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Gene expression profiling reveals a lingering effect of prenatal alcohol exposure on inflammatory-related genes during adolescence and adulthood

Anny Gano[a,b], Laura Prestia[a,c], Frank A. Middleton[a,c], Steven L. Youngentob[a,d], Cherry Ignacio[a,b,c], Terrence Deak[a,b,*]

[a] Developmental Exposure Alcohol Research Center (DEARC), USA
[b] Binghamton University-SUNY, Binghamton, NY 13902-6000, USA
[c] SUNY-Upstate Medical University, Syracuse, NY 13210, USA
[d] University of Tennessee Health Science Center, Memphis, TN 38163, USA

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ABSTRACT

Prenatal Alcohol Exposure (PAE) exerts devastating effects on the Central Nervous System (CNS), which vary as a function of both ethanol load and gestational age of exposure. A growing body of evidence suggests that alcohol exposure profoundly impacts a wide range of cytokines and other inflammation-related genes in the CNS. The olfactory system serves as a critical interface between infectious/inflammatory signals and other aspects of CNS function, and demonstrates long-lasting plasticity in response to alcohol exposure. We therefore utilized transcriptome profiling to identify gene expression patterns for immune-related gene families in the olfactory bulb of Long Evans rats. Pregnant dams received either an *ad libitum* liquid diet containing 35% daily calories from ethanol (ET), a pair-fed diet (PF) matched for caloric content, or free choice (FCL) access to the liquid diet and water from Gestational Day (GD) 11–20. Offspring were fostered to dams fed the FCL diet, weaned on P21, and then housed with same-sex littermates until mid-adolescence (P40) or young adulthood (P90). At the target ages of P40 or P90, offspring were euthanized via brief CO_2 exposure and brains/blood were collected. Gene expression analysis was performed using a Rat Gene 1.0 ST Array (Affymetrix), and preliminary analyses focused on two moderately overlapping gene clusters, including all immune-related genes and those related to neuroinflammation. A total of 146 genes were significantly affected by prenatal Diet condition, whereas the factor of Age (P40 vs P90) revealed 998 genes significantly changed, and the interaction between Diet and Age yielded 162 significant genes. From this dataset, we applied a threshold of 1.3-fold change (30% increase or decrease in expression) for inclusion in later analyses. Findings indicated that in adolescents, few genes were altered by PAE, whereas adults displayed an increase of a wide range of gene upregulation as a result of PAE. Pathway analysis predicted an increase in Nf-κB activation in adolescence and a decrease in adulthood due to prenatal ethanol exposure, indicating age-specific and long-lasting alterations to immune signaling. These data may provide important insight into the relationship between immune-related signaling cascades and long-term changes in olfactory bulb function after PAE.

1. Introduction

A substantial proportion of those affected by alcohol suffer its deleterious effects without choosing to consume it themselves. Prenatal alcohol exposure (PAE) is alarmingly common in the United States (US) and can lead to a range of outcomes collectively termed fetal alcohol spectrum disorder (FASD). The conservative rate of prevalence for FASD is 0.2 to 1.5 cases per 1,000 births in the US, with 1 in 13 American women reporting alcohol use during pregnancy [1]. Within the FASD spectrum, it is easier to identify the more extreme fetal alcohol syndrome (FAS) as it is associated with morphological changes to facial features and pronounced cognitive deficits. However, even abbreviated exposure to low levels of alcohol *in utero* may lead to subtle deficits that can persist into adulthood and affect further development, as well as future alcohol-related behavior. It is difficult to estimate the true range of these disturbances due to variance in approach methodology in the literature, the wide range of possible external and internal characteristics of the outcome, and the varied age at which diagnosis is possible [2].

* Corresponding author at: Behavioral Neuroscience Program, Department of Psychology, Binghamton University-State University of New York, Binghamton, NY 13902-6000, USA.
E-mail address: tdeak@binghamton.edu (T. Deak).

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Fortunately, some of the ambiguity may be dissected via the use of animal models of prenatal alcohol exposure (PAE), providing an opportunity to test for varied effects of *in utero* ethanol exposure by targeting specific developmental epochs and subsequently assessing both behavioral deficiencies and structural abnormalities. For example, PAE models have revealed a relationship between binge alcohol consumption in early pregnancy and changes in stress responsivity in the offspring, particularly via altered hypothalamic–pituitary-adrenal (HPA) reactivity, which may contribute to the increased rate of anxiety in the FASD-diagnosed population [3,4]. Low and moderate levels of ethanol exposure during gestation have also been shown to produce later deficits in cognitive tasks dependent on memory [5]. In addition to imparting deficits that affect function and quality of life, prenatal exposure to ethanol exerts influence over an individual's further interactions with alcohol. It has been shown that prenatal exposure to even moderate (1–2 g/kg intragastric) doses of ethanol affects the endogenous opioid system within the reward circuitry, which has also been shown to influence future alcohol palatability, intake, and reinforcement [6–8].

Neuroimmune signaling has been identified not only as a responder to acute and chronic alcohol exposure, but also a key player in the regulation of drinking behavior [9,10]. Consistent with this, acute alcohol exposure in the form of a single dose administered intraperitoneally or intragastrically as well as chronic ethanol exposure spanning weeks to months affects cytokine signaling across a variety of brain regions [11,12]. There is also some evidence that PAE induces altered neuroimmune profiles across brain areas such as the hypothalamus and hippocampus that persist across development [13]. Global manipulation of neuroimmune genes in the brain, such as deletion of genes for cytokines Interleukin (IL)-6 and IL-1 receptor antagonist, resulted in alterations to drinking behavior in mice [14]. Investigating neuroimmune targets of prenatal alcohol exposure can, therefore, not only provide useful biomarkers for categorizing FASD-associated damage, but also shed light upon the influence of gestational alcohol on future addiction-related behavior.

In these studies, we examined gene expression changes specifically within the olfactory bulb, an area which has received far less attention in published studies. Previous work has shown this area to be responsive to gestational ethanol exposure, as evinced by changes in expression of genes involved in synaptic transmission, plasticity, and neuronal development [15]. There is an extensive literature connecting the prenatal experience of the chemosensory aspects of ethanol and later perception of ethanol, with important implications for consumption and preference [16–18]. Specifically, in the olfactory bulb, effects of fetal alcohol exposure have been implicated as possible causative agents in increased adolescent drinking [19]. Within the current experiments, we specifically examined PAE-induced changes in olfactory bulb neuroinflammatory gene expression during adolescence and adulthood in an unbiased manner using microarray gene expression profiling followed by confirmation with RT-PCR. To our knowledge, this analysis has not previously been performed, and can serve as a useful starting point for understanding the influence of gestational ethanol exposure on FASD pathology, and the development of detrimental drinking patterns in adolescence and adulthood.

2. Methods and materials

2.1. Subjects

Timed pregnant Long-Evans dams were acquired from Harlan (Indianapolis IN), and shipped during the first week of gestation to the AAALAC-accredited facility at SUNY Upstate Medical University. Pregnant dams (N = 18) were housed under standard colony conditions (22 °C; Lights on 0600-1800) with food and water available *ad libitum* at all times except during prenatal alcohol exposure as described below. All experimental procedures were approved by the Committee on Humane Use of Animals (CHUA) at SUNY-Upstate Medical University, and studies were conducted in accordance with the Public Health Service (PHS) policy on the Humane Care and the Use of Laboratory Animals and National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

2.2. Prenatal alcohol exposure

On GD11-20, dams were randomly allocated and exposed to one of three prenatal diet conditions. The Prenatal Alcohol Exposure group (PAE; n = 6 received an *ad libitum* diet containing 35% daily calories from ethanol. Following a previously established protocol, pregnant dams were weaned (from gestational day [G] 6–10) onto an *ad libitum* liquid diet (L10251, Research Diets, New Brunswick, NJ) that provided 35% of daily calories from ethanol from G11-G20 [15,20]. Weight-matched control dams were pair-fed (PF; n = 6) to the ethanol group and received an *iso*-caloric *iso*-nutritive liquid diet (L100252, Research Diets, New Brunswick, NJ) with maltose/dextrin substituted for the calories derived by ethanol. Free choice liquid diet control dams (FCL; n = 6) received *ad libitum* access to liquid diet and water from G11-20. Both we and others have shown that this exposure model yields peak blood ethanol concentration levels that are on the order of 150 mg/dl (when tested on the evening of G17, 3 h after lights out in the animal facility) e.g., [20,21,22]. As such, the method provides for consistent level of ethanol exposure designed to model moderate-to-high ethanol intake e.g., [23,24,25]. Consistent with prior reports, no differences in the size or composition of litters were observed after PAE. An experimental timeline is represented in the schematic shown in Fig. 1A.

2.3. Offspring

Litters were culled to a total of 10 pups per dam, with even numbers of males and females maintained where possible (n = 4–6 for each sex per litter) within 24 h of birth and were cross-fostered to dams fed the FCL diet. This is a standard procedure to ensure that the effects of PAE (and other gestational manipulations) are not confounded by differences in maternal care displayed by PAE dams toward their pups. Litters were weaned on post-natal day (P) 21, and then housed with same-sex littermates until either adolescence (P40) or adulthood (P90). No more than 2 offspring per litter (one male, one female) were utilized in each experimental condition to ensure that experimental effects were not confounded by litter of origin effects. That is, two randomly selected animals of each sex from any particular ET, PF, and FCL litter were utilized, and these animals, in turn, were further randomly allocated to either the P40 (adolescent) or P90 (adult) time points. Thus, a total of 12 ET (6 male, 6 female), 12 PF (6 male, 6 female) and 12 FCL (6 male, 6 female) progeny at P40 and the same number and distribution of animals at P90, participated in this study.

2.4. Tissue collection and processing

Two post-natal ages were selected for analysis in this study, emphasizing mid-adolescence (P40) and young adulthood (P90). At the target ages, offspring were euthanized via brief CO_2 exposure and brains/blood were collected for further analysis. Olfactory Bulbs (OBs) were removed, frozen on dry ice, and stored at −80 °C until the time of tissue processing. RNA extractions were performed as previously described [15]. Briefly, total RNA was purified using the RNeasy kit (Qiagen).

2.5. Microarray analysis

For high-throughput gene expression quantification, RNA samples were prepared using the FlashTag Biotin HSR RNA Labeling Kit (Affymetrix). They were then hybridized to a Rat Gene 1.0 ST Array (Affymetrix). Arrays were hybridized, washed, stained and scanned

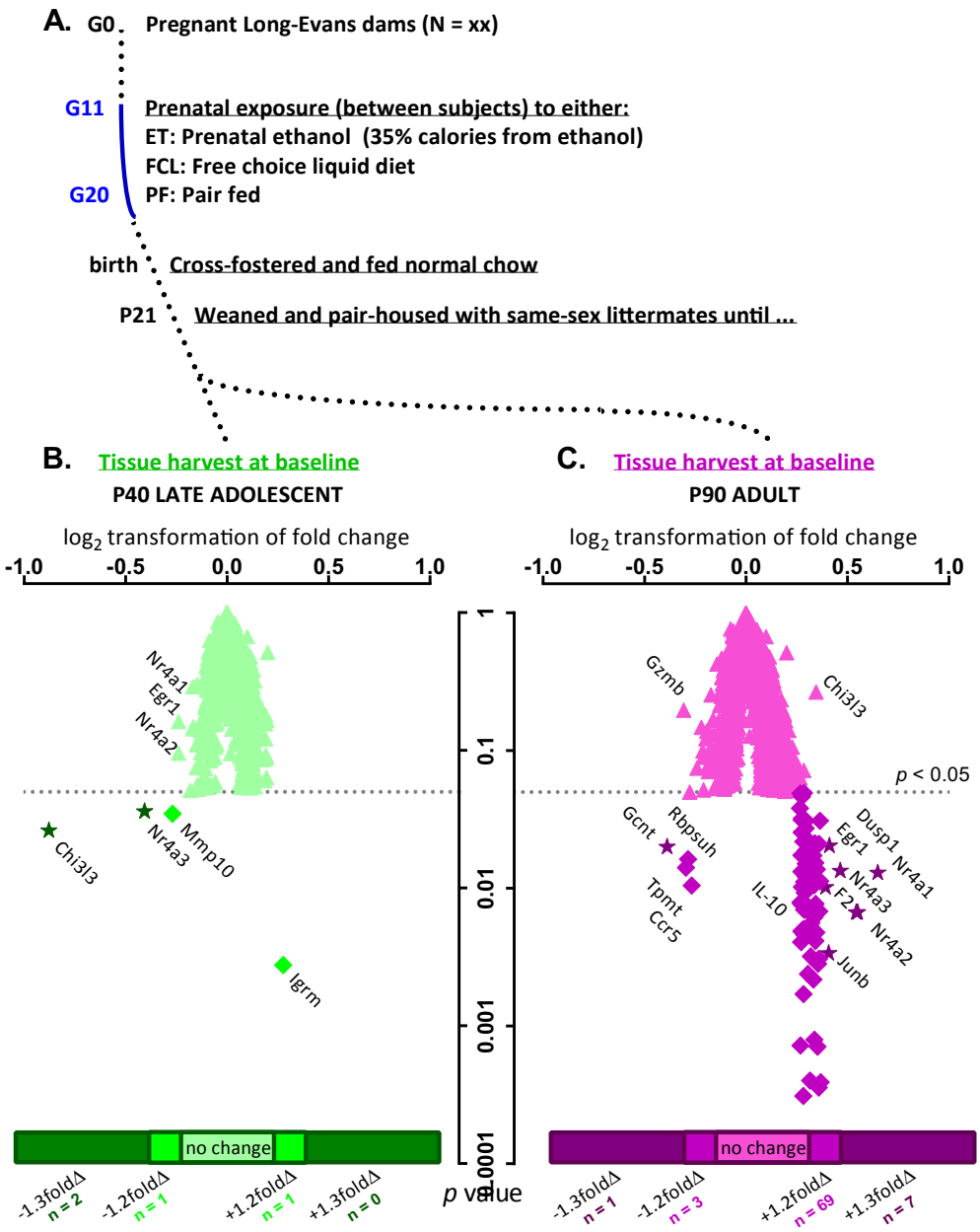

Fig. 1. Experimental timeline and microarray summary. This figure shows the (A) experimental schematic for this experiment detailing the timeline of manipulations. Baseline changes in gene expression revealed via microarray for the (B) adolescents and (C) adults are shown graphed as $\log_2$ transformation of fold change against the significance of findings. Triangles represent genes that did not change significantly, squares are genes that have shown greater than 1.2-fold change with a significance of $p < 0.05$, and stars are genes showing greater than 1.3-fold change with a significance of $p < 0.05$. Selected genes are labeled in the graph based on significance and relevance of results, as follows: Ccr5 (C-C motif chemoki;ne receptor 5); Chi3l3 (chitinase-like 3); Dusp1 (dual specificity phosphatase 1); Egr1 (early growth hormone protein 1); F2 (prothrombin, aka coagulation factor 2); Gcnt (beta 1,6-N-acetylglucosaminyltransferase); Gzmb (granzyme B); Igrm (immunity-related GTPase family); IL-10 (interleukin-10); Junb (junb proto-oncogene, AP-1 transcription factor subunit); Mmp10 (matrix metallopeptidase 10); Nr4a1 (nuclear receptor subfamily group a member 1), 2, and 3; Rbpsuh (recombining binding protein suppressor of hairless); and Tpmt (thiopurine S-methyltransferase).

according to manufacturer instructions. The resulting microarray .CEL files were imported into Partek Genomics Suite (Partek) and normalized using robust multi-array averaging (RMA).

Preliminary analyses focused on two moderately overlapping gene clusters, including all immune-related genes (a total of 1473 genes), and those related to neuroinflammation and vascular function (283 genes examined). Microarray outcomes for the immune-related gene cluster were analyzed using a 3 (Diet: pair-fed [PF] vs. ethanol [ET] vs. free choice liquid diet [FCL]) by 2 (Sex: female vs. male) by 2 (Age: adolescent vs. adult) ANOVA with a Benjamini-Hochberg correction of the false discovery rate (FDR).

2.6. Validation of significant gene expression changes

In addition to completion of the unbiased scan for gene expression changes performed via microarray analysis, we performed follow-up analyses on two separate clusters of genes. The first set of genes examined were those which our previous work (or others) had established as being sensitive to acute ethanol exposure in adults (i.e., a large binge-like dose of ethanol resulting in blood ethanol concentrations approximately 200 mg/dL or higher), and for which *a priori* expectations for directional outcomes were possible. These genes included Interleukin [IL]-1β, IL-6, IL-10, IL-1 Receptor-Like 1 (IL-1RL1), and IL-1 Receptor 2 (IL-1R2). The second set of genes selected for analysis were genes identified by the unbiased scan, which we sought to validate using a secondary method of detection. To accomplish this, we used qRT-PCR on individual animal samples. A total of 40 ng/ul cDNA was reverse transcribed from OB RNA using the QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA). qRT-PCR reactions were then performed using 5 µl SybrGreen I Master Mix (Roche Applied Sciences, Indianapolis, IN), 1 µl mixed F/R primers, 3 µl PCR grade H_2O, and 1 µl OB cDNA as per the LightCycler® 480 SYBR Green I Master Kit instructions. All qPCR reactions were run in duplicate. The number of cycles to pass the detection threshold was determined for each gene using the 2nd derivative maximum algorithm.

2.7. Global gene expression analysis

Additionally, we performed a global analysis of all genes interrogated by the Rat Gene 1.0 Array. We performed ANOVAs to identify genes with a significant main effect on treatment ($p < 0.05$). P-values were uncorrected in order to retain more nominally significant probesets for downstream pathway analysis. Fold changes reported reflect entire group differences irrespective of sex. Genes with $p < 0.05$ for both ages were imported into QIAGEN Ingenuity® Pathway Analysis (IPA) software and overlaid onto three pre-determined canonical pathways built into IPA: Toll-like Receptor, IL-6 and Nf-κB Signaling Pathways. The expression values of genes present in this filtered list was then used to predict overall activity within a given pathway using IPA's Molecular Activity Prediction function.

3. Results

3.1. General information.

After FDR correction, we identified significant main effects of Diet for 146 genes, a main effect of Age causing change to 998 genes, and no effect of sex (based on $p < 0.05$). There was a Diet × Sex × Age interaction affecting 72 genes. As there was no main effect of sex, and sample size was low, outcome was collapsed across sex. Adolescents and adults were then analyzed separately in individual 3-way ANOVAs (Diet: pair-fed [PF] vs. ethanol [ET] vs. free choice liquid diet [FCL]). There were main effects of prenatal Diet in both adults and adolescents. Based on microarray results, genes of interest were selected for secondary verification via RT-PCR. Additionally, genes that have been noted as particularly sensitive to ethanol in previous studies [11,26,27] were selected for *a priori* analysis and RT-PCR confirmation.

3.2. Ethanol vs. PF comparisons

Adolescents in the ET condition showed an **increase** in only one gene in ET as compared to PF: dual specificity phosphatase 1 (Dusp1, +1.233 fold change, $p < 0.05$); no **decreases** were observed. The ET adults showed no **increase** compared to PF, and only the following 3 genes to have **decreased** by over 1.30 fold (30% or more), as compared to PF adults: S100 calcium binding protein A9 (S100a9, −1.69 fold change, 0.59 ratio, $p < 0.001$); proteoglycan 2, bone marrow (Prg2, −1.67 fold change, 0.60 ratio, $p < 0.01$); and periostin, osteoblast specific factor (Postn,-1.33 fold change, 0.75 ratio, $p < 0.01$). Additionally, two genes showed a fold change greater than 1.2 and fit the FDR p value criterion for significance were: hemoglobin subunit beta (Hbb, −1.28 fold change, 0.78 ratio, $p = 0.05$) and thiopurine S-methyltransferase (Tpmt, −1.25 fold change, 0.80 ratio, $p = 0.01$).

3.3. Ethanol vs. FCL comparisons

In adolescents, one gene showed a significant high-fold **increase** in ET as compared to FCL. Compared to the FCL control, ET adolescents showed an increase in immunity-related GTPase M (Irgm, +1.21 fold change, $p < 0.01$). Compared to the FCL control, adolescents in the ET

Table 1
ADOLESCENT (P40) gene microarray and qRT-PCR ANOVA results expressed as ratio comparisons with noted significance (p value) for: dual specificity phosphatase 1 (Dusp1); early growth response protein 1 (Egr1); interferon gamma (Ifnγ); transcription factor jun-B (JunB); matrix metalloproteinase-10 (Mmp10); nuclear receptor subfamily 4 group A members 1 (Nr4a1), 2 (Nr4a2), and 3 (Nr4a3); S100 calcium-binding protein A9 (S100a9); toll-like receptor 11 (Tlr11); Interleukin-1β (IL-1 β); Interleukin-1 receptor-like 1 (IL-1RL1); Interleukin-1 receptor type 2 (IL-1R2); IL-6; and Interleukin-10. Pearson's correlations between microarray and qRT-PCR results were performed to confirm trends. **Bolded** numbers indicate significant effects at the $p < 0.05$ criterion. For microarray data, *underlined italics* indicate effects that fell under the step-up p value of $p < 0.2$, and in PCR, trends that were identified at $p < 0.1$.

		P40: Adolescents						Pearson's R	Trends Validated?
		Microarray			qRT-PCR				
		ET vs. FCL	ET vs. PF	PF vs. FCL	ET vs. FCL	ET vs. PF	PF vs. FCL		
Dusp1	Ratio	0.902	**1.233**	**0.731**	1.113	**1.737**	**0.641**	+0.995	Yes
	p value	0.301	**0.042**	**0.004**	0.619	**0.002**	**0.027**		
Egr1	Ratio	*0.781*	1.095	**0.714**	**1.401**	*1.328*	1.055	+0.468	Yes
	p value	*0.052*	0.461	**0.010**	**0.018**	*0.074*	0.677		
Ifnγ	Ratio	0.980	0.953	1.029	**2.485**	1.549	1.614	−0.103	No
	p value	0.797	0.531	0.711	**0.018**	0.310	0.295		
JunB	Ratio	0.847	*1.249*	**0.678**	0.967	**1.694**	**0.571**	+0.998	Yes
	p value	0.162	*0.065*	**0.002**	0.903	**0.033**	**0.025**		
Mmp10	Ratio	0.830	0.957	0.867	0.872	0.959	0.908	+0.991	Yes
	p value	0.035	0.603	0.100	0.629	0.889	0.719		
Nr4a1	Ratio	**0.743**	1.132	**0.656**	0.831	1.516	0.548	+0.993	Yes
	p value	**0.090**	0.470	**0.020**	0.694	0.304	0.163		
Nr4a2	Ratio	*0.805*	1.096	**0.735**	1.090	1.415	0.770	+0.944	Yes
	p value	*0.106*	0.485	**0.025**	0.745	0.139	0.298		
Nr4a3	Ratio	**0.754**	0.973	0.775	0.894	1.525	0.586	+0.915	Yes
	p value	**0.036**	0.834	0.056	0.819	0.345	0.225		
S100a9	Ratio	0.942	0.953	0.988	1.104	1.059	0.983	−0.989	No
	p value	0.630	0.701	0.921	0.682	0.647	0.886		
Tlr11	Ratio	1.062	1.053	1.009	*1.488*	**1.776**	0.838	+0.894	Yes
	p value	0.513	0.577	0.923	*0.082*	**0.043**	0.541		
IL-1β	Ratio	0.932	1.002	0.930	1.110	1.198	0.926	+0.765	Yes
	p value	0.275	0.970	0.259	0.340	0.175	0.508		
IL-1RL1	Ratio	1.031	1.027	1.004	0.960	1.273	0.754	+0.711	Yes
	p value	0.533	0.588	0.934	0.844	0.271	0.124		
IL-1R2	Ratio	1.000	1.010	0.990	1.238	**1.325**	0.935	+0.952	Yes
	p value	0.999	0.906	0.905	0.130	**0.032**	0.635		
IL-6	Ratio	0.930	1.077	0.861	1.131	*1.308*	0.865	+0.949	Yes
	p value	0.305	0.298	0.045	0.385	*0.061*	0.222		
IL-10	Ratio	1.009	1.112	0.907	1.356	1.070	1.267	−0.675	No
	p value	0.888	0.101	0.131	0.220	0.822	0.387		

condition showed three genes to have **decreased** significantly by more than 1.3-fold: chitinase 3-like 3 (Chi3l3, −1.84 fold change, 0.54 ratio, $p < 0.05$), nuclear receptor subfamily 4, group A, member 3 (Nr4a3, −1.32 fold change, 0.74 ratio, $p < 0.05$), and, using the step-up p value criterion, nuclear receptor subfamily 4, group A, member 1 (Nr4a1, −1.35 fold change, 0.75 ratio, $p = 0.09$). One gene was decreased greater than 1.2 fold change: matrix metallopeptidase 10 (Mmp10, −1.20 fold change, 0.83 ratio, $p < 0.05$). Additionally, two genes showed fold changes greater than 1.2 and fit the step-up p value criterion for significance: early growth response 1 (Egr1, −1.28 fold change, 0.78 ratio, $p = 0.052$) and nuclear receptor subfamily 4, group A, member 2 (Nr4a2, −1.24 fold change, 0.81 ratio, $p = 0.106$).

A much wider variety of genes showed gene expression elevation in ET adults as compared to FCL controls than any other groups. ET adult genes that showed more than a 1.3-fold significant **increase** from FCL are as follows: nuclear receptor subfamily 4, group A, member 3 (Nr4a1: +1.57 fold change, $p < 0.05$), nuclear receptor subfamily 4, group A, member 3 (Nr4a2: +1.47 fold change, $p < 0.01$), and nuclear receptor subfamily 4, group A, member 3 (Nr4a3: + 1.46 fold change, $p < 0.05$), early growth response 1 (Egr1: +1.38 fold change, $p < 0.05$), coagulation factor II (thrombin) (F2: +1.33 fold change, $p < 0.01$), jun B proto-oncogene (Junb: +1.33 fold change, $p < 0.01$), dual specificity phosphatase 1 (Dusp1: +1.31 fold change, $p < 0.05$), Chitinase 3-like 3 (Chi3l3: +1.40 fold change, 1.13 ratio, $p = 0.20$) also had significant fold change over 1.3 and fit the step-up p value criterion.

Sixty-nine genes showed greater than 1.2-fold **increase** from FCL controls and met the criterion ($p < 0.05$) for significance. The top twenty-four genes that were significant at $p < 0.05$ and showed the highest fold change are displayed in Table 3.

Adults in the ET condition showed one gene to have **decreased** significantly by more than 1.3-fold as compared to FCL control: glucosaminyl (N-acetyl) transferase 1, core 2 beta-1,6-N-acetylglucosaminyltransferase (Gcnt, −1.31 fold change, 0.76 ratio, $p < 0.05$). Three genes showed significant change greater than 1.2 fold as compared to FCL controls: thiopurine S-methyltransferase (Tmpt, −1.23 fold change, $p < 0.05$); recombining binding protein suppressor of hairless (RBPJ, aka Rbpsuh, −1.22 fold change, 0.82 ratio, $p < 0.05$); C-C chemokine receptor type 5 (Ccr5, −1.20 fold change, 0.83 ratio, $p < 0.05$). One gene showed change greater than 1.2 fold as compared to FCL controls and met the step-up p value criterion: Granzyme B (Gzmb, −1.24 fold change, 0.81 ratio, $p = 0.20$). A volcano plot highlighting the more significant findings of the microarray can be found in Fig. 1B & C.

3.4. Control comparison: FCL vs. PF

In both adults and adolescents, some genes differed significantly between the control groups FCL and PF. These data are reported in Tables 1 and 2 for adolescents and adults, respectively. Largely, adolescents showed suppression of a few genes in the PF condition as compared to FCL, as confirmed by RT-PCR (Dusp1, JunB), whereas PF adults showed an increase in a subset of genes (Egr1, JunB, Nr4a1, Nr4a2, Nr4a3).

Table 2
ADULT (P90) gene microarray and qRT-PCR ANOVA results expressed as ratio comparisons with noted significance (p value) for: dual specificity phosphatase 1 (Dusp1); early growth response protein 1 (Egr1); interferon gamma (Ifnγ); transcription factor jun-B (JunB); matrix metalloproteinase-10 (Mmp10); nuclear receptor subfamily 4 group A members 1 (Nr4a1), 2 (Nr4a2), and 3 (Nr4a3); S100 calcium-binding protein A9 (S100a9); toll-like receptor 11 (Tlr11); Interleukin-1β (IL-1 β); Interleukin-1 receptor-like 1 (IL-1RL1); Interleukin-1 receptor type 2 (IL-1R2); IL-6; and Interleukin-10. Pearson's correlations between microarray and qRT-PCR results were performed to confirm trends. **Bolded** numbers indicate significant effects at the $p < 0.05$ criterion. For microarray data, *underlined italics* indicate effects that fell under the step-up p value of $p < 0.2$, and in PCR, trends that were identified at $p < 0.1$.

		P90: Adults						Pearson's r	Trends Validated?
		Microarray			qRT-PCR				
		ET vs. FCL	ET vs. PF	PF vs. FCL	ET vs. FCL	ET vs. PF	PF vs. FCL		
Dusp1	Ratio	**1.312**	1.121	1.171	**1.442**	*1.341*	1.075	+0.512	Yes
	p value	**0.010**	0.254	0.119	**0.034**	*0.072*	0.600		
Egr1	Ratio	**1.380**	1.069	**1.291**	**1.546**	1.035	**1.494**	+0.982	Yes
	p value	**0.013**	0.588	**0.045**	**0.006**	0.757	**0.009**		
Ifnγ	Ratio	1.062	1.091	0.973	1.082	2.062	0.525	+0.903	Yes
	p value	0.436	0.262	0.725	0.863	0.173	0.213		
JunB	Ratio	**1.330**	1.150	1.157	**2.452**	*1.400*	**1.751**	+0.955	Yes
	p value	**0.020**	0.236	0.217	**0.000**	*0.053*	**0.008**		
Mmp10	Ratio	1.187	0.970	**1.224**	1.059	0.739	1.433	+0.908	Yes
	p value	0.050	0.718	**0.023**	0.854	0.201	0.223		
Nr4a1	Ratio	**1.569**	1.164	*1.348*	**4.540**	*1.471*	**3.087**	+0.997	Yes
	p value	**0.013**	0.377	*0.089*	**0.000**	*0.086*	**0.005**		
Nr4a2	Ratio	**1.465**	1.084	**1.351**	**2.129**	1.015	**2.098**	+0.963	Yes
	p value	**0.007**	0.538	**0.029**	**0.000**	0.926	**0.000**		
Nr4a3	Ratio	**1.457**	1.009	**1.444**	**2.587**	0.929	**2.784**	+0.993	Yes
	p value	**0.007**	0.941	**0.008**	**0.005**	0.739	**0.004**		
S100a9	Ratio	0.905	**0.592**	**1.530**	0.941	0.888	1.060	+0.967	Yes
	p value	0.426	**0.000**	**0.002**	0.738	0.222	0.738		
Tlr11	Ratio	**1.211**	1.065	1.137	0.892	**1.65**	**0.541**	−0.535	No
	p value	**0.046**	0.497	0.171	0.610	**0.044**	**0.034**		
IL-1β	Ratio	1.021	1.121	0.911	1.050	0.999	1.051	−0.861	No
	p value	0.743	0.081	0.149	0.772	0.992	0.743		
IL-1RL1	Ratio	1.016	0.985	1.032	1.232	0.969	1.271	+0.975	Yes
	p value	0.742	0.749	0.516	0.247	0.797	0.125		
IL-1R2	Ratio	**1.263**	1.141	1.107	0.870	0.986	0.883	−0.405	No
	p value	**0.011**	0.131	0.239	0.487	0.939	0.570		
IL-6	Ratio	1.094	0.949	1.152	1.115	0.943	1.182	+1.000	Yes
	p value	0.210	0.459	0.052	0.658	0.808	0.364		
IL-10	Ratio	**1.291**	1.063	**1.215**	1.501	1.148	1.308	+0.970	Yes
	p value	**0.000**	0.335	**0.004**	0.341	0.789	0.597		

Table 3
The table displays the top (showing highest fold change) 24 genes that were significantly ($p < 0.05$) increased in the Ethanol vs FCL comparison in ADULTS.

Full name	Abbreviated name	Fold change	p-value
Interleukin-10	IL-10	+1.29	<0.01
Oncostatit M	Osm	+1.29	<0.05
NK3 homeobox 5	Nkx2-5	+1.29	<0.01
Interleukin-17c	IL-17c	+1.28	<0.01
Amyloid P component, serum	Apcs	+1.20	<0.05
Programmed cell death 1 ligand 2	Pdcd1lg2	+1.28	<0.01
Activating transcription factor	Atf3	+1.28	<0.01
Somatostatin	Sst	+1.28	<0.01
Collagen type XIII alpha 1 chain	Col13a1	+1.28	<0.0001
Tumor necrosis factor alpha superfamily member 14	Tnfsf14	+1.27	<0.05
Histidine rich glycoprotein	Hrg	+1.27	<0.01
Glial cell derived neurotrophic factor	Gdnf	+1.27	<0.01
Dihydropyrimidinase	Dpys	+1.27	<0.05
Myosin binding protein C, fast type	Mybpc2	+1.27	<0.01
Prilipin	Plin	+1.26	<0.01
Sodium voltage-gated channel alpha subunit 4	Scn4a	+1.26	<0.01
Interleukin-9	IL-9	+1.26	<0.01
ATP binding cassette subfamily C member 4	Abcc4	+1.26	<0.01
Interleukin-1 receptor type 2	Il1r2	+1.26	<0.01
NLR family, pyrin domain containing 6	Nlrp6	+1.26	<0.01
C-C motif chemokine ligand 22	Ccl22	+1.26	<0.01
Protein C, inactivator of coagulation factors Va and VIIIa	Proc	+1.26	<0.05
ELOVL fatty acid elongase 3	Elovl3	+1.25	<0.05
Tumor necrosis factor superfamily member 25	Tnfrsf25	+1.25	<0.05

3.5. qRT-PCR confirmation

A specific subset of genes that showed significant results from the microarray were chosen for PCR validation. Of these, trends were strongly validated using Pearson's correlations performed separately in each age on ratios between groups, with details and exceptions as follows. In adolescents (see Table 1), the suppression of Nra4a1, 2, and 3 observed in the microarray were not supported by PCR confirmation. Two new effects were seen in PCR that did not appear in the microarray; a significant increase in Interferon gamma in ET as compared to FCL adolescents (IFNγ, +2.49 fold change, $p < 0.05$) and a significant increase in Egr1 (+1.40 fold change, $p < 0.05$). In the adults (see Table 2), all trends were confirmed except TLR11, IL-1β, and IL-1R2.

3.6. A priori tests

Based on our previous work identifying key cytokines across other brain regions that were responsive to ethanol, we performed *a priori* comparisons on a small subset of genes. These results can be seen in the lower panels of Tables 1 and 2. Of these, there were no changes observed in adolescents (matching the microarray findings). In adults, effects seen in the microarray (IL-10 and IL-1R2 increases in the ET vs. FCL comparison) were not confirmed by PCR.

3.7. Molecular activation prediction using Ingenuity pathway analysis (IPA)

Using the molecular activation prediction as in our prior work [28] to probe canonical pathways of activation of NF-κB, TLR, and IL-6 pathways resulted in significant predictions for both ages. In adolescents, the NF-κB pathway was predicted to be activated (Fig. 2), whereas the adults showed the opposite results with predicted suppression of the pathway (Fig. 3). No significant predictions were made about TLR activation in adolescents, whereas adults showed predicted TLR inhibition (see Fig. 4) . Likewise, there was no predicted adolescent effect for IL-6 pathway activation, whereas the adults showed a predicted increase in IL-6 and STAT3 activation (see Fig. 5).

4. Discussion

The chemosensory properties of ethanol are an integral part of the alcohol consumption experience, and there is evidence that the olfactory cues related to ethanol exposure play a key role in shaping future drinking. It has been shown that PAE can render ethanol odor more palatable, thus increasing intake and ethanol preference in adolescence [19,29]. The exact circuitry of these effects in the olfactory bulb (OB) and other connecting structures has not been fully described. The OB is an immunologically sensitive site; for instance, cytokine signaling in the OB has been shown to interact with circuitry pertaining to social interaction [30], an effect that may determine approach and avoidance of conspecifics depending on their recent health status such as stress exposure and acute illness [31] or other demographic features of would-be social partners such as sex, hormonal status, or age [32–36]. Indeed, olfactory cues associated with ethanol exposure strongly influence acceptance of ethanol among peers in rodent models [37], further underscoring the importance of neuroimmune- and plasticity-related findings within the OB.

In this set of data, we probed whether prenatal alcohol exposure would create baseline differences in a network of immune-related genes in adolescence or adulthood that could help predict pathways that are vulnerable to early age ethanol effects. It is important to note that the discrete window of ethanol exposure was specific to gestational days 11–20. During this peak period of exposure, the developing olfactory bulb is beginning as an out-pocketing of the rostral end of the cerebral vesicles [E14-E15, [38,39]. Importantly, while prior studies have demonstrated the damaging impact of pre-natal exposure on olfactory development, at the anatomical level [40–42], using the same exposure paradigm as in the present study, we have previously shown alterations in the expression of neurotransmission genes in the olfactory bulb of adolescent rats [15]. In extension to this work, we found changes in the resting state of immune genes following PAE, but perhaps more interestingly, these results were divergent when brains were harvested during adolescence relative to young adults. While some genes were altered in both adolescents and adults, the directionality and magnitude of changes were not the same across age. Indeed, two distinct profiles emerged when comparing adolescents and adults. The adolescents showed very few gene expression changes, though a small number of immune-related genes showed moderate suppression; specifically, Chi3l3, Egr1, Mmp10, and members of the Nr4a family.

Chi3l3, along with IL-10, is an established marker of the alternative M2 microglial phenotype [43]; in this activation state, microglia serve a neuro-protective role in the brain by taking on an anti-inflammatory profile [44]. It has previously been shown that a single bout of binge ethanol administration results in microglial activation that persists into young adulthood, signifying that adolescence is a time period of immune vulnerability [45] and that changes that occur at this time are likely to make a lasting contribution to the adult response to an immune challenge. As the immune system is moldable across prenatal and adolescent time periods, changes to microglial status observed at these times are likely to have long-term repercussions. Egr1 has been implicated in the maintenance of neuronal plasticity [46]. Mmp10 has been shown to have protective immune properties in a lung model of acute infection [47]. The Nr4a family, comprised of Nr4a1, Nr4a2, and Nr4a3, is a set of immediate early genes associated with the acute response to stress and cytokine signaling [48]. Additionally, these receptors are expressed on dopamine neurons have been shown to have involvement in dopaminergic development [49]. The downregulation of these factors may hint at a slackening of neuroimmune protection,

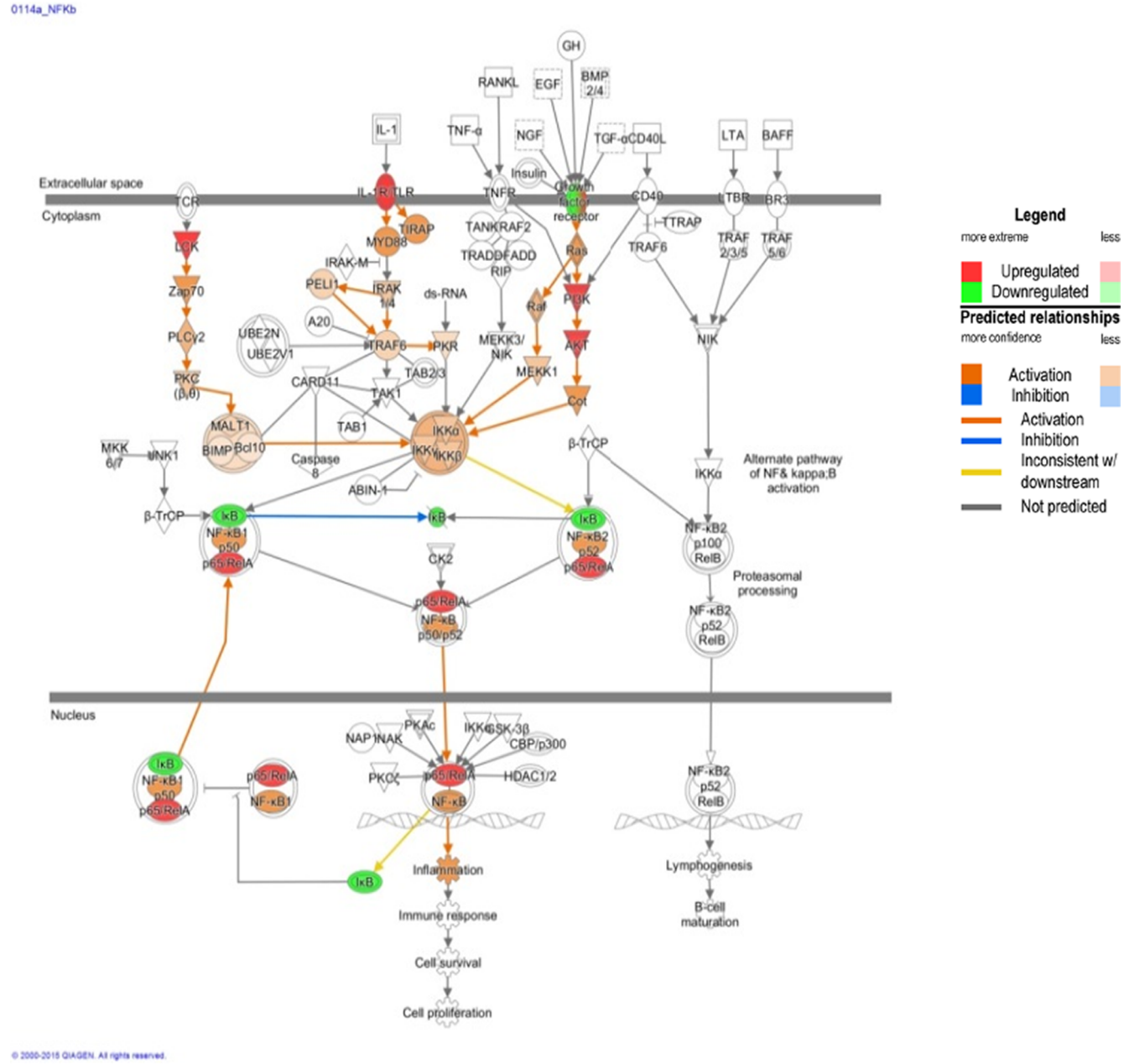

Fig. 2. Nf-κB pathway prediction in adolescents. This figure shows the predicted state of activation for the NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) pathway in adolescents.

but it is not yet possible to determine whether these changes are specifically maladaptive or protective, as this study was designed to identify unbiased networks of immune change without delivering a challenge.

Meanwhile, compared to the mild changes seen in adolescence, the adults showed larger scale upregulation of immune-related genes. Of the few genes that were affected in both age groups, effects were opposite in adolescence and adulthood. For instance, Chi3l3, Egr1, and the Nra4 family showed strong upregulation in adults. Additionally, a large cadre of genes showed mild to moderate upregulation. It is well known that sensitivity to the negative and positive effects of ethanol shifts across ontogeny, and that adolescent insensitivity to the aversive properties of ethanol is a contributing factor to this age group's vulnerability to developing alcohol use disorders (for a recent comprehensive review, see [50]). Immune signaling has emerged as a potential moderator of ethanol consumption, with studies having shown that genetic manipulation of cytokine and chemokine genes and receptors altered ethanol intake behavior [14] and the reinforcing properties of ethanol [51]. More studies will be necessary to examine whether the age differences observed here are related to ethanol's appetitive or aversive properties, or perhaps other developmental factors.

Pathway activation predictions were used to contextualize some of the individual gene effects observed in the microarray. When mapped onto canonical and alternative pathways pertaining to ethanol-induced immune activation, this analysis drew further distinction between the profiles of adolescents and adults after gestational ethanol exposure. In the adolescents, pathway analysis predicted higher activation of TLR pathways and Nf-κB signaling in adolescence as a result of PAE, whereas the adults were likely to show the opposite effects. Several studies report similar findings indicating that a chronic history of ethanol can blunt the later response to immune challenge. For instance, it has been shown that PAE is associated with increased susceptibility to infectious disease [52].

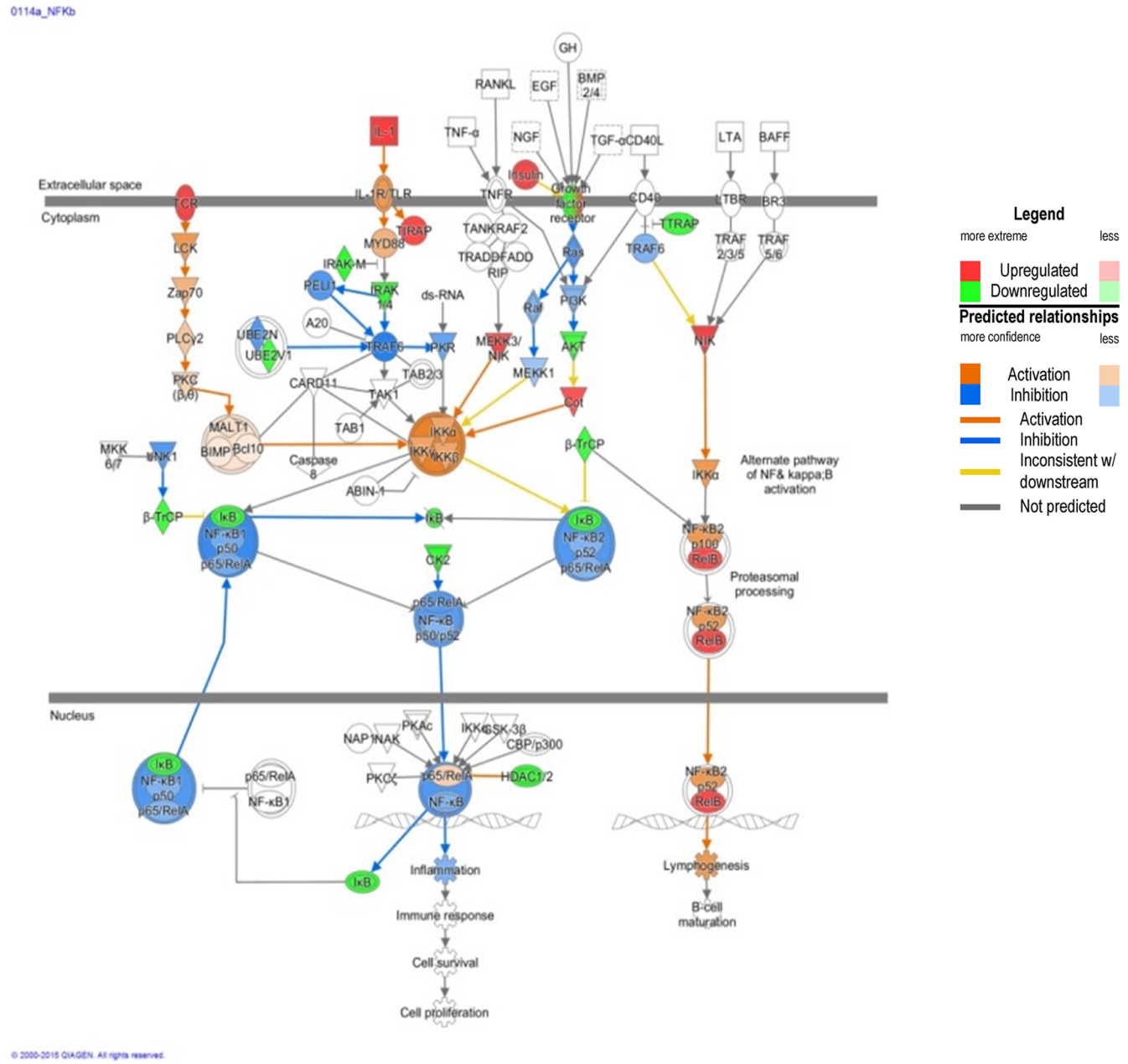

Fig. 3. Nf-κB pathway prediction in adults. This figure shows the predicted state of activation for the NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) pathway in adults.

Also, it has been shown that PAE resulted in a blunted neuroimmune (i.e. prefrontal cortex and hippocampus) response to an immune challenge in young adult rats, as well as altered steady-state levels of brain gene expression [53]. In our own work, we have observed that while ethanol-naïve rats displayed a blunted neuroimmune response to ethanol in adolescence [54], adult rats that have had a history of adolescent chronic intermittent ethanol displayed a blunted peripheral immune response to lipopolysaccharide challenge [55], as well as ethanol challenge [56]. Together, these findings indicate that ethanol exposure during sensitive time periods (i.e. *in utero* or during adolescence) have long-lasting effects on the immune response to a challenge in later development. It is not clear yet why the impact of this manipulation shows more dramatic effects in adults and almost no changes in adolescence, though likely this is because the adolescent immune system is still in active development at this age and effects that are wrought by prenatal ethanol exposure have set the course but not yet fully completed the changes that will later be seen in adulthood.

It is important to note that this set of data describes an altered steady-state in the network of immune genes that, although present in adolescence, emerges more strongly during adulthood. This allows for identification of pathways and genes that are susceptible to ethanol's effect, and while altered baselines allow us to infer the immune state of the PAE rat at different ages, further examination is necessary to fully understand these states. In this sense, it is important to recognize that the changes observed here represent *long-lasting alterations in ambient gene expression*, not stimulus-evoked responses at the age of testing. Probing these altered pathways with a challenge will be necessary to confirm how these PAE-induced changes in immune function will impact later reactivity to immune stimulus, further ethanol exposure, or stress. Other work has shown that extended ethanol exposure in adulthood resulted in changes in gene expression related to microglia in the amygdala and prefrontal cortex at 0 and 8 h following the last of a series of chronic ethanol administrations [57]. In the current study, baseline changes did not differ significantly based on sex, however,

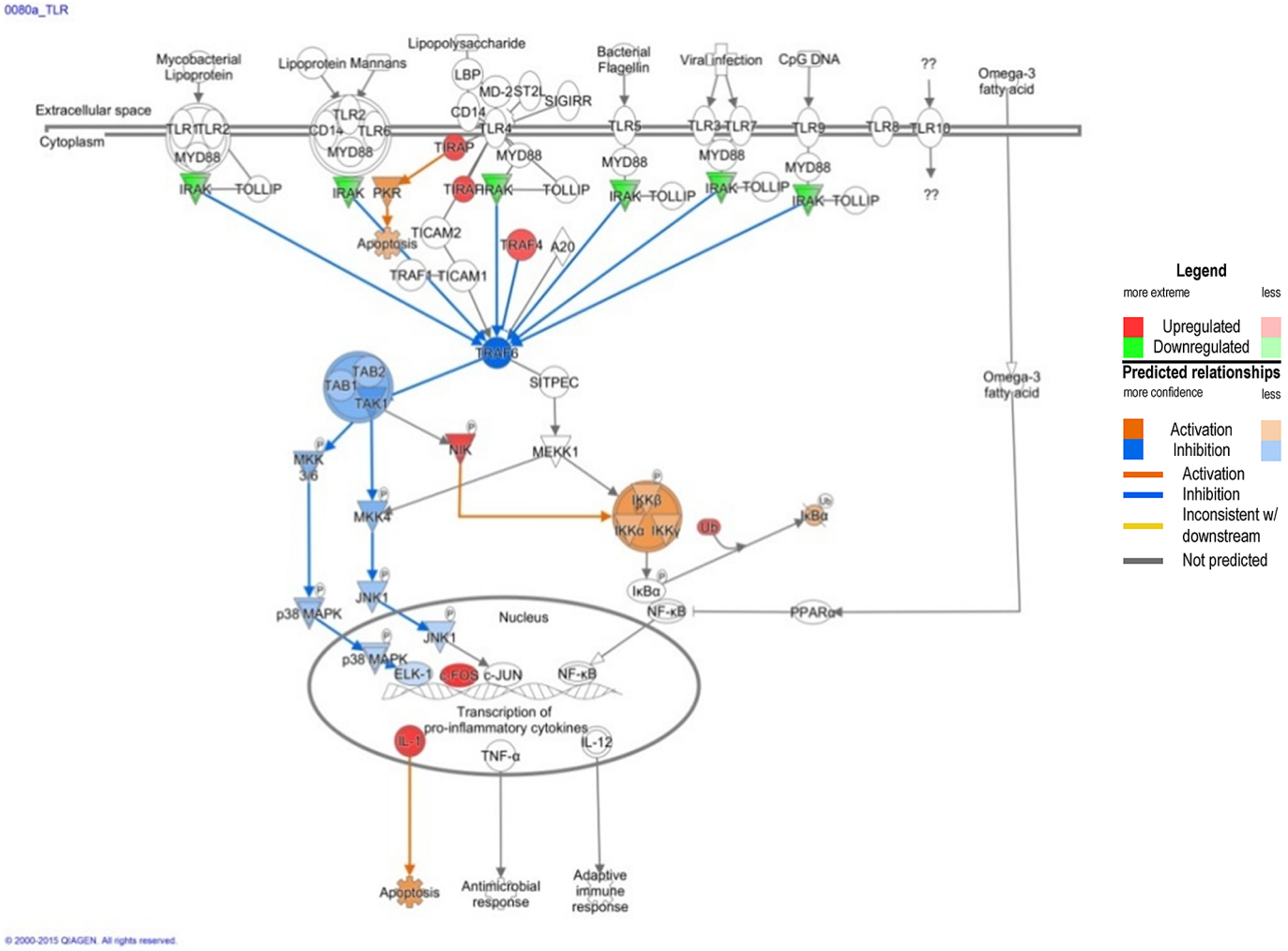

Fig. 4. TLR pathway prediction in adults. This figure shows the predicted state of activation for the TLR (Toll-like receptor) pathway in adults.

other work has shown that the effects of PAE can be sex-specific across development [58]. It is possible that baseline differences belie an effect of sex that may emerge when a challenge is delivered, and therefore future studies will still need to examine males and females.

Overall, this work provides an unbiased analysis of neuroimmune-related gene expression change following prenatal ethanol exposure. PAE resulted in distinct patterns of change across ontogeny, and IPA pathway analysis predicted different patterns of activation in adolescence versus adulthood, suggesting that the preponderance of neuroimmune gene expression changes produced by PAE emerge *after* closure of the adolescent developmental period and become more evident in young adulthood. In future studies, it will be interesting to see whether such changes continue to emerge and/or expand across the lifespan and the overall impact on CNS function in late aging. Together, these data indicate that chronic prenatal alcohol exposure produces little gene expression change in itself in adolescence yet predisposes this age group to a strong Nf-κB activation, which is diametrically opposed to a typical adolescent immune response that has been shown to be blunted in comparison to adult counterparts. In contrast, neuroimmune gene upregulation was found in the adults, along with a predicted dampened TLR and NF-κB activation, commensurate with the literature indicating that early life chronic ethanol exposure produces a locking-in-like neuroimmune effect in adulthood and a suppressed neuroimmune response to challenge. These data provide new information about the olfactory bulb immune response, indicating that PAE alters the immune pathways in this brain area in a manner that is long-lasting and significant.

CRediT authorship contribution statement

Anny Gano: Data curation, Formal analysis, Visualization, Writing - original draft. **Laura Prestia:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Writing - review & editing. **Frank A. Middleton:** Data curation, Formal analysis, Methodology, Project administration, Resources, Software, Supervision, Validation, Writing - review & editing. **Steven L. Youngentob:**

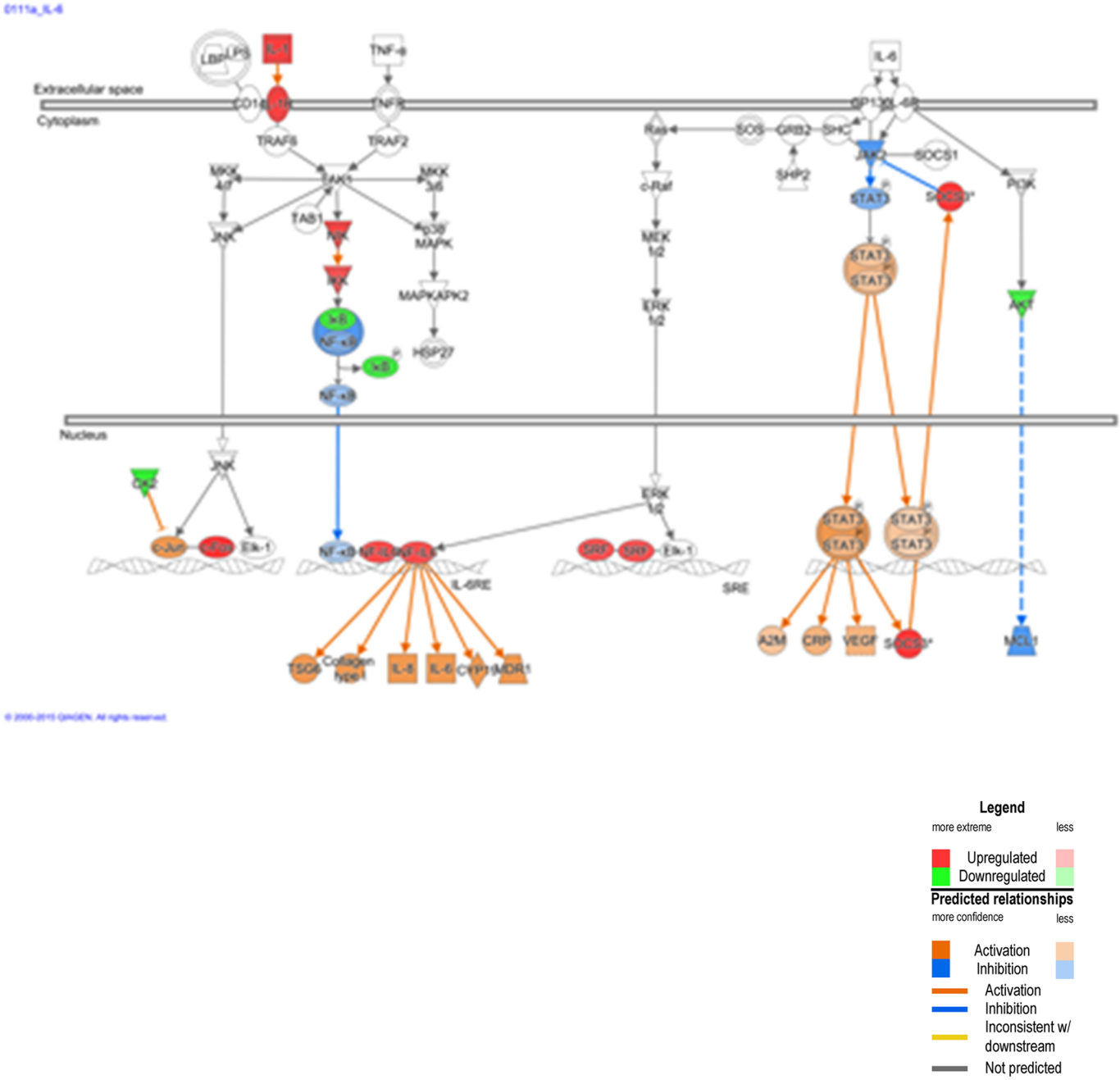

Fig. 5. IL-6 pathway prediction in adults. This figure shows the predicted state of activation for the IL-6 (Interleukin-6) pathway in adults.

Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Software, Supervision, Validation, Writing - review & editing. **Cherry Ignacio:** Data curation, Formal analysis, Software, Writing - review & editing. **Terrence Deak:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

[1] CDC. Fetal Alcohol Spectrum Disorders (FASDs): Data & Statistics. 2017 March 27, 2017 [cited 2017 6/1/2017]; Available from: https://www.cdc.gov/ncbddd/fasd/data.html.
[2] P.A. May, J.P. Gossage, Estimating the prevalence of fetal alcohol syndrome. A summary, Alcohol Res. Health: J. Natl. Inst. Alcohol Abuse Alcohol. 25 (3) (2001) 159–167.
[3] L. Wieczorek, et al., Hypothalamic-pituitary-adrenal axis and behavioral dysfunction following early binge-like prenatal alcohol exposure in mice, Alcohol 49 (3) (2015) 207–217.
[4] C. Raineki, et al., Short- and long-term effects of stress during adolescence on emotionality and HPA function of animals exposed to alcohol prenatally, Psychoneuroendocrinology 74 (2016) 13–23.
[5] U.B. Schambra, C.N. Lewis, T.A. Harrison, Deficits in spatial learning and memory in adult mice following acute, low or moderate levels of prenatal ethanol exposure during gastrulation or neurulation, Neurotoxicol. Teratol. (2017).
[6] C. Arias, M.G. Chotro, Increased palatability of ethanol after prenatal ethanol exposure is mediated by the opioid system, Pharmacol. Biochem. Behav. 82 (3) (2005) 434–442.
[7] K. Bordner, T. Deak, Endogenous opioids as substrates for ethanol intake in the neonatal rat: The impact of prenatal ethanol exposure on the opioid family in the early postnatal period, Physiol. Behav. 148 (2015) 100–110.
[8] H.D. Domínguez, M.F. López, J.C. Molina, Neonatal Responsiveness to Alcohol Odor and Infant Alcohol Intake as a Function of Alcohol Experience During Late Gestation, Alcohol 16 (2) (1998) 109–117.
[9] Y.A. Blednov, et al., Role of interleukin-1 receptor signaling in the behavioral effects of ethanol and benzodiazepines, Neuropharmacology 95 (2015) 309–320.
[10] J. Mayfield, L. Ferguson, R.A. Harris, Neuroimmune signaling: a key component of alcohol abuse, Curr. Opin. Neurobiol. 23 (4) (2013) 513–520.
[11] T.L. Doremus-Fitzwater, et al., Intoxication- and withdrawal-dependent expression of central and peripheral cytokines following initial ethanol exposure, Alcoholism, Clin. Exper. Res. 38 (8) (2014) 2186–2198.
[12] S. Alfonso-Loeches, et al., Pivotal Role of TLR4 Receptors in Alcohol-Induced Neuroinflammation and Brain Damage, J. Neurosci. 30 (24) (2010) 8285–8295.
[13] T.S. Bodnar, L.A. Hill, J. Weinberg, Evidence for an immune signature of prenatal alcohol exposure in female rats, Brain Behav. Immun. 58 (2016) 130–141.
[14] Y.A. Blednov, et al., Neuroimmune regulation of alcohol consumption: behavioral validation of genes obtained from genomic studies, Addict. Biol. 17 (1) (2012) 108–120.
[15] F.A. Middleton, et al., Gestational ethanol exposure alters the behavioral response to ethanol odor and the expression of neurotransmission genes in the olfactory bulb of adolescent rats, Brain Res. 1252 (2009) 105–116.
[16] N.E. Spear, J.C. Molina, Consequences of early exposure to alcohol: How animal studies reveal later patterns of use and abuse in humans, in: M.E.C.J.B. Overmier (Ed.), Animal research and human health: Advancing human welfare through behavioral science, American Psychological Association, Washington, DC, US, 2001, pp. 85–99.
[17] M.C. Fabio, et al., Prenatal ethanol increases ethanol intake throughout adolescence, alters ethanol-mediated aversive learning, and affects μ but not δ or κ opioid receptor mRNA expression, Eur. J. Neurosci. 41 (12) (2015) 1569–1579.
[18] N.E. Spear, J.C. Molina, Fetal or Infantile Exposure to Ethanol Promotes Ethanol Ingestion in Adolescence and Adulthood: A Theoretical Review, Alcohol. Clin. Exp. Res. 29 (6) (2005) 909–929.
[19] S.L. Youngentob, J. Glendinning, Fetal ethanol exposure increases ethanol intake by making it smell and taste better, PNAS 106 (13) (2009) 5359–5364.
[20] S.L. Youngentob, et al., Experience-induced fetal plasticity: The effect of gestational ethanol exposure on the behavioral and neurophysiologic olfactory response to ethanol odor in early postnatal and adult rats, Behav. Neurosci. 121 (6) (2007) 1293–1305.
[21] M.W. Miller, Circadian rhythm of cell proliferation in the telencephalic ventricular zone: effect of in utero exposure to ethanol, Brain Res. 595 (1) (1992) 17–24.
[22] S.L. Youngentob, et al., The effect of gestational ethanol exposure on voluntary ethanol intake in early postnatal and adult rats, Behav. Neurosci. 121 (6) (2007) 1306–1315.
[23] C.D. Driscoll, A.P. Streissguth, E.P. Riley, Prenatal alcohol exposure: Comparability of effects in humans and animal models, Neurotoxicol. Teratol. 12 (3) (1990) 231–237.
[24] E.M. Vavrousek-Jakuba, R.A. Baker, W.J. Shoemaker, Effect of Ethanol on Maternal and Offspring Characteristics: Comparison of Three Liquid Diet Formulations Fed during Gestation, Alcohol. Clin. Exp. Res. 15 (1) (1991) 129–135.
[25] F. Guo, et al., The Lieber-DeCarli Diet—A Flagship Model for Experimental Alcoholic Liver Disease, Alcohol.: Clin. Exper. Res. 42 (10) (2018) 1828–1840.
[26] A. Gano, et al., Rapid alterations in neuroimmune gene expression after acute ethanol: Timecourse, sex differences and sensitivity to cranial surgery, J. Neuroimmunol. 337 (2019) 577083.
[27] L. Richey, et al., Acute illness-induced behavioral alterations are similar to those observed during withdrawal from acute alcohol exposure, Pharmacol. Biochem. Behav. 103 (2) (2012) 284–294.
[28] C. Ignacio, S.M. Mooney, F.A. Middleton, Effects of Acute Prenatal Exposure to Ethanol on microRNA Expression are Ameliorated by Social Enrichment, Front. Pediatr. 2 (103) (2014).
[29] J.H. Hannigan, et al., Prenatal alcohol exposure selectively enhances young adult perceived pleasantness of alcohol odors, Physiol. Behav. 148 (Supplement C) (2015) 71–77.
[30] E.K. Hamasato, et al., Assessment of social behavior directed toward sick partners and its relation to central cytokine expression in rats, Physiol. Behav. 182 (2017) 128–136.
[31] H. Arakawa, S. Cruz, T. Deak, From models to mechanisms: Odorant communication as a key determinant of social behavior in rodents during illness-associated states, Neurosci. Biobehav. Rev. 35 (9) (2011) 1916–1928.
[32] H. Arakawa, K. Arakawa, T. Deak, Acute illness induces the release of aversive odor cues from adult, but not prepubertal, male rats and suppresses social investigation by conspecifics, Behav. Neurosci. 123 (5) (2009) 964–978.
[33] H. Arakawa, S. Cruz, T. Deak, Attractiveness of illness-associated odorant cues in female rats is modulated by ovarian hormones, but not associated with pro-inflammatory cytokine levels, Brain Behav. Immun. 26 (1) (2012) 40–49.
[34] H. Arakawa, K. Arakawa, T. Deak, Oxytocin and vasopressin in the medial amygdala differentially modulate approach and avoidance behavior toward illness-related social odor, Neuroscience 171 (4) (2010) 1141–1151.
[35] A.E. Perkins, et al., A working model for the assessment of disruptions in social behavior among aged rats: The role of sex differences, social recognition, and sensorimotor processes, Exp. Gerontol. 76 (2016) 46–57.
[36] A.E. Perkins, et al., Analysis of c-Fos induction in response to social interaction in male and female Fisher 344 rats, Brain Res. 1672 (2017) 113–121.
[37] A.M. Eade, L. Youngentob, S. Youngentob, The interaction of ethanol ingestion and social interaction with an intoxicated peer on the odor-mediated response to the drug in adolescent rats, Alcohol. Clin. Exp. Res. 40 (4) (2016) 734–742.
[38] A.I. Farbman, J.-P. Mbiene, Early development and innervation of taste bud-bearing papillae on the rat tongue, J. Comparat. Neurol. 304 (2) (1991) 172–186.
[39] A.I. Farbman, The cellular basis of olfaction, Endeavour 18 (1) (1994) 2–8.
[40] S. Barron, E.P. Riley, The effects of prenatal alcohol exposure on behavioral and neuroanatomical components of olfaction, Neurotoxicol. Teratol. 14 (4) (1992) 291–297.
[41] K.K. Sulik, M.C. Johnston, Sequence of developmental alterations following acute ethanol exposure in mice: craniofacial features of the fetal alcohol syndrome, Am. J. Anatomy 166 (3) (1983) 257–269.
[42] D.J. Bonthius, J.R. West, Acute and long-term neuronal deficits in the rat olfactory bulb following alcohol exposure during the brain growth spurt, Neurotoxicol. Teratol. 13 (6) (1991) 611–619.
[43] Zengyang Pei, Shenghao Wang, Qi Li, Sevoflurane suppresses microglial M2 polarization, Neurosci. Lett. 655 (2017) 160–165, https://doi.org/10.1016/j.neulet.2017.07.001.
[44] S. Gordon, Alternative activation of macrophages, Nat. Rev. Immunol. 3 (1) (2003) 23.
[45] J.A. McClain, et al., Adolescent binge alcohol exposure induces long-lasting partial activation of microglia, Brain Behav. Immun. 25 (2011) S120–S128.
[46] F. Duclot, M. Kabbaj, The Role of Early Growth Response 1 (EGR1) in Brain Plasticity and Neuropsychiatric Disorders, Front. Behav. Neurosci. 11 (2017) 35.
[47] R.S. McMahan, et al., Stromelysin-2 (MMP10) Moderates Inflammation by Controlling Macrophage Activation, J. Immunol. 197 (3) (2016) 899–909.
[48] M.A. Maxwell, G.E.O. Muscat, The NR4A subgroup: immediate early response genes with pleiotropic physiological roles, Nucl. Receptor Signaling 4 (2006) e002.
[49] K. Sakurada, et al., Nurr1, an orphan nuclear receptor, is a transcriptional activator of endogenous tyrosine hydroxylase in neural progenitor cells derived from the adult brain, Development 126 (18) (1999) 4017–4026.
[50] T.L. Doremus-Fitzwater, L.P. Spear, Reward-centricity and attenuated aversions: An

adolescent phenotype emerging from studies in laboratory animals, Neurosci. Biobehav. Rev. 70 (2016) 121–134, https://doi.org/10.1016/j.neubiorev.2016.08.015.
[51] Y.A. Blednov, et al., Perturbation of chemokine networks by gene deletion alters the reinforcing actions of ethanol, Behav. Brain Res. 165 (1) (2005) 110–125.
[52] T.W. Gauthier, Prenatal Alcohol Exposure and the Developing Immune System, Alcohol Res.: Curr. Rev. 37 (2) (2015) 279–285.
[53] A.A. Lussier, et al., Prenatal alcohol exposure alters steady-state and activated gene expression in the adult rat brain, Alcohol., Clin. Exper. Res. 39 (2) (2015) 251–261.
[54] T.L. Doremus-Fitzwater, et al., Male adolescent rats display blunted cytokine responses in the CNS after acute ethanol or lipopolysaccharide exposure, Physiol. Behav. 148 (2015) 131–144.
[55] A.S. Vore, et al., Adolescent Ethanol Exposure Leads to Stimulus-Specific Changes in Cytokine Reactivity and Hypothalamic-Pituitary-Adrenal Axis Sensitivity in Adulthood, Front. Behav. Neurosci. 11 (78) (2017).
[56] A. Gano, et al., Assessment of Extracellular Cytokines in the Hippocampus of the Awake Behaving Rat Using Large-Molecule Microdialysis Combined with Multiplex Arrays After Acute and Chronic Ethanol Exposure, Alcohol. Clin. Exp. Res. (2019) 0(0).
[57] E. Osterndorff-Kahanek, et al., Chronic ethanol exposure produces time- and brain region-dependent changes in gene coexpression networks, PLoS ONE (2015) 10(3).
[58] K.A. Uban, et al., Sex differences in associations between white matter microstructure and gonadal hormones in children and adolescents with prenatal alcohol exposure, Psychoneuroendocrinology 83 (Supplement C) (2017) 111–121.