Sex-specific Neurodevelopmental Programming by Placental Insulin Receptors on Stress Reactivity and Sensorimotor Gating

Supplemental Information

CONTENTS

SUPPLEMENTAL METHODS

Hypothalamic-pituitary-adrenal (HPA) Axis Assessment. Plasma corticosterone was assessed at baseline and after 15-min acute restraint stress in a 50 ml conical tube. Restraint was initiated between 2-3 h after lights-on. Blood was collected into EDTA-containing tubes via tail nick at 0, 15, 30, and 120 min after onset of restraint, centrifuged, and plasma stored at -80 C. Plasma corticosterone was quantified in duplicate by radioimmunoassay (MP Biomedicals, Santa Ana, CA).

Prepulse Inhibition of Acoustic Startle (PPI). PPI was recorded in SR-LAB startle chambers (San Diego Instruments, San Diego, CA) between 2-6 h after lights-off as described (1). Test sessions were comprised of 5 min acclimation to background noise (65 dB), 5 consecutive habituation tones (40 ms duration; 120 dB), and ten pseudorandom repetitions of each of the following stimulus types: startle pulse only (40 ms; 120 dB), no stimulus (65 dB background), and prepulses (20 ms; +4, +8, or +16 dB above background) preceding the startle pulse (40 ms; 120 dB) by 100 ms. Acoustic startle response (ASR) was defined as the peak startle magnitude recorded during 65 consecutive 1 ms readings following the startle pulse onset. The percentage PPI was calculated as [1-(average response to prepulse + startle)/(average response to startle only)] x 100.

Barnes Maze Test. The Barnes maze test of spatial learning and memory was performed as described (2). The apparatus consisted of a white circular platform with 24 holes evenly dispersed around the perimeter. A target escape box was located in the same hole throughout acquisition trials (1-6) and was rotated 180 degrees for reversal trials (7-9). Distinct visual cues remained in a fixed position around the maze perimeter. Mice underwent 2 trials per day, separated by a 4 h inter-trial interval, with the exception of day 5, during which only one trial was administered. A trial ended upon entry into the target box or after 4 min of elapsed time. If the mouse failed to locate the target, the investigator guided the mouse into the target box and a latency of 240 s was assigned. Trials were video-recorded and scored by a trained experimenter blinded to genotype. Latency to nose-poke into the target box was determined for each trial.

Auditory Fear Conditioning. Auditory fear conditioning was performed during the light phase in sound-attenuating SR-LAB chambers (San Diego Instruments, San Diego, CA) as described

(3). Mice were pre-exposed to the conditioning context (lights on, fan on, grid floor, 65 dB background noise) for 5 min per day, for 2 consecutive days prior to training. Following 2 min of baseline recording on day 3, mice received 3 tone-shock (US-CS) pairings each comprised of a 30 sec, 75 dB tone co-terminating with a 500 msec, 0.5 mA footshock. Trials were separated by a variable intertribal interval of 60-180 sec. The percentage of time spent freezing was calculated by SR-LAB software (San Diego Instruments, San Diego, CA).

Light-dark Exploration Test. The light-dark exploration test was performed during the dark phase (2-5 h after lights-off) in a Plexiglas apparatus divided into a brightly illuminated chamber (300 lux) and a dark chamber (5 lux) as described (4). Upon placement in the center of the light chamber, activity was video-monitored for the 10-min test duration. Time spent in the light compartment and light-dark transitions were quantified by ANY-maze version 4.75 (Stoelting Co., Kiel, WI).

Glucose Tolerance Test. Mice were fasted overnight and injected intraperitoneally between 1-2 h after lights-on with 2.5 mg/g glucose in 0.9% sterile saline. Blood was obtained via tail nick at 0, 15, 30, 60 and 120 min after injection. Glucose was quantified by OneTouch Ultra (Johnson & Johnson, New Brunswick, NJ).

Fetal Brain Metabolomics. Flash frozen E17.5 whole brains were pulverized to a fine powder over liquid nitrogen and sample weights recorded. Lipids and fatty acids were extracted as described (5), and shipped on dry ice to the University of Pennsylvania Diabetes Research Center/Princeton University Metabolomics Core for quantification by LC/MS as described (5; 6).

Placenta Biogenic Amines. The concentration of biogenic amines in E12.5 placenta hemisections was determined by the University of Pennsylvania Children's Hospital Metabolomics Core using an Agilent 1260 Infinity LC system and normalized to protein content.

Protein Isolation. Following Trizol-chloroform isolation of RNA, protein was extracted from the lower organic phenol-chloroform phase per manufacturer's instructions with slight modifications (Thermo Fisher). DNA was precipitated and pelleted by mixing samples with cold 100% ethanol and centrifuging at 6000 rpm for 15 minutes. The supernatant was mixed with 1:4 ratio of chilled isopropanol and incubated at room temperature for 10 minutes and then centrifuged at 12000 rpm for 15 minutes. The pellets were washed three times with 0.3 M guanidine hydrochloride in

95% ethanol, once with cold 100% ethanol, and centrifuged at 12000 rpm for 5 minutes following each wash. After the last wash, pellets were dried and solubilized in equal parts 8M urea in H_2O and 1% SDS in 1M Tris-HCl pH 8 with 5% protease inhibitor and phosphatase inhibitor cocktails (Sigma) and low sonication. Insoluble material was removed following a 3000 rpm spin for 10 minutes. Protein abundance was quantified using Bradford Reagent (Sigma) according to manufacturer's protocol.

Western Blotting. A total of 30 µg placental protein was loaded per lane for gel electrophoresis onto a NuPAGE 10% Bis-Tris gel (Life Technologies). After running and transfer of proteins to a nitrocellulose membrane (Life Technologies), membranes were blocked with Odyssey blocking buffer (Li-Cor) and probed with rabbit anti-insulin Rβ polyclonal antibody (1:500, Santa Cruz C-19), followed by incubation in IRDye800-conjugated donkey anti-rabbit secondary (1:15000, Li-Cor). The membrane was scanned on an Odyssey Imager (Li-Cor) for quantification of fluorescent signal.

Supplemental Table S1. Number of animals and litters per group by experiment.

	Group N (Litter N)			
Experiment	**pWT ♂**	**pKO ♂**	**pWT ♀**	**pKO ♀**
E12.5 placenta InsR mRNA & protein	4 (4)	4 (3)	4 (4)	4 (3)
E17.5 liver & skeletal muscle InsR mRNA	5 (5)	5 (4)	5 (5)	5 (4)
E17.5 tissue weights*	24 (12)	11 (7)	12 (10)	14 (9)
Behavior cohort 1 (LD, HPA, PPI, GTT, PFC)	14 (14)	9 (9)	14 (12)	12 (10)
Behavior cohort 2 (Barnes maze, fear cond.)	8 (8)	10 (10)	10 (10)	10 (10)
CCO assay	7 (7)	8 (8)	8 (8)	8 (8)
E17.5 placenta GSEA	6 (6)	6 (6)	6 (6)	6 (6)
E12.5 brain GSEA	6 (6)	6 (5)	6 (6)	6 (6)

*For analysis of tissue weights, all littermates of the same genotype and sex were averaged.

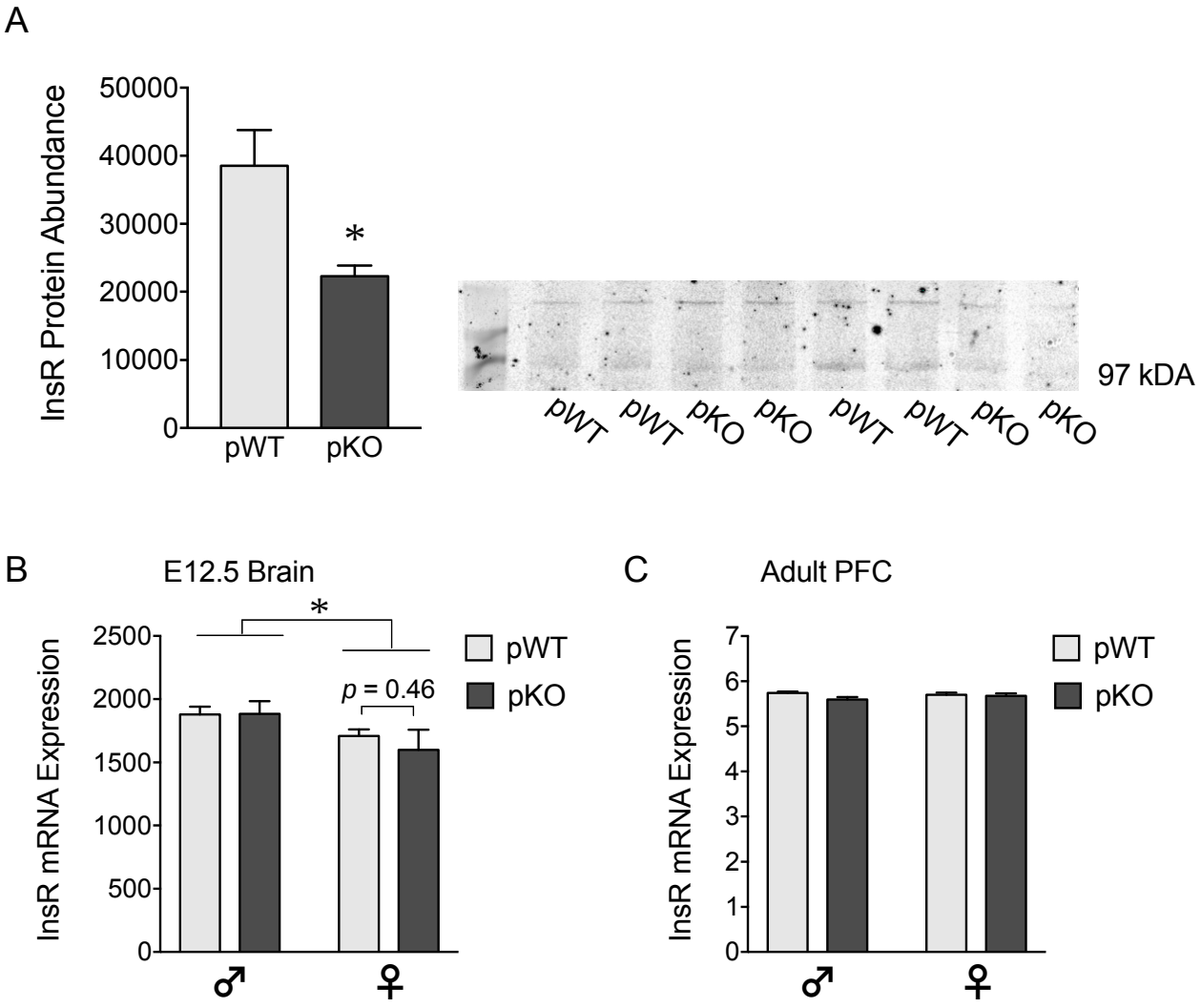

Figure S1. Additional validation of placental trophoblast-specific InsR targeting. (A) In the E12.5 placenta, InsR protein was reduced by approximately 40% in pKO mice relative to pWT [t(6) = 2,97, p = 0.025]. **(B)** In the E12.5 brain, there was no effect of genotype on InsR mRNA expression as detected by whole-genome mRNA-seq [$F_{geno}(1, 20) = 0.26$, $p = 0.62$; $F_{int}(1, 20) = 0.31$, $p = 0.58$]. Regardless of genotype, InsR expression was lower in females at this gestational stage [$F_{sex}(1, 20) = 4.98$, $p = 0.037$]. **(C)** In the adult prefrontal cortex (PFC), there were no significant effects of genotype or sex on InsR mRNA expression [$F_{geno}(1, 24) = 1.55$, $p = 0.090$; $F_{sex}(1, 24) = 0.20$, $p = 0.66$; $F_{int}(1, 24) = 1.55$, $p = 0.22$]. * $p < 0.05$.

Supplemental Table S2. Gene sets downregulated in InsR-deficient male placentas.

Gene set	Type	Size	ES	NES	p	FDR
Lipid homeostasis						
Reactome_Lipoprotein_Metabolism*	c2.cp	26	0.809	2.394	0.000	0.000
Reactome_Lipid_Digestion_Mobilization_and_Transport	c2.cp	41	0.710	2.300	0.000	0.000
Reactome_Chylomicron_Mediated_Lipid_Transport	c2.cp	16	0.832	2.188	0.000	0.001
Lipid_Transport	c5.bp	27	0.724	2.128	0.000	0.001
Regulation_of_Protein_Stability	c5.bp	19	0.732	1.971	0.000	0.011
Lipid_Homeostasis	c5.bp	15	0.744	1.888	0.002	0.026
Vascular function						
BioCarta_AMI_Pathway	c2.cp	19	0.819	2.265	0.000	0.000
Reactome_Formation_of_Fibrin_Clot_Clotting_Cascade	c2.cp	29	0.753	2.223	0.000	0.001
KEGG_Complement_and_Coagulation_Cascades	c2.cp	65	0.633	2.207	0.000	0.001
Reactome_Response_to_Elevated_Platelet_Cytosolic_CA2	c2.cp	73	0.587	2.128	0.000	0.001
BioCarta_Intrinsic_Pathway	c2.cp	22	0.752	2.085	0.000	0.002
Reactome_Platelet_Aggregation_Plug_Formation	c2.cp	34	0.640	1.983	0.000	0.009
PID_Integrin2_Pathway	c2.cp	27	0.667	1.924	0.000	0.019
Reactome_Integrin_AlphaIIb_Beta3_Signaling	c2.cp	26	0.655	1.923	0.000	0.018
PID_UPA_UPAR_Pathway	c2.cp	41	0.593	1.895	0.000	0.026
PID_Integrin3_Pathway	c2.cp	43	0.568	1.852	0.002	0.038
Reactome_Intrinsic_Pathway	c2.cp	15	0.711	1.832	0.002	0.045
PID_EphrinB_Rev_Pathway	c2.cp	29	0.603	1.828	0.000	0.045
Steroid hormone metabolism						
Reactome_Metabolism_of_Steroid_Hormones_Vitamins_A_and_D	c2.cp	34	0.662	2.052	0.000	0.003
Reactome_Steroid_Hormones	c2.cp	28	0.681	1.990	0.000	0.009
Insulin-like growth factor activity						
Reactome_Regulation_of_IGF_Activity_by_IGFBPS	c2.cp	15	0.773	1.993	0.002	0.009
Mitochondrial function						
KEGG_Proximal_Tube_Biocarbonate_Reclamation	c2.cp	22	0.711	1.962	0.000	0.012
Amino acid transport & metabolism						
Reactome_Amino_Acid_Transport_Across_the_Plasma_Membrane	c2.cp	30	0.630	1.855	0.002	0.038
KEGG_Glycine_Serine_and_Threonine_Metabolism	c2.cp	28	0.627	1.845	0.004	0.040
Regulation of transcription						
PID_HNF3B_Pathway	c2.cp	43	0.649	2.129	0.000	0.001
V$HNF1_Q6	c3.tft	227	0.527	2.211	0.000	0.001

* Gene set also downregulated in females

Supplemental Table S3. Gene sets downregulated in InsR-deficient female placentas.

Gene set	Type	Size	ES	NES	p	FDR
Lipid homeostasis						
Reactome_Lipoprotein_Metabolism	c2.cp	26	0.677	2.026	0.002	0.046

* Gene set also downregulated in males

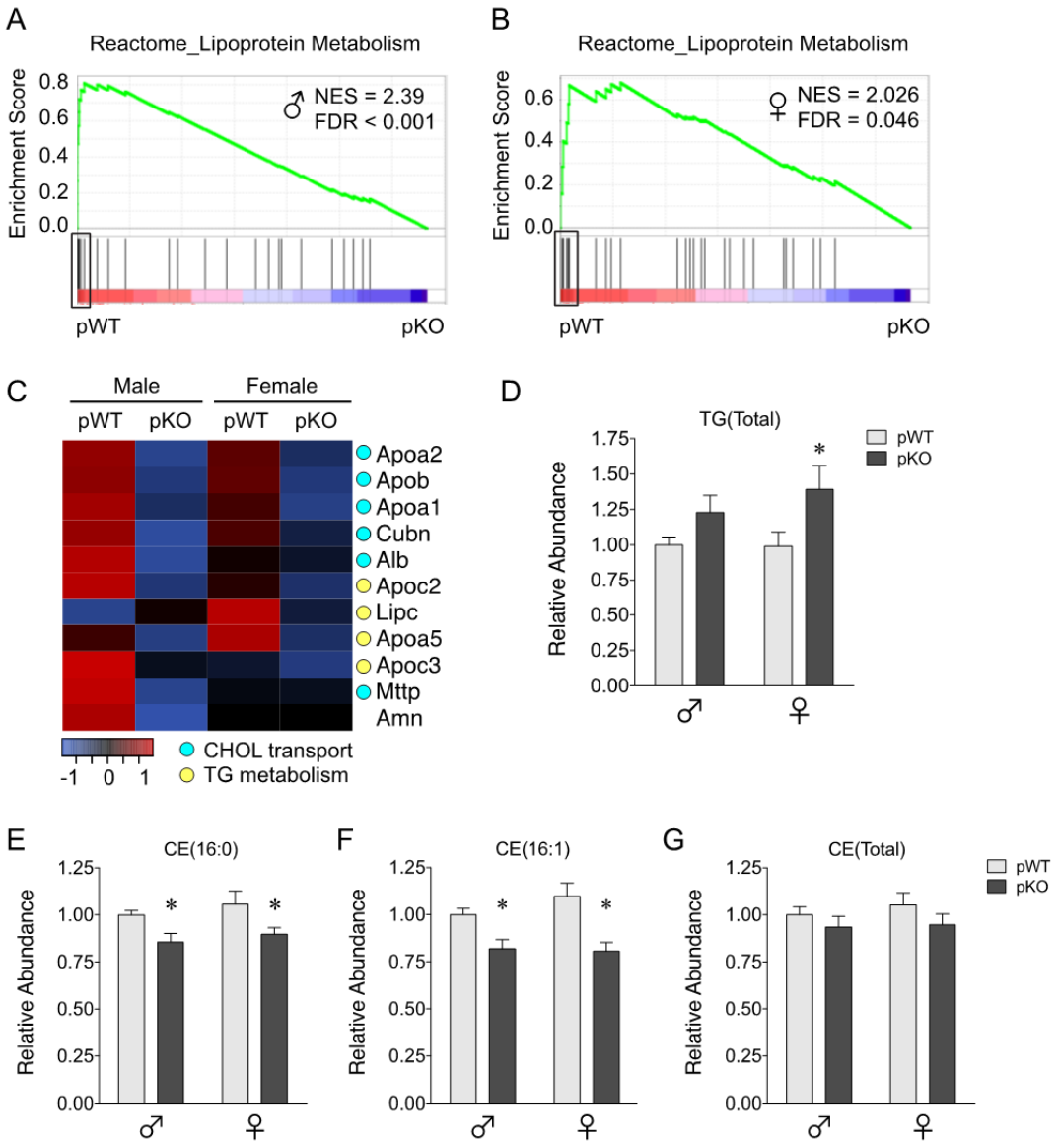

Figure S2. InsR deficiency disrupts lipid transfer in male and female placentas. (A,B) GSEA of E17.5 placenta trancriptome identified one gene set affected in both males **(A)** and females **(B)**, indicating decreased expression of lipoprotein metabolism genes in pKO placentas. Plots depict enrichment score (ES), normalized enrichment score (NES), distribution of gene set members across the GSEA-ranked gene list, and the leading edge subset (boxes). **(C)** Heat map of leading-edge subsets, where blue indicates lower and red indicates higher expression across groups. Notably, the majority of leading-edge genes were suppressed by pKO in both sexes, including mediators of placental cholesterol transport (aqua circles) and triglyceride metabolism (yellow circles). **(D-E)** To evaluate the impact of these placenta transcriptional changes on the developing brain, we performed unsupervised metabolomics analysis of lipids and fatty acids in the E17.5 brain. **(D)** Placental InsR deletion increased triglyceride (TG) levels [$F_{geno}(1, 24) = 7.31$, $p = 0.012$]; however, the post hoc comparison in males did not reach statistical significance ($p = 0.16$). In males and females, pKO decreased levels of 16-carbon cholesteryl esters [CE(16:0): $F_{geno}(1, 24) = 10.21$, $p = 0.0039$; CE(16:1): $F_{geno}(1, 24) = 20.12$, $p = 0.002$], without significantly impacting total CE content [$F_{geno}(1, 24) = 2.20$, $p = 0.15$]. Data are mean (+/- SEM). CHOL, cholesterol. TG, triglyceride. * $p < 0.05$.

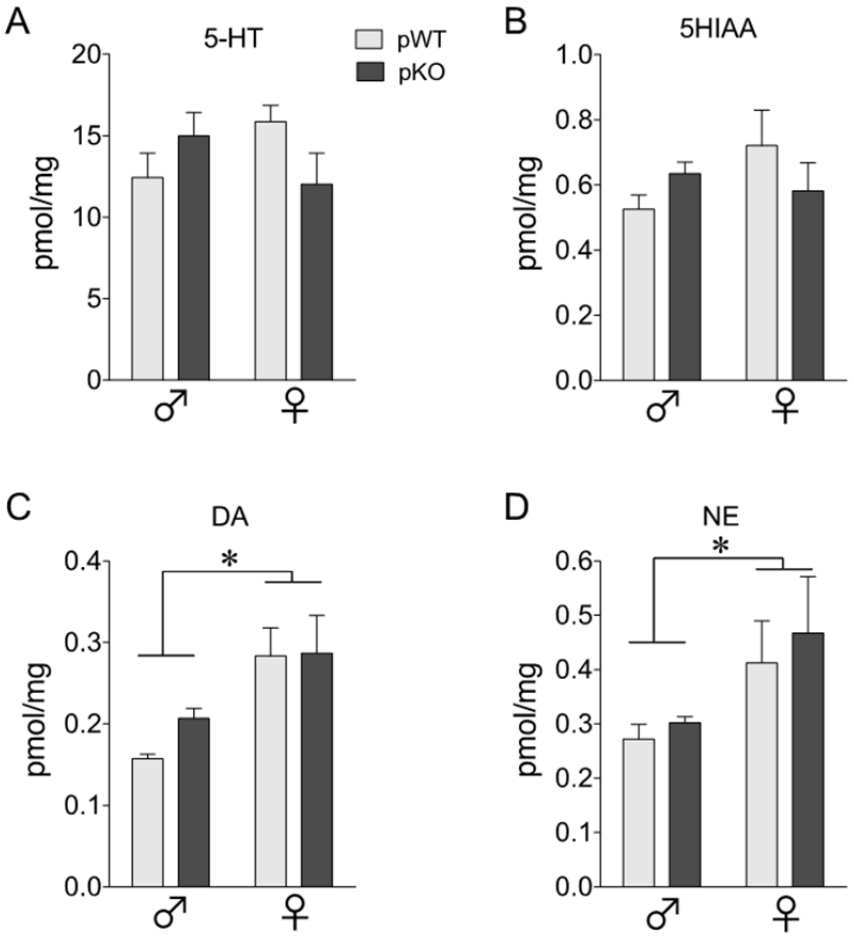

Figure S3. Sex differences in placental dopamine and norepinephrine levels. Concentrations of biogenic amines in the E12.5 placenta were determined by LC/MS. **(A)** While ANOVA detected a significant interaction between the effects of pKO and sex on 5-HT [$F_{int}(1, 20) = 4.54$, $p = 0.046$], post hoc tests did not reach statistical significance. **(B)** There were no effects of genotype or sex on 5HIAA, the major 5-HT metabolite. **(C, D)** Dopamine (DA) and norepinephrine (NE) concentrations were significantly higher in female placentas. ANOVA detected a main effect of sex on DA [$F(1, 19) = 11.12$, $p = 0.0035$] and NE [$F(1, 20) = 5.35$, $p = 0.032$]. Data are mean (+/- SEM). * $p < 0.05$.

Supplemental Table S4. Differentially expressed genes in E12.5 brain

	Male pWT vs. pKO			Female pWT vs. pKO		
Gene	**log2 Fold Change**	***p***	**FDR**	**log2 Fold Change**	***p***	**FDR**
Male-specific						
Pttg1	-0.580	8.00E-12	9.28E-09	--	--	--
Myh8	-0.427	3.90E-06	2.62E-03	--	--	--
Myo7a	-0.351	2.03E-06	1.44E-03	--	--	--
Atp2a1	-0.342	9.02E-05	4.26E-02	--	--	--
Mybph	-0.324	6.35E-05	3.24E-02	--	--	--
Actn2	-0.308	1.64E-05	9.95E-03	--	--	--
Actc1	-0.297	1.46E-07	1.33E-04	--	--	--
Myh3	-0.291	1.41E-06	1.06E-03	--	--	--
Hbb-bh1	0.277	3.74E-05	2.08E-02	--	--	--
Gm20746	0.278	5.48E-05	2.91E-02	--	--	--
Fut10	0.374	6.85E-06	4.37E-03	--	--	--
Pcsk1n	0.465	5.46E-09	5.81E-06	--	--	--
Hddc3	0.689	1.09E-13	1.54E-10	--	--	--
Rps15a-ps8	1.337	1.25E-43	1.59E-39	--	--	--
Males & Females						
Afp	2.098	2.93E-29	1.87E-25	0.978	1.68E-12	3.57E-09
Ctse	-0.408	9.25E-07	7.38E-04	0.855	2.48E-08	2.44E-05
Gm12816	-0.625	3.32E-18	7.07E-15	-0.562	3.84E-09	4.45E-06
Hoxa5	-1.044	1.42E-18	4.55E-15	-0.768	2.37E-11	4.32E-08
Hoxb5	-1.005	9.02E-23	3.84E-19	-0.815	5.64E-15	1.44E-11
Hoxb6	-1.268	7.75E-16	1.24E-12	-0.728	5.97E-08	5.08E-05
Hoxb8	-0.693	2.62E-13	3.34E-10	-0.498	5.78E-07	4.10E-04
Hoxc4	-0.448	7.21E-05	3.54E-02	-0.726	2.67E-09	4.26E-06
Hoxc5	-0.680	5.85E-08	5.75E-05	-0.686	1.22E-07	9.14E-05
Prap1	-2.165	7.93E-18	1.45E-14	-1.050	8.04E-09	8.55E-06
Rpl3	-0.625	3.32E-18	7.07E-15	-0.562	3.84E-09	4.45E-06
Rps3a1	-0.245	2.54E-07	2.16E-04	0.723	6.02E-16	1.92E-12
Ubc	0.320	2.65E-05	1.54E-02	0.403	2.54E-05	1.30E-02
Female-specific						
Hoxb4	--	--	--	-0.813	3.30E-08	3.01E-05
Hoxa4	--	--	--	-0.761	7.31E-08	5.83E-05
Dennd1c	--	--	--	-0.592	5.10E-05	2.18E-02
Lnpep	--	--	--	-0.551	7.86E-06	4.56E-03
Zim1	--	--	--	-0.505	2.00E-05	1.06E-02
Gabrp	--	--	--	-0.491	5.30E-05	2.18E-02
Lcor	--	--	--	-0.464	5.73E-05	2.29E-02
Hoxb3	--	--	--	-0.427	1.19E-05	6.62E-03
Nrk	--	--	--	-0.414	5.17E-05	2.18E-02
Rnf213	--	--	--	0.443	3.94E-05	1.79E-02
Erdr1	--	--	--	0.546	8.68E-07	5.83E-04
Slc15a2	--	--	--	0.625	5.79E-06	3.52E-03

	Male pWT vs. pKO			Female pWT vs. pKO		
Gene	**log2 Fold Change**	***p***	**FDR**	**log2 Fold Change**	***p***	**FDR**
Scand1	--	--	--	0.654	3.26E-05	1.60E-02
Cd99	--	--	--	0.660	3.60E-05	1.70E-02
Bst2	--	--	--	0.777	3.75E-09	4.45E-06
Oasl2	--	--	--	0.867	2.84E-06	1.81E-03
Mir6236	--	--	--	1.296	2.91E-42	1.86E-38
Lars2	--	--	--	1.341	1.86E-49	2.38E-45
Rn7sk	--	--	--	2.392	6.16E-35	2.62E-31

SUPPLEMENTAL REFERENCES

1. Bronson SL, Bale TL (2014): Prenatal stress-induced increases in placental inflammation and offspring hyperactivity are male-specific and ameliorated by maternal antiinflammatory treatment. *Endocrinology*. 155: 2635–2646.

2. Mueller BR, Bale TL (2007): Early prenatal stress impact on coping strategies and learning performance is sex dependent. *Physiol Behav*. 91: 55–65.

3. Dias BG, Ressler KJ (2014): Parental olfactory experience influences behavior and neural structure in subsequent generations. *Nat Neurosci*. 17: 89–96.

4. McEuen JG, Semsar KA, Lim MA, Bale TL (2009): Influence of sex and corticotropin-releasing factor pathways as determinants in serotonin sensitivity. *Endocrinology*. 150: 3709–16.

5. Kamphorst JJ, Fan J, Lu W, White E, Rabinowitz JD (2011): Liquid chromatography-high resolution mass spectrometry analysis of fatty acid metabolism. *Anal Chem*. 83: 9114–22.

6. Clasquin MF, Melamud E, Rabinowitz JD (2012): LC-MS data processing with MAVEN: a metabolomic analysis and visualization engine. *Curr Protoc Bioinformatics*. Chapter 14: Unit14.11.