-7.52
Guca2a	4.38E-01	9.61E-01	-0.89	-1.86	NA	NA	-4.45	-21.92
Mmp3	8.02E-01	9.79E-01	-0.18	-1.13	3.54E-04	6.04E-01	-3.06	-8.37
Rimklb	2.40E-01	9.61E-01	-0.54	-1.46	4.55E-04	6.67E-01	-2.37	-5.19
Sfrp4	4.01E-01	9.61E-01	-0.81	-1.76	NA	NA	-5.00	-32.11

*While some genes appear to be different based on maternal BPS exposure X sex interactions, none of them are different based on a false discovery rate (FDR).
**Not applicable (NA) values are reported by DESeq2 when an extreme count outlier is detected.

Table S5. Fold change of placental gene expression based on maternal treatment X sex interactions in qPCR assays.

	Treatment					
	Control		BPA		BPS	
Gene	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**
Actn2	0.83 ± 0.05	1.21 ± 0.09	0.27 ± 0.03	0.32 ± 0.03	0.36 ± 0.04	0.33 ± 0.03
Ascl2	1.03 ± 0.02	0.97 ± 0.03	0.72 ± 0.02	0.77 ± 0.04	0.80 ± 0.02	0.83 ± 0.03
Calm4	1.06 ± 0.17	0.94 ± 0.17	0.29 ± 0.08	0.27 ± 0.16	0.32 ± 0.17	0.21 ± 0.09
Coch	1.05 ± 0.13	0.96 ± 0.13	0.26 ± 0.10	0.55 ± 0.13	0.60 ± 0.12	0.26 ± 0.06
Cxcl14	0.99 ± 0.16	1.01 ± 0.18	0.27 ± 0.12	0.46 ± 0.11	0.43 ± 0.11	0.28 ± 0.08
Ear2	0.99 ± 0.16	1.01 ± 0.18	0.27 ± 0.12	0.46 ± 0.11	0.43 ± 0.11	0.28 ± 0.08
Epdr	0.89 ± 0.10	1.13 ± 0.09	0.49 ± 0.07	0.39 ± 0.06	0.54 ± 0.05	0.37 ± 0.05
Gdf10	0.96 ± 0.04	1.04 ± 0.08	0.38 ± 0.03	0.57 ± 0.03	0.76 ± 0.02	0.42 ± 0.02
GM9513	1.12 ± 0.06	0.89 ± 0.05	0.23 ± 0.05	0.30 ± 0.08	0.11 ± 0.10	0.09 ± 0.06
Guca2	1.31 ± 0.16	0.77 ± 0.13	0.35 ± 0.07	0.25 ± 0.12	0.27 ± 0.15	0.16 ± 0.08
Mmp3	1.08 ± 0.91	0.97 ± 0.47	0.16 ± 0.05	0.32 ± 0.25	0.30 ± 0.11	0.11 ± 0.03
Rimklb	1.01 ± 0.27	0.99 ± 0.22	0.31 ± 0.10	0.38 ± 0.17	0.45 ± 0.19	0.27 ± 0.08
Sfrp4	0.85 ± 0.11	1.18 ± 0.12	0.30 ± 0.09	0.23 ± 0.10	0.60 ± 0.11	0.27 ± 0.04

Table S6. Concentrations of gabba aminobutyric acid (GABA) in placental samples (mean ± SEM).

Treatment Group	**GABA (pmol/mg)**
Control	21.59 ± 0.92
BPA	24.89 ± 1.09
BPS	24.23 ± 0.99

Table S7. Placental concentrations of other steroid hormones (mean ± SEM).

Treatment Group	**Corticosterone (pg/mg)**	**Estrone (pg/mg)**	**Progesterone (pg/mg)**	**Testosterone (pg/mg)**
Control	229.70 ± 57.80	1.62 ± 0.11	610.02 ± 255.01	4.91 ± 3.32
BPA	139.64 ± 26.09	1.45 ± 0.16	408.36 ± 107.06	2.96 ± 0.85
BPS	172.62 ± 24.9	1.71 ± 0.16	370.63 ± 70.06	7.54 ± 4.30

Table S8. Primer sequences for test genes and housekeeping gene (*Polr2a*, *Rpl7*, and *Ubc*) analyzed via qPCR in placental tissues. The average efficiency for all primers was 100.3 ± 2.2%.

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
Actn2	CATCGAGGAGGATTTCAGGAAC	CAATCTTGTGGAACCGCATTTT
Ascl2	CCGTGAAGGTGCAAACGTC	CCCTGCTACGAGTTCTGGTG
Calm4	GCGGGCTGTGTTCAATGTC	GGGACTCGCCTAACTTGGAC
Coch	TGCTGTCCATAGGGGAGTGAT	CCGAGGAGTAGTTCTCTCGAC
Cxcl14	AGTGTAAGTGTTCCCGGAAGG	GCAGTGTGGGTACTTTGGCTT
Ear2	ACAAGTCCAGTGGAAAGCATTAC	CGGCAGGTGTAGCTGAGATT
Epdr1	TGTGCAGCTAGGCATTAAGGA	GAGCAGCCGTCACTCATCTT
Gdf10	CAGGACATGGTCGCTATCCAC	ACAGGCTTTTGGTCGATCATTTC
Gm9513	CTCGGAGAATTTGTGAGGGGA	GAAGCAAAGGTCAGCACATCT
Guca2a	CAGACTGGTGAGTCACAAGAAG	CAGAGTGGCTACTACATAGCTGT
Mmp3	GTTCTGGGCTATACGAGGGC	TTCTTCACGGTTGCAGGGAG
Polr2a	CTTTGAGGAAACGGTGGATGTC	TCCCTTCATCGGGTCACTCT
Rimklb	CGGATCAGTGGAGAGCTAATCT	GTGGCGCAAAACAGTAATATCAC
Rpl7	AGCTGGCCTTTGTCATCAGAA	GACGAAGGAGCTGCAGAACCT
Sfrp4	TCCATCCTGGTGGCGTTATG	GCATCCGGGTGATGTTCCA
Ubc	ACCAGCAGAGGCTGATCTTT	ACCTCTGAGGCGAAGGACTA

Table S9. MRM and other parameters for quantification of neurotransmitters and internal standard.

Compound	Start Time (minute)	End Time (minute)	Precursor (m/z)	Product (m/z)	Collision Energy (V)	RF Lens (V)
GABA	0	0.9	104.03	45.00	22.06	32
GABA	0	0.9	104.03	69.00	16.45	32
GABA	0	0.9	104.03	87.00	10.23	32
DA	0	0.9	154.05	91.00	24.86	34
DA	0	0.9	154.05	118.92	19.21	34
DA	0	0.9	154.05	137.00	10.87	34
5-HT	0.9	2	177.01	115.00	29.60	32
5-HT	0.9	2	177.01	132.00	22.70	32
5-HT	0.9	2	177.01	160.00	10.23	32
5-HIAA	1.8	3	191.93	91.00	38.02	57
5-HIAA	1.8	3	191.93	118.00	30.70	57
5-HIAA	1.8	3	191.93	146.00	16.98	57
Lidocaine	2	6	235.22	58.00	32.97	61
Lidocaine	2	6	235.22	86.00	17.76	61

Table S10. MRM and other criteria of steroid hormones*.

Q1 Mass (Da)	Q3 Mass (Da)	Retention Time (minute)	Steroid Hormone*	DP (volts)	CE (volts)	CPX (volts)
347	105	6.4	Corticosterone	80	32	12
315	109	9.2	Progesterone	80	32	14
289	97	7.6	Testosterone	100	27	12
506	171	12.4	E2-derivatized	80	32	12
269	145	7.3	Estrone	-130	-48	-17

*Two transitions were considered for each hormone, one for quantitation and the second for qualification. DP, Declustering potential; CE, Collision energy; CPX, Collision cell exit potential

Supplementary Datasets

Dataset S1. Complete list of all identified metabolites in the BPA vs. control and BPS vs. control comparisons. Those that differ for these two comparisons are highlighted.

References Cited in Supplementary Appendix:

1. P. Koopman, J. Gubbay, N. Vivian, P. Goodfellow, R. Lovell-Badge, Male development of chromosomally female mice transgenic for Sry. *Nature* **351**, 117-121 (1991).
2. M. Martin, Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal; Vol 17, No 1: Next Generation Sequencing Data AnalysisDO - 10.14806/ej.17.1.200* (2011).
3. D. Kim, B. Langmead, S. L. Salzberg, HISAT: a fast spliced aligner with low memory requirements. *Nat Methods* **12**, 357-360 (2015).
4. S. Anders, P. T. Pyl, W. Huber, HTSeq--a Python framework to work with high-throughput sequencing data. *Bioinformatics* **31**, 166-169 (2015).
5. B. Li, C. N. Dewey, RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* **12**, 323 (2011).
6. M. I. Love, W. Huber, S. Anders, Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* **15**, 550 (2014).
7. A. Jain, G. Tuteja, TissueEnrich: Tissue-specific gene enrichment analysis. *Bioinformatics* **35**, 1966-1967 (2019).
8. Y. Shen *et al.*, A map of the cis-regulatory sequences in the mouse genome. *Nature* **488**, 116-120 (2012).
9. D. Szklarczyk *et al.*, STRING v10: protein-protein interaction networks, integrated over the tree of life. *Nucleic Acids Res* **43**, D447-452 (2015).
10. J. Wang, D. Duncan, Z. Shi, B. Zhang, WEB-based GEne SeT AnaLysis Toolkit (WebGestalt): update 2013. *Nucleic Acids Res* **41**, W77-83 (2013).

11. M. E. Solano, K. Thiele, M. K. Kowal, P. C. Arck, Identification of suitable reference genes in the mouse placenta. *Placenta* **39**, 7-15 (2016).
12. J. M. Halket *et al.*, Deconvolution gas chromatography/mass spectrometry of urinary organic acids--potential for pattern recognition and automated identification of metabolic disorders. *Rapid Comm Mass Spec* **13**, 279-284 (1999).
13. L. Lei, L. H., J. Chang, P. X. Zhao, L. W. Sumner, MET-IDEA version 2.06; improved efficiency and additional functions for mass spectrometry-based metabolomics data processing. *Metabolomics* **8**, 105-110 (2012).
14. C. D. Broeckling, I. R. Reddy, A. L. Duran, X. Zhao, L. W. Sumner, MET-IDEA: data extraction tool for mass spectrometry-based metabolomics. *Anal Chem* **78**, 4334-4341 (2006).
15. J. Vitku *et al.*, Development and validation of LC-MS/MS method for quantification of bisphenol A and estrogens in human plasma and seminal fluid. *Talanta* **140**, 62-67 (2015).
16. S. W. Blue *et al.*, Simultaneous quantitation of multiple contraceptive hormones in human serum by LC-MS/MS. *Contraception* **97**, 363-369 (2018).
17. N. W. Gaikwad, Ultra performance liquid chromatography-tandem mass spectrometry method for profiling of steroid metabolome in human tissue. *Anal Chem* **85**, 4951-4960 (2013).
18. D. A. Armbruster, T. Pry, Limit of blank, limit of detection and limit of quantitation. *Clin Biochem Rev* **29 Suppl 1**, S49-52 (2008).
19. R. E. Nelson, S. K. Grebe, O. K. DJ, R. J. Singh, Liquid chromatography-tandem mass spectrometry assay for simultaneous measurement of estradiol and estrone in human plasma. *Clin Chem* **50**, 373-384 (2004).
20. B. K. Matuszewski, M. L. Constanzer, C. M. Chavez-Eng, Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. *Anal Chem* **75**, 3019-3030 (2003).
21. F. Rohart, B. Gautier, A. Singh, K.-A. Le Cao, mixOmics: An R package for 'omics feature selection and multiple data integration. *PLoS Comput Biol* **13**, e1005752 (2017).
22. I. González, K.-A. Lê Cao, M. J. Davis, S. Déjean, Visualising associations between paired 'omics' data sets. *BioData Mining* **5**, 19 (2012).
23. http://www.chemspider.com/