# Prenatal Alcohol Exposure Alters Steady-State and Activated Gene Expression in the Adult Rat Brain

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**Background:** Prenatal alcohol exposure (PAE) is associated with alterations in numerous physiological systems, including the stress and immune systems. We have previously shown that PAE increases the course and severity of arthritis in an adjuvant-induced arthritis (AA) model. While the molecular mechanisms underlying these effects are not fully known, changes in neural gene expression are emerging as important factors in the etiology of PAE effects. As the prefrontal cortex (PFC) and hippocampus (HPC) play key roles in neuroimmune function, PAE-induced alterations to their transcriptome may underlie abnormal steady-state functions and responses to immune challenge. This study examined brains from adult PAE and control females from our recent AA study to determine whether PAE causes long-term alterations in gene expression and whether these mediate the altered severity and course of arthritis in PAE females.

**Methods:** Adult females from PAE, pair-fed (PF), and ad libitum-fed control (C) groups were injected with either saline or complete Freund's adjuvant. Animals were terminated at the peak of inflammation or during resolution (Days 16 and 39 postinjection, respectively); cohorts of saline-injected PAE, PF, and C females were terminated in parallel. Gene expression was analyzed in the PFC and HPC using whole-genome mRNA expression microarrays.

**Results:** Significant changes in gene expression in both the PFC and HPC were found in PAE compared to controls in response to ethanol exposure alone (saline-injected females), including genes involved in neurodevelopment, apoptosis, and energy metabolism. Moreover, in response to inflammation (adjuvant-injected females), PAE animals showed unique expression patterns, while failing to exhibit the activation of genes and regulators involved in the immune response observed in control and pair-fed animals.

**Conclusions:** These results support the hypothesis that PAE affects neuroimmune function at the level of gene expression, demonstrating long-term effects of PAE on the central nervous system response under steady-state conditions and following an inflammatory insult.

**Key Words:** Prenatal Alcohol Exposure, Ethanol, Inflammation, Arthritis, Gene Expression, Rat.

THE PREVALENCE OF fetal alcohol spectrum disorder (FASD) in North America is estimated at 2 to 5% of live births, making prenatal alcohol exposure (PAE) a leading cause of neurodevelopmental disorders (May et al., 2009). In addition to lasting neurocognitive deficits, impairments in self-regulation, and deficits in adaptive functioning, children with FASD also display changes in a number of physiological systems, including the immune system, with adverse impacts on both innate and adaptive immunity (Gauthier et al., 2005; Johnson et al., 1981; Streissguth et al., 1985).

Animal models have corroborated clinical findings, with PAE animals displaying behavioral and cognitive deficits, including delays in learning and memory, and altered responsivity to stressors (reviewed in Hellemans et al., 2010). Moreover, PAE animals also exhibit altered development of the thymus, decreased lymphocyte proliferative responses to mitogens, increased susceptibility to infections, and greater vulnerability to immune and inflammatory challenges (reviewed in Bodnar and Weinberg, 2013). PAE animals also show larger increases in plasma levels of pro-inflammatory cytokines, as well as reduced proliferative responses of B cells to

lipopolysaccharide, and splenic T cells and T lymphoblasts to concanavalin A and/or interleukin-2 (Weinberg and Jerrells, 1991; Zhang et al., 2005). Likewise, in an adjuvant-induced arthritis (AA) paradigm, we recently demonstrated that PAE animals show increased severity of joint inflammation and a prolonged course of disease (39 days postinjection, higher incidence of arthritis in PAE compared pair-fed [PF] and control [C] animals; Zhang et al., 2012). These findings suggest that although PAE causes deficits in adaptive immunity, PAE offspring show increased responses to some immune/inflammatory challenges.

The immune, neuroendocrine, and central nervous systems have extensive bidirectional communication, sharing numerous ligands and receptors. Brain regions, such as the prefrontal cortex (PFC) and hippocampus (HPC), not only play a role in the regulation of neuroendocrine function, but also respond to immune/inflammatory molecules, including cytokines and neuropeptides (Crofford et al., 1992). For example, adjuvant injection induces c-Fos expression in the HPC for up to 4 months, suggesting a role for this region in AA (Carter et al., 2011). Thus, long-term changes in gene expression may modulate AA manifestation and progression. Indeed, mounting evidence suggests a role for altered gene expression in the etiology of FASD (Kobor and Weinberg, 2011). Widespread changes to gene expression levels in fetal and neonatal brains following PAE, as well as long-lasting alterations to the neural transcriptome following alcohol exposure during the neonatal (third-trimester equivalent) period or across all 3 trimesters, have been reported (Green et al., 2007; Hard et al., 2005; Kleiber et al., 2012, 2013; Zhou et al., 2011).

Using saline-injected (steady-state) animals as a baseline, the current study examined brains from adult PAE and control females from our recent AA study to determine whether long-term alterations in gene expression mediate the altered severity and course of arthritis observed in PAE females (Zhang et al., 2012). As the PFC and HPC play key roles in both neuroendocrine and neuroimmune processes and show altered function following PAE, PAE-induced alterations in the transcriptome of these regions could result in marked downstream effects, including dysregulation of the immune response and neuroendocrine–neuroimmune interactions (Norman et al., 2009). Whole-genome microarrays were utilized to assess gene expression in the PFC and HPC of adult PAE, PF, and C females terminated at the peak or during resolution of inflammation (Days 16 and 39 postadjuvant injection, respectively); cohorts of saline-injected PAE, PF, and C females were terminated in parallel. Under steady-state conditions, we identified changes in gene expression and altered activation states of upstream regulators specific to PAE. Furthermore, at the peak of inflammation, we found not only changes in genes related to PAE, but also a failure of PAE animals to mount appropriate responses to the immune challenge, showing no change in the activation or

inhibition of inflammation-related genes and upstream regulators identified in control groups.

## MATERIALS AND METHODS

### Breeding and Prenatal Breeding and Prenatal Diets

All animal protocols were approved by the University of British Columbia Animal Care Committee and are consistent with the NIH Guide for the Care and Use of Laboratory Animals (National Research Council, 2011). Details of the breeding and feeding procedures have been published (Zhang et al., 2012). Briefly, male and female Sprague–Dawley rats (Animal Care Center, University of British Columbia) were paired; presence of a vaginal plug indicated gestation day (GD) 1. Pregnant dams were singly housed and assigned to experimental groups: prenatal ethanol (EtOH) exposure (PAE; ad libitum access to liquid EtOH diet, 36% EtOH-derived calories); pair-fed (PF; liquid-control diet, maltose-dextrin isocalorically substituted for EtOH, in the amount consumed by a PAE partner, g/kg body weight/GD); or ad libitum-fed control (C; laboratory chow redundant). All animals had ad libitum access to water. Experimental diets (Weinberg/Kiever Ethanol Diet #710324, Weinberg/Kiever Control Diet #710109; Dyets Inc., Bethlehem, PA) were fed from GD 1 to 21 and then replaced with laboratory chow. Litters were weighed and culled at birth to 5 males and 5 females, when possible. Following weaning (postnatal day [PND] 22), offspring were group-housed by litter and sex. Female offspring were used in the present study due to their increased susceptibility to arthritis (Whitacre, 2001).

### Induction of Arthritis and Termination of Animals

Details of the AA paradigm have been published (Zhang et al., 2012). Female offspring (50 to 65 days of age) from C, PF, and PAE groups received an intradermal injection of 0.1 ml of a 12 mg/ml suspension of complete Freund's adjuvant or 0.1 ml physiological saline at the base of the tail. Animals were single-housed postinjection and monitored for clinical signs of arthritis under light anesthesia with isoflurane. Paws were scored individually for redness and swelling on Days 7, 10, and every other day thereafter until Day 39 following injection (Zhang et al., 2012).

Animals were terminated by decapitation, following brief exposure to $CO_2$, in 2 cohorts: Day 16 postinjection or Day 39 postinjection (peak or resolution phase of AA, respectively). Each cohort contained 9 adjuvant-injected animals and 5 saline-injected animals for each group (C, PF, and PAE). Brains were rapidly removed, immediately frozen on dry ice, and stored at $-70°C$.

### Tissue Dissection and RNA Extraction

Brains were thawed to 4°C, and the PFC and HPC were dissected, placed in RNAlater, and stored at $-20°C$. Total RNA and DNA were simultaneously extracted from the tissues (Qiagen All-Prep DNA/RNA Mini kit; QIAGEN Inc., Toronto, ON). RNA integrity was determined using the Agilent BioAnalyzer mRNA Nano assay (Agilent Technologies, Santa Clara, CA).

### Whole-Genome Gene Expression Microarray and Quality Control

The Ambion Illumina TotalPrep RNA Amplification kit (Life Technologies, Carlsbad, CA) was used to generate cRNA (750 ng) from total RNA (250 ng) for each sample. Expression data were obtained using the Illumina RatRef-12 Expression BeadChip microarray with the Illumina iScan, which provides probe-level data for all expressed genes (~1 probe per gene). Samples and hybridization replicates were highly correlated (Table S2). Data sets were filtered

to remove control probes and probes with a detection $p$-value >0.05 in comparison to negative control probes. After filtering, 20,215 and 20,069 probes remained in the PFC and HPC, respectively (out of a total of 23,350 probes). The filtered, log2-transformed gene expression profiles were quantile-normalized within each tissue. Raw and normalized data were submitted to GEO (GSE63561).

### Differential Gene Expression Analysis

Gene expression analysis utilized the *sva* and *limma* packages in the statistical program R (Leek and Storey, 2007; Smyth, 2005). Using *sva*, surrogate variables representative of heterogeneity from sources other than experimental treatments (e.g., batch effects) were generated. These were included in linear modeling of gene expression with *limma*, which uses moderated F- and *t*-statistics to identify significant differences. Gene expression changes were modeled in 2 ways using separate sample means: effects of prenatal treatment alone on steady-state levels of gene expression (saline-injected animals, $n = 5$ per C, PF, PAE group) and interaction of prenatal treatment with an inflammatory challenge (adjuvant- vs. saline-injected animals; $n = 5$ for saline, $n = 9$ for adjuvant per C, PF, PAE group). Each probe received a moderated *F*-statistic, and their $p$-values were corrected for multiple testing using Benjamini–Hochberg correction. The false discovery rate (FDR) was controlled at <25% due to the moderate alcohol-exposure paradigm and its relatively subtle effects. Significant changes in PAE compared to controls had a moderated *t*-statistic $p$-value < 0.05. Sequences for significant probes were queried against the RefSeq database for *Rattus norvegicus* to identify target transcripts.

### Verification of Microarray Results

Differentially expressed genes were verified using reverse-transcription quantitative real-time PCR (RT-qPCR) on the Corbett Rotorgene 6000 (Corbett Research, Australia) for both PFC and HPC, with the same RNA used for microarray analysis ($n = 4$ in both tissues for each C, PF, and PAE). Primers were designed using well-established guidelines to obtain gene-level data, and multiple reference genes were used to normalize expression data (Nolan et al., 2006). Three reference genes across a spectrum of expression levels and no evidence for differences across groups (*F*-statistic $p$-value > 0.05) were selected for each tissue (Table S4). The normalization factor for each sample was calculated using the geometric mean of cycle threshold values (Vandesompele et al., 2002). Expression levels relative to the factor were determined, and analysis of variance (ANOVA) was conducted to test for significant differences between groups (Schmittgen and Livak, 2008). See Fig. S5 for overview of analyses and main findings of gene expression analyses.

### Gene Ontology and Pathway Analysis

Gene ontology (GO) analysis was conducted to identify "biological processes" enriched for the effects of prenatal treatment and adjuvant exposure using the gene-score resampling (GSR) method in ErmineJ (Lee et al., 2005). The set of candidate FASD genes from the curated NeuroCarta database was included in the analysis as a custom GO term (Portales-Casamar et al., 2013; Table S1). Benjamini–Hochberg correction was used with an FDR of 1% within single brain regions and 10% when comparing overlapping effects. Where many GO categories were identified, these were mapped to their parent GO Slim terms using CateGOrizer to determine common categories of altered function (Zhi-Liang et al., 2008). Following GO analysis, the Ingenuity© Upstream Regulator Analysis tool (URA; Ingenuity Systems Inc., Redwood City, CA) was used to predict master transcriptional regulators that explain the observed expression changes within the data set. Genes with a fold change ≥1.2 and $p < 0.05$ between treatments were analyzed for effects of PAE and adjuvant injection. For steady-state effects of PAE, prenatal groups were compared, while adjuvant effects were assessed by comparing adjuvant- to saline-injected animals in each prenatal group. Significantly activated and inhibited genes were identified through a *Z*-score ≥2 or ≤−2, respectively, as well as an overlap $p$-value ≤ 0.1, calculated by Fisher's exact test.

## RESULTS

### Developmental Data

As expected, body weights of PAE dams were lower than those of controls ($p < 0.001$) by the end of pregnancy (GD21), Group × Day interaction, $F(6, 99) = 17.2$, $p < 0.0001$, with PF dams intermediate to PAE and C; dams no longer differed in weight by lactation Day 8. At birth, PAE ($5.7 \pm 0.17$ g) females weighed less than their C ($6.5 \pm 0.18$ g) counterparts, main effect of group, $F(2, 66) = 7.02$, $p < 0.01$, which persisted until weaning (PAE, $51.2 \pm 1.4$ g; PF, $55.3 \pm 1.6$ g; C, $55.2 \pm 1.5$ g), (Group × Day, $F(6, 99) = 1.96$, $p = 0.079$). Blood alcohol levels (BALs) for dams in this paradigm typically average ~100 to 150 mg/dl (Lan et al., 2009; Sliwowska et al., 2008).

### PAE Altered Steady-State Levels of Gene Expression in PFC and HPC

PAE effects on steady-state levels of gene expression were examined in saline-injected females on days 16 and 39 postinjection (~ PND 75 and 95, respectively). On Day 16, $p$-value distributions were skewed toward zero for contrasts of PAE versus C and PAE versus PF, suggesting gene expression differences in PAE compared to C and PF females (Fig. S1). Following Benjamini–Hochberg correction, significant effects of prenatal treatment were found for 80 and 30 genes in the PFC and HPC, respectively, at 25% FDR (Fig. 1). While many genes (43% in PFC, 37% in HPC) showed significant effects of EtOH exposure against both control groups, only a subset (15 in PFC, 4 in HPC; $p < 0.05$) showed changes specific to PAE, in that levels were similar between C and PF animals (Table 1, Fig. 2). These had a number of annotated functions in common, including neurodevelopment, differentiation, neuronal signaling, and regulation of cell death and transcription.

By contrast, on Day 39 postinjection, no relationship between gene expression and PAE was apparent in either brain region, according to $p$-value distributions (Fig. S1). Moreover, only 2 probes met a 25% FDR, but were not specific to PAE effects (data not shown). Thus, subsequent analyses focused on brains from Day 16 postinjection.

### Verification of Results Related to PAE with RT-qPCR

Of 19 probes showing differential expression due to PAE (Table 1), 17 aligned to a sequence in the *Rattus norvegicus* RefSeq database (*ILMN_1372701* and *ILMN_1374168* were the exceptions). Specific RT-qPCR primers were successfully
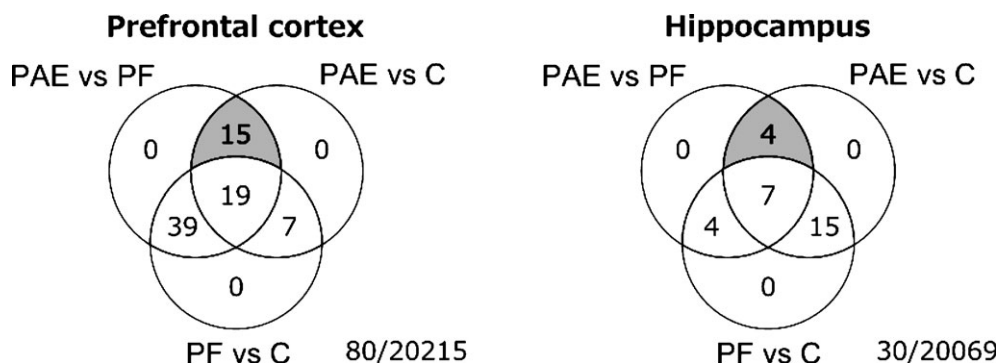
## Prefrontal cortex

PAE vs PF    PAE vs C

0    **15**    0

19

39    7

0

PF vs C    80/20215

## Hippocampus

PAE vs PF    PAE vs C

0    **4**    0

7

4    15

0

PF vs C    30/20069

**Fig. 1.** Prenatal treatment alters gene expression patterns under steady-state conditions. Venn diagram of the number of probes significantly altered in each contrast at Day 16 post saline injection, with moderated *F*-statistic *q* < 0.25 and moderated *t*-statistic *p* < 0.05 (80 in the prefrontal cortex, 30 in the hippocampus). The number of probes with unique effects in prenatal alcohol-exposed (PAE) versus both pair-fed (PF) and control (C) animals is highlighted in gray and listed in Table 1. The center of each Venn diagram shows the number of probes differentially expressed among all 3 prenatal treatment groups. The intersection on the left of each diagram shows the number of probes with a common effect of prenatal ethanol exposure and pair-feeding. The intersection on the right of each diagram shows the number of probes with a unique effect of pair-feeding.

**Table 1.** Genes with Significant Expression Changes Under Steady-State Conditions in Prenatal Alcohol-Exposed (PAE) Compared to Both Control (C) and Pair-Fed (PF) Animals (*p* < 0.05) in the Prefrontal Cortex (PFC) (a) and Hippocampus (HPC) (b) at D16 Post saline Injection

| | | | | | | Fold change | | |
|---|---|---|---|---|---|---|---|---|
| Gene symbol | Gene name | Average expression | F | *p*-Value | *q*-Value | Ethanol/Control | Ethanol/Pair-fed | Pair-fed/Control |
| (a) Genes differentially expressed in PFC of PAE versus both PF and C animals under steady-state conditions in the microarray | | | | | | | | |
| H2afv | *Rattus norvegicus* similar to H2A histone family, member V isoform 1 (LOC685909) | 10.6 | 18.7 | 4.8E-05 | 0.11 | **0.65** | **0.76** | 0.86 |
| Tcf4 | Transcription factor 4 | 11.2 | 11.4 | 7.0E-04 | 0.23 | **0.67** | **0.66** | 1.01 |
| Rnasek | Ribonuclease, RNase K | 13.2 | 11.1 | 8.0E-04 | 0.23 | **0.68** | **0.57** | 1.19 |
| Ppp1r14a | Protein phosphatase 1, regulatory (inhibitor) subunit 14A | 10.0 | 12.6 | 4.1E-04 | 0.23 | **0.68** | **0.64** | 1.05 |
| Rps8 | Ribosomal protein S8 | 13.0 | 11.1 | 7.9E-04 | 0.23 | **0.69** | **0.74** | 0.93 |
| ILMN_1372701 | na | 9.4 | 11.3 | 7.3E-04 | 0.23 | **0.71** | **0.79** | 0.90 |
| ILMN_1374168 | na | 9.1 | 10.7 | 9.4E-04 | 0.25 | **0.77** | **0.73** | 1.05 |
| Pex11 g | Peroxisomal biogenesis factor 11 gamma | 7.0 | 11.5 | 6.7E-04 | 0.23 | **0.82** | **0.71** | 1.16 |
| Ndfip1 | Nedd4 family interacting protein 1 | 11.4 | 12.1 | 5.1E-04 | 0.23 | **1.32** | **1.37** | 0.97 |
| Acsl3 | Acyl-CoA synthetase long-chain family member 3 | 10.2 | 12.2 | 4.9E-04 | 0.23 | **1.36** | **1.36** | 1.00 |
| Dusp6 | Dual specificity phosphatase 6 | 9.9 | 12.5 | 4.4E-04 | 0.23 | **1.41** | **1.21** | 1.17 |
| Rpl7 | Ribosomal protein L7 | 11.6 | 13.7 | 2.7E-04 | 0.22 | **1.44** | **1.36** | 1.05 |
| Med28 | Mediator complex subunit 28 | 9.2 | 11.1 | 7.9E-04 | 0.23 | **1.48** | **1.29** | 1.15 |
| Atp6ap1 | ATPase, H+ transporting, lysosomal accessory protein 1 | 11.0 | 10.6 | 9.8E-04 | 0.25 | **1.50** | **1.35** | 1.11 |
| Ap1s2 | Adaptor-related protein complex 1, sigma 2 subunit | 9.7 | 12.4 | 4.6E-04 | 0.23 | **1.60** | **1.35** | 1.19 |
| (b) Genes differentially expressed in HPC of PAE versus both PF and C animals under steady-state conditions in the microarray | | | | | | | | |
| Cnih2 | Cornichon homolog 2 (Drosophila) | 11.1 | 16.0 | 8.1E-05 | 0.14 | **0.61** | **0.60** | 1.01 |
| Caap1 | Caspase activity and apoptosis inhibitor 1 | 9.2 | 15.2 | 1.1E-04 | 0.14 | **0.68** | **0.71** | 0.95 |
| LOC688637 | Similar to WD repeat domain 36 | 8.8 | 15.4 | 1.0E-04 | 0.14 | **1.46** | **1.36** | 1.08 |
| Rgs3 | Regulator of G-protein signaling 3 | 9.1 | 14.6 | 1.4E-04 | 0.15 | **1.71** | **1.83** | 0.93 |

Bold = *p* < 0.05; na = Probe had no specific alignment to current RefSeq RNA database.

designed for 15 of the 17 genes (Table S3; *Rps8* and *Rpl7* were not analyzed).

Despite differences with microarray technology, RT-qPCR verified 2 genes in the PFC (*Ap1s2*, *Dusp6*) and 1 in the HPC (*Rgs3*), all of which showed increased expression (*p* < 0.1; Fig. 3*A*). Moreover, for 7 significantly up-regulated genes in the microarray, changes trended in the same direction by RT-qPCR (Fig. 3*B*). No down-regulated genes from microarray analysis showed significant differences in PAE animals by RT-qPCR, but 1 gene (*Cnih2*) also trended downward. Importantly, positive correlation between microarray and RT-qPCR data was obtained for PAE effects ($r^2$ = 0.35,
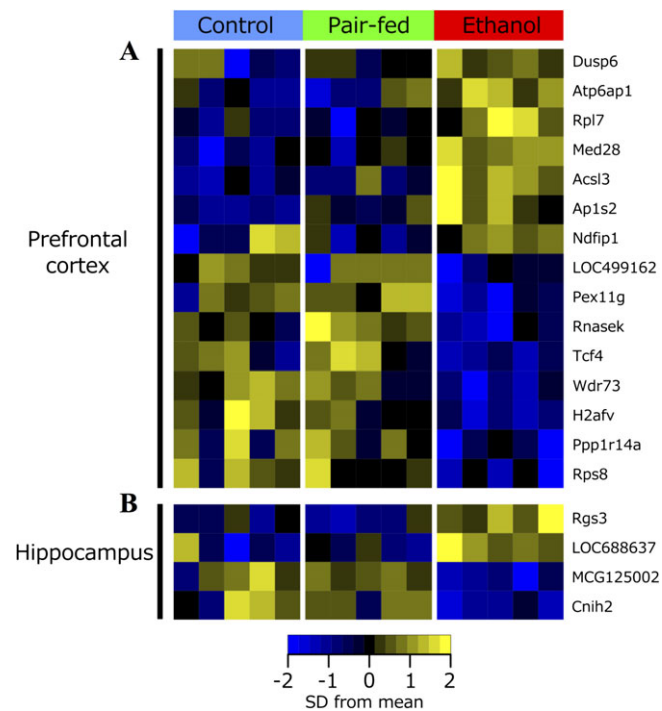
**Fig. 2.** Prenatal alcohol exposure altered steady-state gene expression at Day 16 post saline injection. In the prefrontal cortex (**A**), 15 genes were differentially expressed in response to ethanol. In the hippocampus (**B**), 4 genes were differentially expressed in response to ethanol. *F*-statistic *q*-value < 0.25 for all genes identified.

$p < 0.02$; Fig. 3*B*), and significant genes were corroborated by the small differences between methods shown in the Bland–Altman plot (Fig. 3*C*). No correlation was found for PF animals (Fig. S4). Collectively, the general agreement between qPCR and microarray data suggested that PAE caused persistent alterations to gene expression in the PFC and HPC.

*GO and Upstream Regulator Analysis of PAE Effects Under Steady-State Conditions*

GO analysis was performed to ascertain the broad functional impact of PAE-induced changes in gene expression. Following multiple test correction, 6 processes were altered in the PFC of PAE compared to PF and C animals at a 1% FDR (Fig. S2a): positive regulation of cell projection organization, chemical/ion homeostasis, response to virus, and regulation of intracellular transport. In the HPC, GSR identified 79 processes specific to PAE, which were involved in metabolism (24%), cell communication (18%), development (18%), transport (15%), and signal transduction (10%) (Fig. S2a). At a 10% FDR, several PAE-specific biological processes overlapped between brain regions: positive regulation of neuron differentiation, dorsal/ventral pattern formation, circadian rhythm, regulation of lymphocyte differentiation, and regulation of lipase activity (Fig. S2b). Moreover, GSR also identified the NeuroCarta candidate gene list for FASD in the PFC of PAE females (Portales-Casamar et al., 2013).
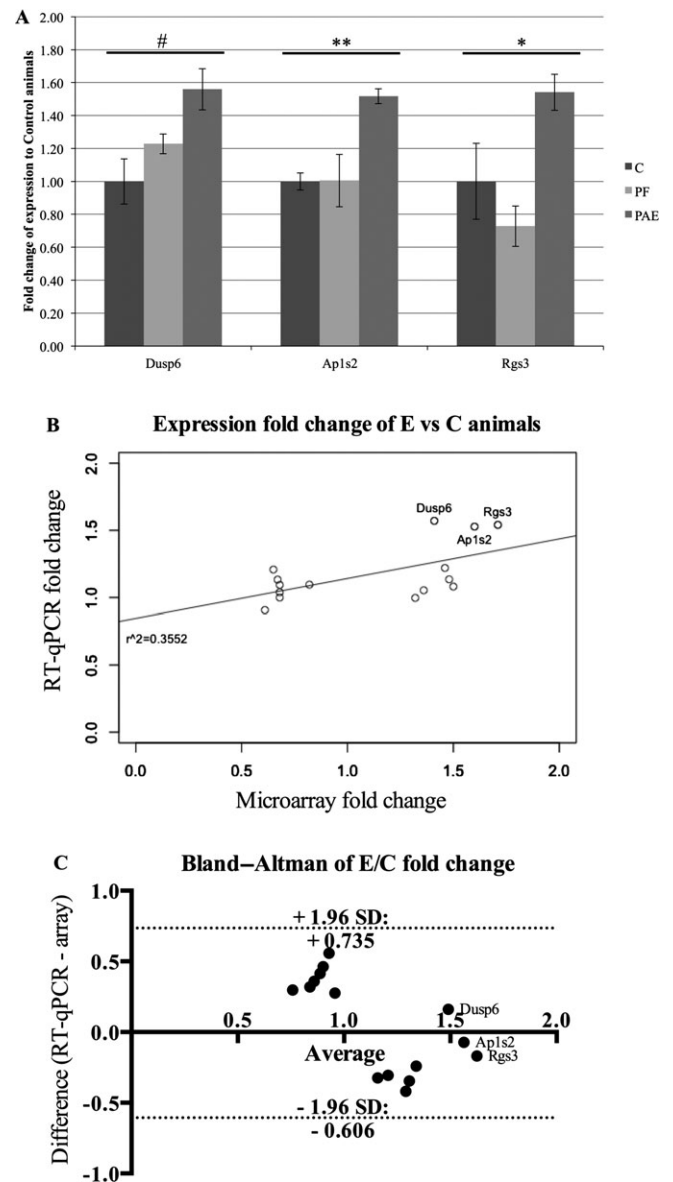


**Fig. 3.** RT-qPCR verification of genes altered by prenatal alcohol exposure. (**A**) Three genes were significantly up-regulated in prenatal alcohol exposed (PAE) animals compared to pair-fed (PF) and controls (C) (*Dusp6* and *Ap1s2* in prefrontal cortex; *Rgs3* in hippocampus). Graphs were plotted as fold change to control animals (where C animals expression = 1) ± SEM. \*\*$p < 0.01$, \*$p < 0.05$, #$p < 0.1$. (**B**) Fold changes in expression were positively correlated between microarray and RT-qPCR results for PAE versus C animals ($r^2 = 0.3552$, $p < 0.02$). Annotated data points represent genes identified as significant in both methods. (**C**) Bland–Altman plot of genes identified by microarray analysis. Dotted lines represent the 95% limits of agreement (Bias = 0.06467), and annotated data points represent genes identified as significant in both methods.

As noted, gene sets were then analyzed using URA to predict master regulators driving the observed expression changes within the data set. In the PFC, a significant activation of *Gast* and an activation of *Lep* that approached statistical significance were identified in PAE compared to PF and C animals (Table 2a), whereas in the HPC, significant differential activation of *Laminin* and *Ifng* was observed (Table 2b).

**Table 2.** Genes Identified Using Ingenuity Upstream Regulator Analysis in the Prefrontal Cortex (PFC) (a) and Hippocampus (HPC) (b) of Steady-State Animals

| Gene symbol | Gene name | Predicted status | Z-score | | | Overlap p-value | | |
|---|---|---|---|---|---|---|---|---|
| | | | PAEvC | PAEvPF | PFvC | PAEvC | PAEvPF | PFvC |
| (a) Upstream regulator analysis in the PFC of animals under steady-state conditions | | | | | | | | |
| Gast | Gastrin | Activated | **2.1** | **2.2** | NA | **0.05** | **0.009** | 1.00 |
| Lep | Leptin | Activated | **2.5** | **2.6** | NA | 0.12 | **0.04** | 1.00 |
| (b) Upstream regulator analysis in the HPC of animals under steady-state conditions | | | | | | | | |
| Ifng | Interferon-gamma | Activated | **3.8** | **2.5** | NA | **0.05** | **0.04** | 1.00 |
| Laminin | Laminin | Activated | **2.0** | **2.0** | NA | **0.01** | **0.04** | 1.00 |

Genes with a Z-score $\geq 2$ or $\leq -2$ and an overlap p-Value $\leq 0.1$ are considered significant (bold). Those with no overlap had a p-value of 1 and no Z-score (NA). The groups used for contrasts were as follows: prenatal alcohol exposure (PAE), pair-fed (PF), control (C).

## Prenatal Treatments Resulted in Common, Graded, and Differential Effects Under Steady-State Conditions

A number of prenatal group effects not specific to PAE were observed in the microarray analysis (Fig. 1). Of the probes affected by prenatal treatment, many showed the same levels of expression in PAE and PF compared to C animals (Table S5), while a handful were altered in opposite directions by EtOH exposure and pair-feeding (Table S6). Conversely, several genes exhibited graded effects of prenatal treatment, with effects of EtOH greater than those of pair-feeding (PAE > PF > C), or vice versa (PF > PAE > C) (Table S6). Pair-feeding also had some unique effects, particularly in the HPC (Table S7), on genes involved in small molecule metabolism, transport, signal transduction, and stress responses. At a 10% FDR, GSR identified 2 PF-related processes overlapping between the PFC and HPC: negative regulation of neuron projection development and positive regulation of epithelial cell migration

(Fig. S2c). Moreover, the curated list of candidate FASD genes from NeuroCarta was also identified in the HPC of the PF group (Portales-Casamar et al., 2013).

## PAE Altered Neural Gene Expression in Response to an Inflammatory Challenge

Consistent with the findings on steady-state gene expression, the greatest effects of immune challenge were observed on Day 16 postinjection (peak of inflammation). The dominant neural response to adjuvant across prenatal treatments was an up-regulation of mRNA levels. However, some genes (8 in PFC and 4 in HPC) were differentially expressed in PAE compared to PF and C animals (Table 3 and Fig. 4). For all hippocampal genes identified, C and PF animals showed a significant up-regulation of expression, while PAE animals showed no change in expression levels between the saline and adjuvant conditions (Fig. 5). These genes (Ctgf, Lcn2, Sgk1, Vwf) were multifunctional, with roles in growth,

**Table 3.** Genes with a Significantly Different Response to Adjuvant in Prenatal Alcohol Exposure (PAE) Compared to Both Control and Pair-Fed Animals ($p < 0.05$) in Prefrontal Cortex (PFC) (a) and Hippocampus (HPC) (b) at the Peak of Inflammation (D16)

| Gene symbol | Gene name | Average expression | F | p-Value | q-Value | Fold change (adjuvant/saline) | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | Control | Pair-fed | Ethanol |
| (a) Genes differentially expressed in PFC of PAE animals in response to adjuvant | | | | | | | | |
| ILMN_1351665 | na | 7.0 | 7.9 | 3.4E-04 | 0.17 | **0.80** | **0.80** | 1.12 |
| Ghrhr | Growth hormone releasing hormone receptor | 7.0 | 8.2 | 2.5E-04 | 0.14 | 0.87 | **0.78** | **1.23** |
| ILMN_1354124 | na | 6.9 | 7.1 | 7.0E-04 | 0.24 | 0.94 | 0.99 | **1.34** |
| ILMN_1364624 | na | 8.4 | 7.2 | 6.3E-04 | 0.24 | 1.22 | 1.06 | **0.51** |
| ILMN_1372588 | na | 11.1 | 8.7 | 1.7E-04 | 0.13 | **1.38** | 1.10 | **0.67** |
| ILMN_1351971 | na | 11.9 | 9.8 | 6.5E-05 | 0.08 | **1.40** | **1.23** | **0.71** |
| Flna | Filamin A, alpha | 8.6 | 7.1 | 7.1E-04 | 0.24 | **1.33** | **1.27** | 0.99 |
| Bhlhe40 | Basic helix-loop-helix family, member e40 | 9.5 | 8.1 | 2.8E-04 | 0.15 | **1.42** | **1.45** | 1.02 |
| (b) Genes differentially expressed in HPC of PAE animals in response to adjuvant | | | | | | | | |
| Sgk1 | Serum-/glucocorticoid-regulated kinase 1 | 11.4 | 9.1 | 1.1E-04 | 0.18 | **1.63** | **1.67** | 1.01 |
| Vwf | Von Willebrand factor | 8.9 | 15.6 | 7.3E-07 | 0.00 | **1.76** | **1.70** | 1.06 |
| Lcn2 | Lipocalin 2 | 7.4 | 18.6 | 1.1E-07 | 0.00 | **1.55** | **1.92** | 1.03 |
| Ctgf | Connective tissue growth factor | 10.4 | 11.4 | 1.6E-05 | 0.05 | **1.77** | **2.14** | 0.85 |

Bold = $p < 0.05$; na = probe had no specific alignment to current RefSeq RNA database.
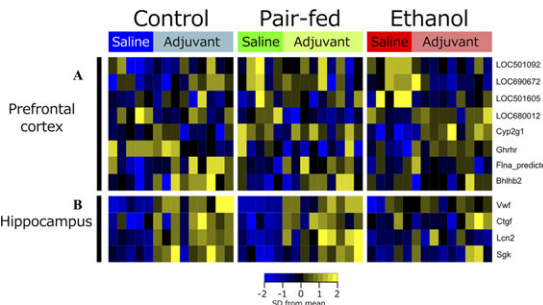
**Fig. 4.** Adjuvant exposure altered gene expression at Day 16 postinjection. Eight genes showed significant changes in expression among treatment groups in prefrontal cortex (**A**). Four genes demonstrated significant changes among treatment groups in the hippocampus (**B**). F-statistic q-value < 0.25 for all genes identified.

proliferation, adhesion, structural organization, and cellular response to immunological or stressful stimuli.

*GO and Upstream Regulator Analysis of PAE Effects in Response to Adjuvant*

GSR identified numerous biological processes altered in response to adjuvant at a 1% FDR. In both the PFC and

HPC, PAE animals had the fewest uniquely altered categories (8% in PFC and 11% in HPC), while C animals had the most (25% in PFC and 30% in HPC) (Fig. S3a). Four PAE-specific processes overlapped between brain regions (Fig. S3b): regulation and positive regulation of epithelial cell proliferation, cellular protein complex assembly, and regulation of hormone level. In categories identified only in PF and C (normal response to adjuvant exposure), 6 overlapped between the PFC and HPC: response to organic nitrogen, actin filament-based process, actin cytoskeleton organization, regulation of cell morphogenesis, developmental growth, and mRNA metabolic process (Fig. S3c).

Moreover, URA of gene sets for both the PFC and HPC predicted several master regulators of PAE-specific response to adjuvant, as well as some present only in PF and C animals. In the PFC, 2 PAE-specific genes (*Fn1*, *Dicer1*) and 4 PF-/C-specific genes (*Agt*, *Foxo3*, *P38 Mapk*, *Osm*) were significantly activated, while a single PAE-specific gene, *Calmodulin*, was significantly inhibited (Table 4a). In the HPC, 2 PAE-specific genes (*Adcyap1*, *Prl*) showed significant inhibition, and one, *Nr1i3*, showed marginally significant activation (Table 4b). As well, PF-/C-specific effects were found for *Adamts12* (inhibited) and *Foxo4* (activated). Of note,
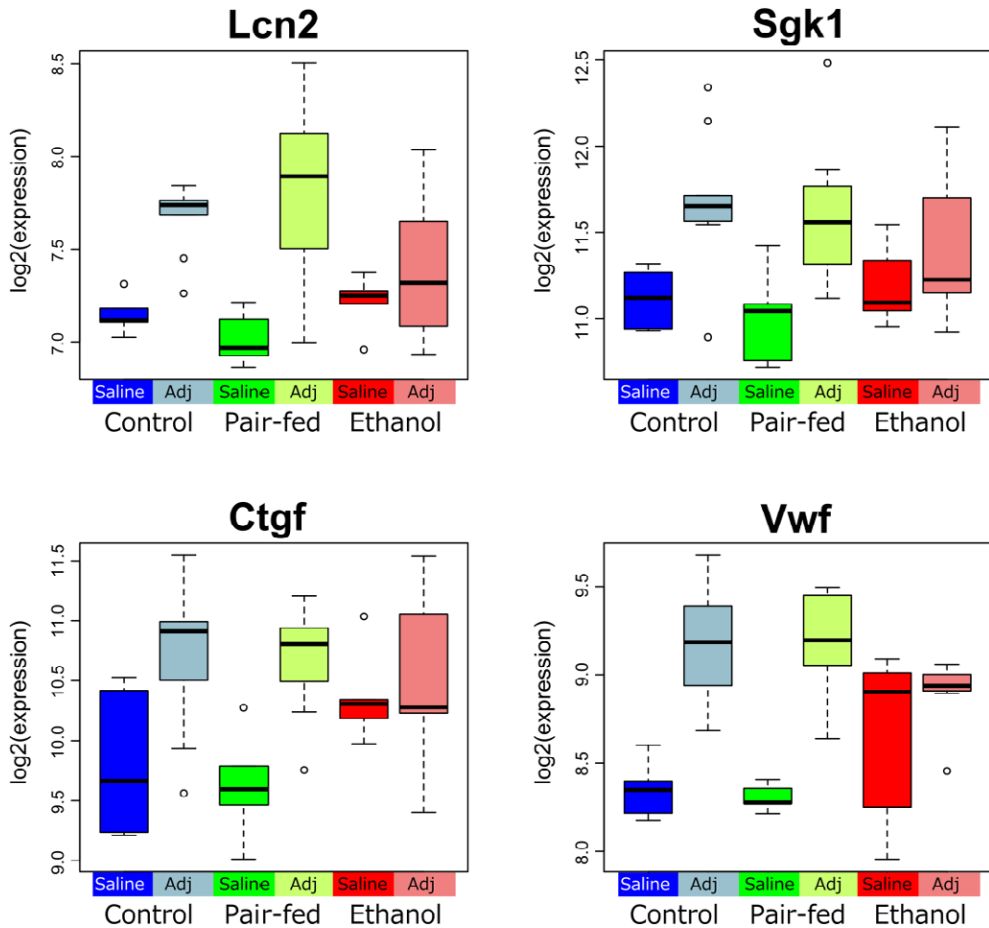


**Fig. 5.** Prenatal alcohol-exposed (PAE) animals show altered response to adjuvant. In a subset of genes, PAE animals showed no response to adjuvant, although pair-fed and control animals responded with an up-regulation of the gene (*Lcn2, Sgk1*). In others, gene expression levels in ethanol animals were already elevated compared to pair-feds and controls, but did not change to the extent of their control counterparts (*Ctgf, Vwf*).

**Table 4.** Genes Identified Using Ingenuity Upstream Regulator Analysis in the Prefrontal Cortex (PFC) (a) and Hippocampus (HPC) (b) of Adjuvant Versus Saline Animals

| Gene symbol | Gene name | Predicted status | Z-score | | | Overlap p-value | | |
|---|---|---|---|---|---|---|---|---|
| | | | C | PF | PAE | C | PF | PAE |
| (a) Upstream regulator analysis of the PFC in adjuvant- versus saline-injected animals | | | | | | | | |
| PAE-specific | | | | | | | | |
| *Calmodulin* | Calmodulin | Inhibited | NA | 0.4 | **−2.0** | 1.00 | 0.02 | **0.02** |
| *Dicer1* | Dicer 1, ribonuclease type III | Activated | NA | NA | **2.0** | 1.00 | 1.00 | **0.09** |
| *Fn1* | Fibronectin 1 | Activated | 1.3 | 1.1 | **2.1** | 0.03 | 0.0002 | **0.03** |
| Non-PAE | | | | | | | | |
| *Agt* | Angiotensinogen | Activated | **2.5** | **2.2** | NA | **0.02** | **0.002** | 1.00 |
| *Foxo3* | Forkhead box O3 | Activated | **2.3** | **3.1** | 0.2 | **0.02** | **0.002** | 1.00 |
| *Osm* | Oncostatin M | Activated | **2.9** | **2.7** | NA | **0.1** | **0.07** | 1.00 |
| *P38 Mapk* | p38 mitogen-activated protein kinase | Activated | **2.0** | **3.2** | NA | **0.04** | **0.005** | 1.00 |
| (b) Upstream regulator analysis in the HPC of adjuvant- versus saline-injected animals | | | | | | | | |
| PAE-specific | | | | | | | | |
| *Adcyap1* | Adenylate cyclase-activating polypeptide 1 | Inhibited | NA | NA | **−2.2** | 1.00 | 1.00 | **0.09** |
| *Nr1i3* | Nuclear receptor subfamily 1, group I, member 3 | Activated | NA | NA | **2.2** | 1.00 | 1.00 | 0.12 |
| *Prl* | Prolactin | Inhibited | 2.3 | NA | **−2.0** | 0.24 | 1.00 | **0.05** |
| Non-PAE | | | | | | | | |
| *Adamts12* | ADAM metallopeptidase with thrombospondin type 1 motif, 12 | Inhibited | **−2.4** | **−2.0** | NA | **0.0003** | **0.001** | 1.00 |
| *Foxo4* | Forkhead box O4 | Activated | **2.0** | **2.0** | NA | **0.07** | **0.04** | 1.00 |
| *Foxo3* | Forkhead box O3 | Activated | **2.6** | **2.6** | NA | 0.13 | 0.13 | 1.00 |

Genes with a Z-score $\geq 2$ or $\leq -2$ and an overlap p-value $\leq 0.1$ are considered significant (bold). Those with no overlap had a p-value of 1 and no Z-score (NA). The groups used for contrasts were as follows: prenatal alcohol exposure (PAE), pair-fed (PF), control (C).

*Foxo3* approached significance in the HPC of PF and C animals, representing the only overlap between brain regions.

## DISCUSSION

PAE altered patterns of neural gene expression under both steady-state and immune challenge conditions. In saline-injected females, we identified PAE-induced changes in the expression of *Rgs3*, *Dusp6*, and *Ap1s2*, as well as activation of upstream regulators involved in metabolism and immune function. At the peak of inflammation, adjuvant injection caused PAE-specific changes in gene expression and uncovered a failure to mount appropriate responses to inflammatory challenge in PAE animal, as evidenced by the absence of changes in inflammation-related genes and upstream regulators identified in controls.

### PAE Altered Neural Gene Expression Under Steady-State Conditions

Microarray analysis identified unique effects of PAE on 15 and 4 genes in the PFC and HPC, respectively. These had roles in neurodevelopment, cell death, differentiation, transcriptional regulation, and neuronal signaling. Using RT-qPCR, we successfully verified the significant up-regulation of *Dusp6* and *Ap1s2* in the PFC, as well as *Rgs3* in the HPC. Furthermore, the majority of genes not verified by RT-qPCR trended in the same direction as the microarray. The discrepancy in technical verification may arise from the different methods of measurement between the technologies and the underpowered analysis resulting from a relatively low num-

ber of samples. Additional large-scale experiments will be required to fully validate these results at the biological level.

It is tempting to speculate that these genes play important roles in the cognitive and behavioral deficits observed in FASD. *Ap1s2* is involved in neurodevelopment and associated with intellectual disability and autism spectrum disorder, while *Dusp6* promotes apoptosis and is linked to bipolar disorder (Borck et al., 2008; Kim et al., 2012). Activation of *Laminin* could also be involved in the altered neuronal migration patterns observed in PAE brains (Ozer et al., 2000). Moreover, inappropriate feeding behavior in children with FASD, as well as altered glucose metabolism and insulin tolerance in PAE animals, has been reported (Harper et al., 2014; Werts et al., 2014). As *Rgs3* negatively regulates glucose output via cAMP production in hepatic cells, it may also play a role in altered energy metabolism within the brain when combined with the activation of gastrin and leptin in the PFC (Raab et al., 2005). Furthermore, the activation of interferon-γ in the HPC supports a role for this cytokine in the altered immune system activity and response to challenge in PAE offspring.

Previous studies on fetal and neonatal brains have uncovered EtOH-induced alterations in the expression of genes related to energy metabolism, adhesion, cytoskeletal remodeling, cell cycle, proliferation, differentiation, apoptosis, as well as neuronal growth and survival (Green et al., 2007; Hard et al., 2005; Zhou et al., 2011). Long-term PAE studies in brains of adult male mice identified networks related to cellular development, free radical scavenging, and small molecule metabolism, as well as genes involved in cognitive function, anxiety, attention deficit hyperactivity disorder,

and mood disorders (Kleiber et al., 2012). Interestingly, none of the genes found here directly overlapped with those previously identified. These disparities are likely due to species- and sex-specific effects, differences between exposure paradigms, and different gene expression patterns in whole brains versus specific regions. As such, these discrepancies highlight the importance of examining both sexes and targeted brain regions to gain deeper insight into PAE effects. It is also possible that immediate changes in gene expression in response to PAE may not persist or that environmental influences cause alterations over the course of development. Moreover, the relatively moderate levels of EtOH exposure (BALs ~120 to 150 mg/dl) in this paradigm are consistent with those reported for children with FASD who show functional and cognitive deficits (Mattson et al., 2011). Perhaps most importantly, the genes identified here have not previously been examined in gene expression studies, suggesting that we have uncovered novel candidates for the effects of PAE in females. Whether our specific changes are mediated through epigenetic mechanisms remains to be investigated (Kobor and Weinberg, 2011).

A limitation of this study is that estrous stages were not determined at the time of termination. We have previously shown that PAE induces changes in basal levels of hippocampal glucocorticoid and serotonin type 1A (5-HT1A) receptor mRNA as a function of estrous stage, which likely has widespread effects on global expression patterns in the brain (Sliwowska et al., 2008). While most females in the present study were likely in diestrus, estrous cycle variation might partially explain intragroup differences in gene expression (Lan et al., 2009).

### PAE Altered the Gene Expression Response to Adjuvant

PAE-specific responses to adjuvant were found for 8 and 4 genes in the PFC and HPC, respectively. These had roles in growth, proliferation, adhesion, structural organization, and cellular response to immunological or stressful stimuli. Across all prenatal treatments, adjuvant caused a global increase in gene expression compared to saline-injected animals. Importantly, PAE animals failed to exhibit the up-regulation in expression observed in controls for genes related to immune and cellular responses to stressful stimuli (*Ctgf*, *Lcn2*, *Sgk1*, *Vwf*). Up-regulation of immune-related genes normally occurs in the central nervous system in response to peripheral inflammatory stimuli or neuroinflammation, which occurs in AA (reviewed in Liu et al., 2012; Ousman and Kubes, 2012). PAE animals may fail to detect these immune changes and/or launch the appropriate neuroendocrine/neuroimmune response, which could contribute to the prolonged inflammation observed in our previous AA study (Zhang et al., 2012). Consistent with this finding, most master regulators identified in the upstream regulator analysis were involved in the immune response. For example, *P38 Mapk* plays a role in signal transduction within the normal inflammatory cascade and is only activated in PF and C ani-

mals (Cuadrado and Nebreda, 2010). Moreover, *Adamts12* modulates neutrophil apoptosis during inflammation, while *Osm* attenuates the inflammatory response (Dumas et al., 2012; Moncada-Pazos et al., 2012). Thus, inhibition of *Adamts12* and activation of *Osm* in control animals may blunt their responses to adjuvant. Furthermore, *Adcyap1* modulates anti-inflammatory responses and is neuroprotective in neurons following inflammation (Waschek, 2013). Its inhibition in PAE animals suggests a lower level of protection against inflammation than the one that would occur in controls. In turn, as *Prl* promotes pro-inflammatory responses, its PAE-specific activation suggests an altered response to adjuvant (Brand et al., 2004). Failure of PAE animals to activate *Foxo*-related pathways may also play a role in their unique response to adjuvant, as knockdown of *Foxo3* or *Foxo4* increases inflammatory responses (Hwang et al., 2011; Zhou et al., 2009). The possibility that *Foxo3* is already up-regulated in PAE animals and thus may not change further after adjuvant injection remains to be investigated (Kleiber et al., 2013). The activation of fibronectin in PAE animals is interesting, as it is involved in the development of inflammatory arthritis (Barilla and Carsons, 2000). Greater production or sensitivity to this protein could underlie the altered course and severity of AA in PAE animals. Finally, activation of *Dicer1* in PAE animals suggests alterations to microRNA processing under stress conditions, previously demonstrated following PAE (Guo et al., 2012).

### Effects of Pair-Feeding on Neural Gene Expression: Pair-Feeding is a Treatment in Itself

A number of genes were similarly altered or showed graded and differential effects in PAE and PF compared to C animals (Fig. 2). These may respond to common effects of EtOH exposure and pair-feeding, such as reduced caloric availability or altered stress system regulation. While both PAE and PF animals receive the same number of calories, PAE dams eat ad libitum whereas PF dams receive a reduced ration, likely resulting in hunger and stress (Harris and Seckl, 2011). Moreover, PF dams tend to consume their daily ration within a few hours and are deprived until the next feeding, which may have unique metabolic effects associated with "disordered" eating. Our results suggest that the HPC may be susceptible to fetal programming in response to energy- and stress-related environmental factors. Interestingly, the curated list of candidate FASD genes from NeuroCarta was identified in the HPC of PF animals, suggesting that these genes are potentially related to common mechanisms underlying PAE, nutrition, and stress (Portales-Casamar et al., 2013). Studies such as ours are critical to separate the effects of prenatal stress and PAE at the level of gene expression.

## SUMMARY AND CONCLUSIONS

Our results support the hypothesis that PAE has long-term effects on gene expression patterns in the brain, as

well as on the response to a systemic inflammatory insult. As both the PFC and HPC play important roles in cognitive, neuroendocrine, and immune function, the identified changes in steady-state and activated expression likely contribute to immune-related alterations, as well as cognitive and behavioral deficits arising from PAE. Moreover, an inability to mount appropriate response to immune/inflammatory challenges may contribute to the increased vulnerability of individuals with FASD to infections and immune problems. These findings extend our previous data demonstrating that PAE animals exhibit increased susceptibility to and impaired recovery from an inflammatory challenge (Zhang et al., 2012) and suggest that the adverse impact of PAE on the neural transcriptome may underlie the long-term health and developmental outcomes observed in individuals with FASD.

## ACKNOWLEDGMENTS

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Candidate genes involved in the etiology of FASD, catalogued in NeuroCarta.

**Table S2.** (a) Correlation of expression profiles among all samples and among replicates in PFC microarray data set. (b) Correlation of expression profiles among all samples and among replicates in HPC microarray data set.

**Table S3.** Sequences of primers used for RT-qPCR.

**Table S4.** (a) Microarray expression results for common reference genes in PFC of Day 16 saline animals. (b) Microarray expression results for common reference genes in HPC of Day 16 saline animals.

**Table S5.** (a) Genes showing common change in expression in PFC of PAE and PF compared to C animals under steady-state conditions. (b) Genes showing common change in expression in HPC of PAE and PF compared to C animals under steady-state conditions.

**Table S6**. (a) Genes differentially expressed in PFC among all 3 prenatal treatment groups under steady-state conditions. (b) Genes differentially expressed in HPC among all 3 prenatal treatment groups under steady-state conditions.

**Table S7.** (a) Genes differentially expressed in PFC of Pair-fed versus both PAE and C animals under steady-state conditions. (b) Genes differentially expressed in HPC of Pair-fed versus both PAE and C animals under steady-state conditions.

**Table S8.** Accessions and probe sequences for differentially expressed genes.

**Fig. S1.** Plot of *p*-value distributions for gene expression differences among prenatal treatment groups, within the steady-state conditions, in (a) PFC at Day 16 post saline injection, (b) PFC at Day 39 post saline injection, (c) HPC at Day 16 post saline injection, and (d) HPC at Day 39 post saline injection.

**Fig. S2.** Venn diagrams demonstrating the number of biological processes significant for each contrast in Day 16 animals under steady-state conditions, and overlap of processes between different contrasts for PFC and HPC at FDR < 1% (a). FDR was increased to 10% to identify biological processes that showed overlapping changes in both tissues, specific to prenatal alcohol exposure (b) and pair-feeding (c). FDR < 10%.

**Fig. S3.** Venn diagrams demonstrating the number of biological processes significantly altered in the response to adjuvant within each prenatal treatment group, and the overlap of processes enriched between groups (a). Many biological processes showed changes specific to prenatal alcohol exposure, and several overlapped between tissues (b). Other processes were common to the PF and C response to adjuvant, and several overlapped between tissues (c). FDR < 1%.

**Fig. S4.** Fold changes in expression were not correlated between microarray and RT-qPCR results for PF versus C animals ($r^2 = 0.02528$, $p = 0.5714$).

**Fig. S5**. Overview of analyses and main findings for gene expression analysis.