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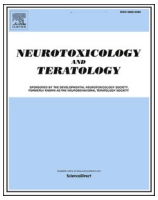

Ethanol modulation of hippocampal neuroinflammation, myelination, and neurodevelopment in a postnatal mouse model of fetal alcohol spectrum disorders

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ABSTRACT

Fetal alcohol spectrum disorders (FASD) are alarmingly common and result in significant personal and societal loss. Neuropathology of the hippocampus is common in FASD leading to aberrant cognitive function. In the current study, we evaluated the effects of ethanol on the expression of a targeted set of molecules involved in neuroinflammation, myelination, neurotransmission, and neuron function in the developing hippocampus in a postnatal model of FASD. Mice were treated with ethanol from P4-P9, hippocampi were isolated 24 h after the final treatment at P10, and mRNA levels were quantitated by qRT-PCR. We evaluated the effects of ethanol on both pro-inflammatory and anti-inflammatory molecules in the hippocampus and identified novel mechanisms by which ethanol induces neuroinflammation. We further demonstrated that ethanol decreased expression of molecules associated with mature oligodendrocytes and greatly diminished expression of a *lacZ* reporter driven by the first half of the myelin proteolipid protein (PLP) gene (*PLP1*). In addition, ethanol caused a decrease in genes expressed in oligodendrocyte progenitor cells (OPCs). Together, these studies suggest ethanol may modulate pathogenesis in the developing hippocampus through effects on cells of the oligodendrocyte lineage, resulting in altered oligodendrogenesis and myelination. We also observed differential expression of molecules important in synaptic plasticity, neurogenesis, and neurotransmission. Collectively, the molecules evaluated in these studies may play a role in ethanol-induced pathology in the developing hippocampus and contribute to cognitive impairment associated with FASD. A better understanding of these molecules and their effects on the developing hippocampus may lead to novel treatment strategies for FASD.

1. Introduction

Fetal Alcohol Spectrum Disorders (FASD) result from ethanol exposure to the developing fetus. They are alarmingly prevalent, occurring in 2–5% of births, and result in approximately 40,000 new cases annually in the United States (Wozniak et al., 2019). There is no cure for FASD which have tremendous impact at both an individual and societal level. Damage to the central nervous system (CNS) is common in FASD, which are the leading cause of intellectual disability (Abel and Sokol, 1986). FASD associated neuropathology occurs in multiple brain regions and can result in learning and memory deficits, impulsive behavior, mood disturbances, and increased prevalence of substance abuse (Glass et al., 2014; Joseph et al., 2014; Mattson et al., 2011; Norman et al., 2009; Streissguth et al., 2004). One such brain region, the hippocampus, plays a critical role in learning and memory as well as spatial navigation. Imaging studies demonstrate hippocampal abnormalities in people with FASD, which are believed to contribute to learning and memory deficits (Autti-Rämö et al., 2002; Coles et al., 2011; Dodge et al., 2020; Howell et al., 2006; Krueger et al., 2020; Willoughby et al., 2008). Animal models accurately reproduce pathology in the hippocampus and associated cognitive deficits commonly observed in individuals with FASD. Ethanol exposure in these models results in loss of hippocampal

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pyramidal and granule neurons as well as altered hippocampal synaptic plasticity, both of which contribute to aberrant learning and memory that are dependent on hippocampal function (Bonthius and West, 1991; Everett et al., 2012; Gil-Mohapel et al., 2010; Greene et al., 1992; Livy et al., 2003a; Murawski et al., 2012; Puglia and Valenzuela, 2010; Sadrian et al., 2014; Tran and Kelly, 2003; Zink et al., 2011; Zucca and Valenzuela, 2010).

Ethanol induced neuroinflammation in the developing CNS is believed to contribute to neuropathology observed in individuals with FASD and has been demonstrated in both gestational and postnatal animal models of FASD with widespread occurrence observed in the cerebral cortex, cerebellum, hippocampus, hypothalamus, midbrain, and spinal cord (Aghaie et al., 2020; Ahlers et al., 2015; Bodnar et al., 2016; Boschen et al., 2016; Cantacorps et al., 2020; Chastain et al., 2019; Drew et al., 2015; Kane et al., 2011; Komada et al., 2017; H. Li et al., 2019; Lussier et al., 2015; Pascual et al., 2017; Raineki et al., 2017; Ren et al., 2019; Ruggiero et al., 2018; Shrivastava et al., 2017; Terasaki and Schwarz, 2016; Tiwari and Chopra, 2011; Topper et al., 2015; K. Zhang et al., 2018). The early postnatal period in rodents is developmentally equivalent to the third trimester of pregnancy in humans (Clancy et al., 2001). Ethanol administered postnatally activates microglia in the developing hippocampus demonstrated by altered morphology toward a more amoeboid appearance, as well as increased expression of pro-inflammatory cytokines TNF-α and IL-1β and chemokines CCL2 and CCL4 (Boschen et al., 2016; Drew et al., 2015; Ruggiero et al., 2018; Tiwari and Chopra, 2011; Topper et al., 2015). Interestingly, the expression of the anti-inflammatory cytokine TGF-β was also increased by ethanol exposure in some of these studies (Boschen et al., 2016; Tiwari and Chopra, 2011). Despite these observations, the mechanisms by which ethanol induces neuroinflammation in the developing hippocampus are largely unknown. In one study, ethanol induced the expression of the transcription factor NF-κB, which is known to activate the expression of genes encoding pro-inflammatory cytokines and chemokines, in the hippocampus, suggesting one mechanism by which ethanol may induce neuroinflammation (Tiwari and Chopra, 2011).

Imaging studies demonstrate white matter abnormalities in children which can persist in adolescents and adults with FASD. These white matter abnormalities are believed to contribute to behavioral and cognitive deficits in individuals with FASD (Archibald et al., 2001; L. Li et al., 2009; Ma et al., 2005; Sowell et al., 2008; Treit et al., 2013; Wozniak et al., 2019). Myelin is a lipid rich structure that extends from oligodendrocytes to surround the axon of neurons, forming a sheath, which provides trophic support, insulates the axon, and facilitates neuron-to-neuron communication, in the form of propagation of action potentials. Mature myelinating oligodendrocytes are generated upon differentiation of oligodendrocyte progenitor cells (OPCs) (Baydyuk et al., 2020; Elbaz and Popko, 2019; Kuhn et al., 2019). Importantly, ethanol affects oligodendrocyte lineage cells and myelination in animal models of FASD. For example, ethanol caused hypoplasia of the optic nerve as well as aberrant myelin structure and reduced numbers of myelinated axons in the developing optic nerve in rodents (Parson et al., 1995; Phillips and Krueger, 1992; Pinazo-Duran et al., 1997; Samorajski et al., 1986). Postnatal ethanol exposure also caused myelin deficits in the cerebellum (Rufer et al., 2012) and decreased the number of OPCs and mature oligodendrocytes in the corpus callosum (Newville et al., 2017). Relatively little is known concerning the effects of ethanol on myelination in the hippocampus of FASD animal models, which we have investigated in the current studies.

The brain undergoes a late phase of histiogenesis during early postnatal development in mice. A number of critical developmental events occur during this period including later stages of neurogenesis, cell migration, and synapse formation (Camarillo and Miranda, 2008; Rice & Barone, 2000; Wilhelm and Guizzetti, 2015). Ethanol is known to alter synaptic plasticity and neurotransmission during this period of development which can result in long-term pathology associated with FASD (Fontaine et al., 2016; Valenzuela et al., 2011).

The present studies were designed to evaluate ethanol effects on the developing hippocampus using a postnatal mouse model of FASD, equivalent to third trimester exposure in humans. We specifically analyzed the hippocampus because of its susceptibility to the toxic effects of ethanol during this stage of development, and its role in memory, learning, and behavior, which are commonly aberrant in individuals with FASD. We evaluated the effects of ethanol on the expression of pro-inflammatory and anti-inflammatory molecules, molecules expressed by oligodendrocytes and OPCs, and molecules critical to hippocampal development and function. These studies suggest additional mechanisms by which ethanol mediates neurotoxicity in the developing hippocampus, and provide insight concerning potential novel targets for treatment of FASD.

2. Materials and methods

2.1. Animals

C57BL/6 J mice were obtained from Jackson Laboratories (Bar Harbor, ME; Cat. #000664; RRID:IMSR_JAX:000664). C57BL/6 J mice were used to evaluate the effects of ethanol on specific mRNAs by qRT-PCR as described below. *PLP1-lacZ* transgenic mice, which use the first half of the mouse *PLP1* gene to drive a *lacZ* expression cassette, were obtained from our breeding colony of PLP(+)Z mice (line 26H) (Wight et al., 1993). PLP1-*lacZ* transgenic mice or their wild-type littermate controls were used to evaluate the effects of ethanol on β-galactosidase activity which reflects PLP1 gene expression. Mice were housed in a federally approved Division of Laboratory Animal Medicine facility at the University of Arkansas for Medical Sciences and all experimental studies were conducted following protocols approved by the Institutional Animal Care and Use Committee. Mice were bred in-house to generate neonates used in the studies. Breeders were housed with 1 male and 2 females per cage on ventilated cage racks until females became visibly pregnant at which time they were removed and housed individually in static cages on an open-air rack. Pregnant females were monitored daily for birth of pups, with the day of birth being designated as P0. Food and water were provided ad libitum for the duration of breeding and subsequent experimental time periods. In addition to standard pine-chip bedding, all cages were supplemented with transparent polycarbonate igloos and cotton nestlets, and animals were only handled during cage changes or as necessary on experimental days. Furthermore, animals were housed in controlled temperature (22 °C set point) and humidity (30–50%) conditions on a 14:10 light:dark cycle. Experimental animals from Ethanol animals (Ethanol or E) were treated with 4 g/kg ethanol by intragastric gavage using 30% (*w/v*) ethanol diluted in 20% Intralipid while vehicle treated control animals (Vehicle or V) were similarly administered an equal volume of Intralipid in which water was substituted for ethanol. Treatment occurred daily from P4–9, during which time each litter was removed from its respective dam and placed into a warm cage with clean bedding. Each pup was marked, weighed, and dosed accordingly. After all pups from a given litter had been dosed over a period of 10–15 min total, the entire litter was returned to the home cage with its dam. Peak blood ethanol concentrations occurred 90 min following administration of ethanol and were 401 +/− 16 (mean +/− SD) mg/dl (Drew et al., 2015). These BECS are relatively high. However, this ethanol treatment paradigm recapitulates most of the neuropathology observed in FASD in humans (Petrelli et al., 2018). In addition, humans with alcohol use disorders can exhibit similar or higher BECs which are well-tolerated (Adachi et al., 1991). Also, mice metabolize ethanol more rapidly than rats and humans (Livy et al., 2003b), which likely moderates the effects of ethanol exposure. Tissue was harvested for gene expression analysis on P10, 24 h after the final treatment. Tissue was harvested for determination of β-galactosidase activity on P10, 24 h after the final treatment or on P15, as indicated in the Figure legends. To avoid possible litter effects, experimental animals from multiple litters were distributed as evenly as possible into

Table 1
TaqMan® gene expression assays. 20× primer/probe sets (FAM-MGB) were purchased from Thermo Fisher Scientific, Cat. #4331182, and were used at a final concentration of 1× for qRT-PCR. Assays were selected to span an exon-exon junction where possible.

Gene name	Assay ID	Gene name	Assay ID
β-actin	Mm00607939_s1	IL-4	Mm00445259_m1
AVPR1a	Mm00444092_m1	KCNC2	Mm01234232_m1
CACNG3	Mm00517091_m1	KCNJ9	Mm00434622_m1
CAR7	Mm01247656_m1	MAG	Mm00487538_m1
CCL2	Mm00441242_m1	MBP	Mm01266402_m1
CD24a	Mm00782538_m1	MOG	Mm00447824_m1
CD38	Mm00483143_m1	Neu4	Mm00620597_m1
Connexin 30	Mm00433661_m1	NG2	Mm00507257_m1
CX3CL1	Mm00436354_m1	P2Y12R	Mm00446026_m1
CX3CR1	Mm0260111_s1	PDGFR-α	Mm00440701_m1
CXCL12	Mm0044553_m1	PDYN	Mm00457573_m1
GABRD	Mm01266203_g1	PGC-1a	Mm01208835_m1
GPR34	Mm02620221_s1	PLP	Mm01297210_m1
GPR83	Mm00439103_m1	PPAR-γ	Mm01184322_m1
GRIN2c	Mm00439180_m1	Siglec-H	Mm00618627_m1
IL-1α	Mm00439620_m1	TGF-β	Mm01178820_m1
IL-1β	Mm00434228_m1	TNF-α	Mm00443258_m1
IL-33	Mm00505403_m1	TRIM67	Mm01253530_m1
IL-34	Mm01243248_m1	VIP	Mm00660234_m1

Vehicle (V) treated control or Ethanol (E) treated groups and also distributed based on sex. For gene expression analysis, samples were derived from 5 male and 3 female Vehicle treated animals, and 5 male and 3 female Ethanol treated animals, which were distributed from a total of 5 litters. For β-galactosidase activity, samples were derived from 4 male and 4 female wild-type Vehicle treated animals, 2 male and 5 female transgenic Vehicle treated animals, and 6 male and 5 female transgenic Ethanol treated animals, which were distributed from 5 total litters for the P10 time-point. For the P15 time-point, samples were derived from 2 male and 6 female wild-type Vehicle treated animals, 9 male and 5 female transgenic Vehicle treated animals, and 8 male and 7 female transgenic Ethanol treated animals, which were distributed from 6 total litters.

2.2. Isolation of RNA and cDNA Synthesis

Mice were anesthetized with an overdose of isoflurane vapor followed by transcardial perfusion with 1× phosphate buffered saline (PBS) containing 5 U/ml of heparin. Following perfusion, mice were decapitated, the brain was removed, and the hippocampus was microdissected, flash frozen immediately with liquid nitrogen, and stored at −80 °C. A Qiagen RNeasy Mini Kit (Valencia, CA; Cat. #74104) was used to isolate RNA. Briefly, the frozen hippocampal tissue was disrupted in kit lysis buffer using a BBX24B Bullet Blender homogenizer (Next Advance, Averill Park, NY) for 2 min intervals at a setting of 8 in the presence of 0.5 mm glass beads until intact tissue was no longer visible (2–4 min total). Tissue homogenates were placed into columns and RNA was further purified according to the manufacturer's instructions. DNA was removed from the RNA isolate using a Qiagen RNase-Free DNase Set (Cat. #79254) following the optional on-column digestion steps. The concentration of the isolated RNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE; RRID:SCR_018042) and cDNA was synthesized using a Bio-Rad iScript cDNA synthesis kit (Hercules, CA; Cat. #1708891). Briefly, 2 μg of RNA was diluted to 100 ng/μl in 20 μl of nuclease-free water and mixed with 20 μl of reverse transcriptase mastermix prepared according to the manufacturer's instructions. Samples were placed into a thermocycler and run using the kit suggested conditions. Following synthesis completion, cDNA was diluted to 25 ng/μl and stored at 4 °C until further use.

2.3. Real-time quantitative PCR analysis

A Bio-Rad CFX96 Real-time PCR detection system (RRID: SCR_018064) was utilized for qualitative real-time PCR (qRT-PCR) to compare mRNA expression levels among samples. cDNA was amplified with TaqMan Gene Expression Assays (ThermoFisher Cat. #4331182, Table 1) and SsoAdvanced Universal Probes Supermix (Bio-Rad Cat. #1725285) in duplicate 20 μl reactions with an input of 25 ng cDNA, or 75 ng for IL-1β, TNF-α, and IL-4 which had an anticipated low abundance. Following a hot start of 95 °C for 30 s, reactions were run for 40 cycles (95 °C for 5 s, 60 °C for 10 s) in 96-well PCR plates (Bio-Rad Cat. #HSP9601B). For each sample, mean CT values were generated for duplicate reactions and expressed as mean ΔCT relative to duplicate β-actin control reactions on the same plate. The ΔΔCT method was used to calculate fold differences between experimental groups.

2.4. β-galactosidase enzyme assay

Following anesthetization with isoflurane, hemizygous PLP(+)Z transgenic mice or their wild-type littermates were decapitated, the brain was removed, the hippocampus was microdissected for the β-galactosidase assay, and a small tail snip was taken for genotyping. Tail snips were submerged in lysis buffer (25 mM NaOH and 0.5 mM EDTA) and incubated at 95 °C for 1 h. Lysates were neutralized with an equal volume of 40 mM Tris-HCl and vortexed for approximately 5 s to ensure sufficient homogenization and DNA accessibility. An aliquot from the resulting tail snip lysate was then used for genotyping by PCR. LacZ forward (5'-GTTGCAGTGCACGGCAGATACACTTGCTGA-3′), and reverse (5'-GCCACTGGTGTGGGCCATAATTCAATTCGC -3′) primers were obtained from Integrated DNA Technologies (Coralville, IA). Each PCR reaction consisted of 10 μL Jumpstart™ Red Taq® ReadyMix™ Reaction Mix (Sigma Cat. #P0982), 1 μl of 10 μM forward primer, 1 μl of 10 μM reverse primer, 6 μl of water, and 2 μl of tail lysate for a total of 20 μl per reaction. Reactions were placed into a thermocycler and run for 30 cycles (93 °C for 20 s, 68 °C for 1 min) following a hot start of 93 °C for 1 min. Samples were visualized on a 1% agarose gel run at 150 V for approximately 25–30 min with SYBR® Safe DNA gel stain in 1× TAE (Thermo Fisher Cat. #S33111). Genotypes were determined and recorded for each sample. The presence of a 389 bp band was used to identify PLP(+)Z transgenic mice.

The β-galactosidase assays were performed using homogenates from freshly isolated hippocampal tissue. Briefly, hippocampi were homogenized separately in lysis buffer [100 mM potassium phosphate buffer, 0.2% Triton X-100, 1 mM dithiothreitol (DTT), 5 μg/ml lupeptin, and 0.2 mM phenylmethylsulfonyl fluoride (PMSF)] using a Pro Scientific BioGen Series PRO200 homogenizer with a 5 mm × 75 mm generator (Oxford, CT) for approximately 15 s on speed 2. Homogenates were centrifuged for 10 min at 16,595 rcf and the supernatant was removed and incubated for 1 h at 48 °C to inactivate any endogenous β-galactosidase activity. Following incubation, samples were centrifuged for an additional 10 min at 16,595 rcf and the subsequent supernatant was collected. For the β-galactosidase assay, a 10 μl aliquot of the supernatant (lysate) was incubated with 70 μl of a 1:99 dilution of Galacton Plus: Galacton Reaction Buffer solution (Thermo Fisher Cat. #T2118 and #T2073). Each sample was incubated for 1 h in the dark at room temperature. Following incubation, 100 μl of Galacton Accelerator Solution (Thermo Fisher Cat. #T2222) was added to each sample and the relative light units (RLU) were measured in an Autolumat LB 953 luminometer (EG&G, Gaithersburg, MD). The β-galactosidase assay was run in triplicate for each sample. In addition, the protein concentration was determined for each lysate using the Pierce BCA Protein Assay kit according to the manufacturer's instructions (Thermo Fisher Cat. #23227). Results are presented as the mean β-galactosidase activity (RLU) per μg of protein.

Table 2
Statistical analysis of gene expression. Student's t-tests (two-tailed, parametric, 95% confidence) were used to identify statistically significant differences in fold differences between experimental groups. Statistical outliers were identified using the ROUT method (Q = 1%). F tests were used to indicate the presence of any variance between experimental groups. Ordinary two-way ANOVA (alpha = 0.05) was used to investigate the presence or absence of any interactions between sex and treatment among experimental animals. GraphPad Prism 9 software was used for these analyses.

	Student's t-test				F test		Two Way ANOVAs					
	n (Vehicle)	*n* (Ethanol)	*P* Value	*t* Statistic	*P* Value	F, DFn, Dfd	Interaction		Treatment		Sex	
							F Statistic	*P* Value	F Statistic	*P* Value	F Statistic	*P* Value
IL-1β	8	8	0.0034	$t = 3.518$, df = 14	<0.0001	77.41, 7, 7	F (1,12) = 0.4067	0.5356	F (1, 12) = 9.643	0.0091	F (1, 12) = 0.4565	0.5121
TNF-α	8	8	0.0112	$t = 2.918$, df = 14	0.0001	35.68, 7, 7	F (1, 12) = 0.1518	0.7036	F (1, 12) = 7.459	0.0182	F (1, 12) = 0.0139	0.9081
CCL2	7	8	0.0115	$t = 2.941$, df = 13	0.0003	36.62, 7, 6	F (1,11) = 0.8504	0.3762	F (1, 11) = 9.259	0.0112	F (1, 11) = 0.5596	0.4701
IL-1α	8	8	0.0326	$t = 2.371$, df = 14	0.0009	19.23, 7, 7	F (1, 12) = 0.4739	0.5043	F (1, 12) = 4.027	0.0679	F (1, 12) = 0.1323	0.7224
CD24a	8	8	0.0043	$t = 3.401$, df = 14	0.2672	2.417, 7, 7	F (1, 12) = 0.0014	0.9708	F (1, 12) = 11.23	0.0058	F (1, 12) = 2.579	0.1343
PPAR-γ	8	8	0.0007	$t = 4.300$, df = 14	0.1508	3.172, 7, 7	F (1, 12) = 0.8494	0.3749	F (1, 12) = 20.34	0.0007	F (1, 12) = 1.942	0.1888
PGC-1a	8	8	0.0003	$t = 4.758$, df = 14	0.5423	1.615, 7, 7	F (1, 12) = 1.635	0.2252	F (1, 12) = 17.93	0.0012	F (1, 12) = 0.0450	0.8357
IL-33	8	7	0.0037	$t = 3.534$, df = 13	0.4817	1.823, 7, 6	F (1, 11) = 0.0081	0.9301	F (1, 11) = 10.90	0.0071	F (1, 11) = 1.097	0.3173
GPR83	8	8	<0.0001	$t = 7.854$, df = 14	0.1642	3.051, 7, 7	F (1, 12) = 2.823	0.1188	F (1, 12) = 55.07	<0.0001	F (1, 12) = 0.0613	0.8087
IL-4	8	8	0.9206	$t = 0.1016$, df = 14	0.2346	2.579, 7, 7	F (1, 12) = 0.6324	0.4419	F (1, 12) = 0.0854	0.7751	F (1, 12) = 0.0144	0.9064
TGF-β	8	8	0.9906	$t = 0.0119$ df = 14	0.6235	1.471, 7, 7	F (1, 12) = 0.3363	0.5727	F (1, 12) = 0.0245	0.8783	F (1, 12) = 1.529	0.2399
P2Y12R	8	8	<0.0001	$t = 6.422$, df = 14	0.7801	1.245, 7, 7	F (1, 12) = 2.184	0.1652	F (1, 12) = 43.30	<0.0001	F (1, 12) = 3.305	0.0941
Siglec-H	8	8	0.0003	$t = 4.710$, df = 14	0.4889	1.725, 7, 7	F (1, 12) = 0.9386	0.3518	F (1, 12) = 19.23	0.0009	F (1, 12) = 1.473	0.2483
GPR34	8	8	0.0264	$t = 2.481$, df = 14	0.9842	1.016, 7, 7	F (1, 12) = 0.2078	0.6566	F (1, 12) = 5.195	0.0417	F (1, 12) = 1.683	0.2189
CD38	8	8	<0.0001	$t = 6.425$, df = 14	0.0411	5.380, 7, 7	F (1, 12) = 1.734	0.2125	F (1, 12) = 34.14	<0.0001	F (1, 12) = 0.0458	0.8342
CX3CL1	8	8	0.0007	$t = 4.362$, df = 14	0.0171	7.395, 7, 7	F (1, 12) = 0.2712	0.6120	F (1, 12) = 15.01	0.0022	F (1, 12) = 0.3191	0.5826
CX3CR1	8	8	0.1832	$t = 1.400$, df = 14	0.8077	1.210, 7, 7	F (1, 12) = 0.0052	0.9439	F (1, 12) = 2.624	0.1312	F (1, 12) = 8.425	0.0133
CXCL12	8	7	<0.0001	$t = 7.574$, df = 13	0.7991	1.210, 6, 7	F (1, 11) = 0.3581	0.5617	F (1, 11) = 40.81	<0.0001	F (1, 11) = 0.0065	0.9370
IL-34	8	8	<0.0001	$t = 5.514$, df = 14	0.3267	2.176, 7, 7	F (1, 12) = 0.2634	0.6171	F (1, 12) = 23.84	0.0004	F (1, 12) = 0.0725	0.7923
MAG	8	7	0.0019	$t = 3.877$, df = 13	0.1113	4.006, 7, 6	F (1, 11) = 0.5058	0.4918	F (1, 11) = 13.22	0.0039	F (1, 11) = 0.0395	0.8461
MBP	8	7	0.0019	$t = 3.874$, df = 13	0.1485	3.495, 7, 6	F (1, 11) = 0.4768	0.5042	F (1, 11) = 13.26	0.0039	F (1, 11) = 0.3131	0.5870
MOG	8	7	0.0013	$t = 4.090$, df = 13	0.0402	6.235, 7, 6	F (1, 11) = 0.0686	0.7982	F (1, 11) = 12.94	0.0042	F (1, 11) = 0.0778	0.7855
PLP	8	7	0.0032	$t = 3.601$, df = 13	0.199	3.022, 7, 6	F (1, 11) = 0.2826	0.6055	F (1, 11) = 10.91	0.0070	F (1, 11) = 0.0055	0.9423
PDGFR-α	8	7	0.0004	$t = 4.774$, df = 13	0.7729	1.245, 6, 7	F (1, 11) = 0.9227	0.3574	F (1, 11) = 16.19	0.0020	F (1, 11) = 0.1630	0.6942
NG2	8	8	0.0023	$t = 3.727$, df = 14	0.9016	1.102, 7, 7	F (1, 12) = 0.3200	0.5824	F (1, 12) = 13.25	0.0034	F (1, 12) = 3.049	0.1063
GABRD	8	8	0.0006	$t = 4.415$, df = 14	0.0652	4.506, 7, 7	F (1, 12) = 3.295	0.0945	F (1, 12) = 54.12	<0.0001	F (1, 12) = 21.21	0.0006
CAR7	8	8	0.0028	$t = 3.611$, df = 14	0.6366	1.449, 7, 7	F (1, 12) = 0.0267	0.8730	F (1, 12) = 15.81	0.0018	F (1, 12) = 5.714	0.0341
GRIN2c	8	8	0.0169	$t = 2.711$, df = 14	0.7105	1.338, 7, 7	F (1, 12) = 0.1579	0.6981	F (1, 12) = 7.349	0.0189	F (1, 12) = 1.696	0.2172
KCNJ9	8	8	<0.0001	$t = 6.577$, df = 14	0.4829	1.738, 7, 7	F (1, 12) = 1.174	0.2999	F (1, 12) = 51.61	<0.0001	F (1, 12) = 3.323	0.0933
KCNC2	8	8	0.0025	$t = 3.669$, df = 14	0.7993	1.221, 7, 7	F (1, 12) = 0.5375	0.4775	F (1, 12) = 10.82	0.0065	F (1, 12) = 0.8399	0.3775
CACNG3	8	8	<0.0001	$t = 5.699$, df = 14	0.0573	4.738, 7, 7	F (1, 12) = 3.507	0.0857	F (1, 12) = 49.11	<0.0001	F (1, 12) = 4.162	0.0640
Connexin 30	8	8	0.0046	$t = 3.372$, df = 14	0.8609	1.147, 7, 7	F (1, 12) = 0.3521	0.5640	F (1, 12) = 8.802	0.0118	F (1, 12) = 0.3923	0.5428
TRIM67	8	8	0.0005	$t = 4.501$, df = 14	0.2957	2.294, 7, 7	F (1, 12) = 2.053	0.1774	F (1, 12) = 16.42	0.0016	F (1, 12) = 0.2848	0.6033
Neu4	8	8	<0.0001		0.9801			0.6551		<0.0001		0.0879

(*continued on next page*)

Table 2 (*continued*)

	Student's t-test				F test		Two Way ANOVAs					
	n (Vehicle)	*n* (Ethanol)	*P* Value	*t* Statistic	*P* Value	F, DFn, Dfd	Interaction		Treatment		Sex	
							F Statistic	*P* Value	F Statistic	*P* Value	F Statistic	*P* Value
				$t = 6.087$, df = 14		1.020, 7, 7	F (1, 12) = 0.2098		F (1, 12) = 37.44		F (1, 12) = 3.452	
VIP	8	8	0.0025	$t = 3.683$, df = 14	0.4524	1.809, 7, 7	F (1, 12) = 0.4634	0.5089	F (1, 12) = 16.40	0.0016	F (1, 12) = 4.108	0.0655
AVPR1a	8	8	0.0003	$t = 4.762$, df = 14	0.0268	6.300, 7, 7	F (1, 12) = 0.2042	0.6594	F (1, 12) = 21.35	0.0006	F (1, 12) = 2.551	0.1362
PDYN	8	8	0.0229	$t = 2.555$, df = 14	0.8158	1.200, 7, 7	F (1, 12) = 0.3945	0.5417	F (1, 12) = 5.194	0.0418	F (1, 12) = 1.183	0.2982

Table 3
Statistical analysis of PLP(+)Z transgene expression (β-galactosidase activity). Ordinary one-way ANOVA (alpha = 0.05) with Tukey's post-hoc test was used to identify statistically significant differences between experimental groups. Statistical outliers were identified using the ROUT method (Q = 1%). Ordinary Two-way ANOVA (alpha = 0.05) was used to investigate the presence or absence of any interactions between sex and treatment among experimental animals. GraphPad Prism 9 software was used for these analyses.

	One-Way ANOVAs		Tukey's Post-hoc Test		Two-way ANOVAs					
	F Statistic	*P* Value	q, n1, n2	*P* Value	Interaction		Treatment Group		Sex	
					F Statistic	*P* Value	F Statistic	*P* Value	F Statistic	*P* Value
P10	F (2, 23) = 0.7870	0.4671			F (2,20) = 0.1014	0.9041	F (2, 20) = 0.7644	0.4787	F (1,20) = 0.02135	0.8853
Tg E vs. Tg V			1.612, 11, 7	0.5000						
Tg E vs. WT V			1.330, 11, 7	0.6209						
Tg V vs. WT V			0.3122, 7, 8	0.9735						
P15	F (2, 34) = 56.03	P < 0.0001			F (2,31) = 1.144	0.3316	F (2, 31) = 56.37	<0.0001	F (1,31) = 0.6864	0.4137
Tg E vs. Tg V			13.86, 15, 14	<0.0001						
Tg E vs. WT V			0.5125, 15, 8	0.9303						
Tg V vs. WT V			11.11, 14, 8	<0.0001						

2.5. Statistical analysis

Statistical analysis and data visualization of gene expression fold differences were completed using GraphPad Prism 9 software (San Diego, CA). Any statistical outliers were identified using the ROUT method (Q = 1%) and subsequently excluded from further analysis. Student's *t*-tests (two-tailed, parametric, 95% confidence) were used to identify statistically significant differences between experimental groups. F tests, reported as part of the Student's t-test, were used to indicate the presence of any variance between experimental groups. Additionally, ordinary Two-way ANOVA (alpha = 0.05) was used to investigate the presence or absence of any interactions between sex and treatment among experimental animals. Sex differences by ANOVA were only noted in 3 transcripts (CX3CR1, GABRD, and CAR7) but had no significant interaction effect with treatment. It should be noted that the small number of males and females is a limitation of the current study and likely makes it underpowered to identify any but the most obvious sex-effects. Statistical analysis of gene expression is summarized in Table 2. Prism 9 was also used to determine statistically significant differences in β-galactosidase activity via ordinary One-way ANOVA (alpha = 0.05) with Tukey's post-hoc multiple comparison test. Ordinary Two-way ANOVA (alpha = 0.05) was used to investigate the presence or absence of any interactions between sex and experimental treatment groups. Though no sex differences were observed, it should be noted that the study is likely underpowered to identify sex-effects. Statistical analysis of β-galactosidase activity is summarized in Table 3. Furthermore, Potential litter effects in all studies were appropriately controlled for by distributing experimental animals of mixed sex, treatment, and genotype as evenly as possible over 5–6 litters for each endpoint and was therefore not included as a factor in the analysis.

3. Results

We demonstrate that ethanol increased the expression of the pro-inflammatory cytokines IL-1β (Fig. 1a: $t_{14} = 3.518$, $P = 0.0034$, Table 2) and TNF-α (Fig. 1b: $t_{14} = 2.918$, $P = 0.0112$, Table 2), and the chemokine CCL2 (Fig. 1c: $t_{13} = 2.941$, $P = 0.0115$, Table 2) which is consistent with our previous observations (Drew et al., 2015). In addition, we demonstrate that ethanol also increased the expression of the pro-inflammatory IL-1α, which like IL-1β, is a member of the IL-1 family of cytokines (Fig. 1d: $t_{14} = 2.371$, $P = 0.0326$, Table 2), and CD24a which contributes to pathogenesis in a variety of autoimmune diseases (Fig. 1e: $t_{14} = 3.401$, $P = 0.0043$, Table 2). The effects of ethanol on the expression of anti-inflammatory molecules in postnatal models of FASD have not been adequately evaluated. We previously demonstrated that the peroxisome proliferator-activated receptor (PPAR)-γ agonist Pioglitazone acted as a potent anti-inflammatory agent to block ethanol induction of pro-inflammatory cytokines and chemokines in the developing hippocampus (Drew et al., 2015). In the current study, we demonstrated that ethanol suppressed the expression of PPAR-γ (Fig. 1f: $t_{14} = 4.3$, $P = 0.0007$, Table 2) as well as the PPAR-γ co-activator molecule PGC-1a (Fig. 1g: $t_{14} = 4.758$, $P = 0.0003$, Table 2). This suggests ethanol induces neuroinflammation by decreasing anti-inflammatory PPAR-γ signaling. We also demonstrated that ethanol suppressed the expression of the immunosuppressive cytokine IL-33 (Fig. 1h: $t_{13} = 3.534$, $P = 0.0037$, 1 E outlier excluded, Table 2) and

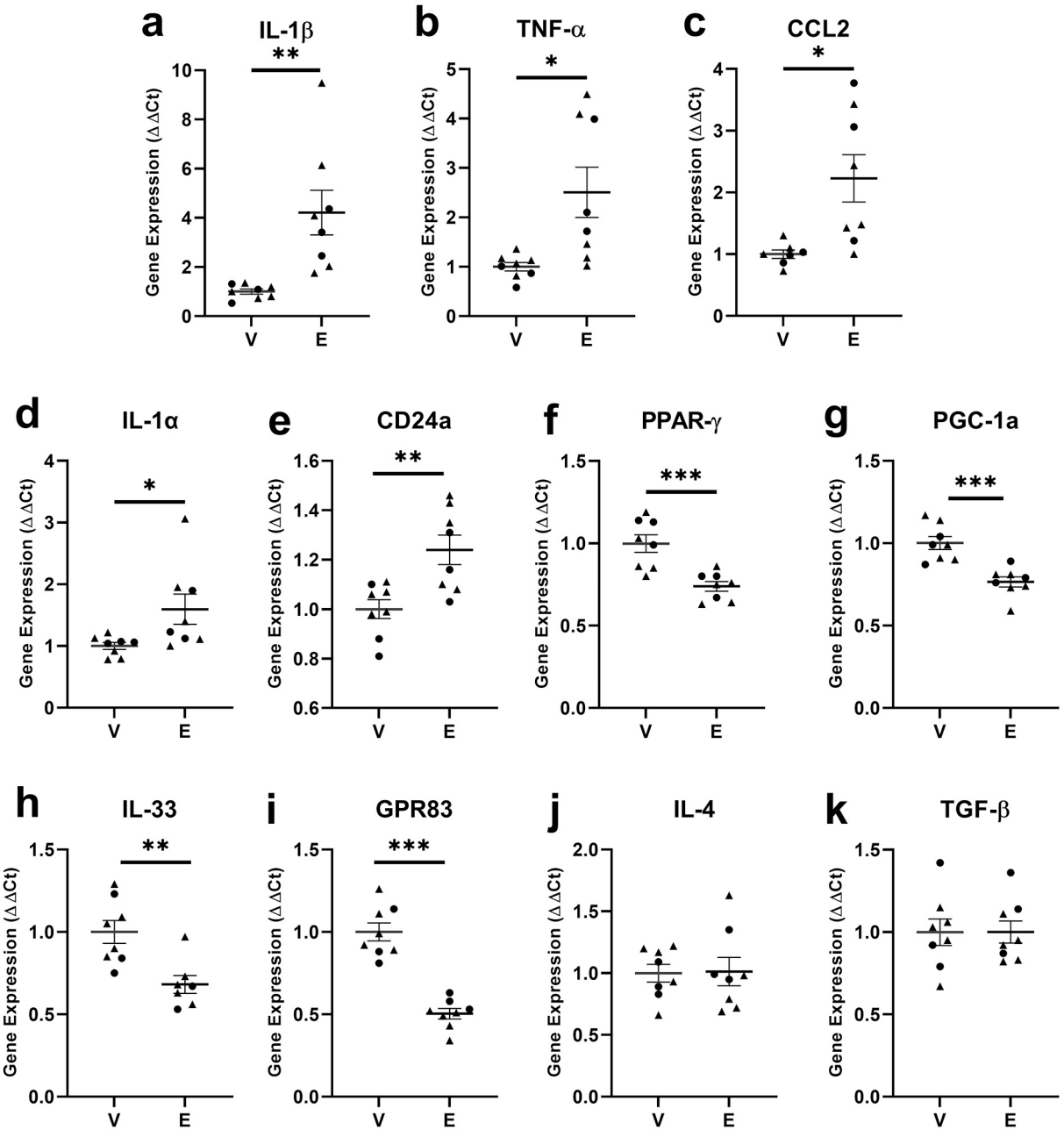

Fig. 1. Effect of ethanol on inflammation related genes. Neonates were gavaged with either 4 g/kg/day ethanol (E) or vehicle (V) from P4-P9 and sacrificed on P10. Expression of (a) IL-1β, (b) TNF-α, (c) CCL2, (d) IL-1α, (e) CD24a, (f) PPAR-γ, (g) PGC-1a, (h) IL-33, (i) GPR83, (j) IL-4, and (k) TGF-β mRNA was measured using qRT-PCR as described in the Methods. Results are expressed as a fold change in the ethanol group relative to the vehicle control group. Values are mean +/− SEM. N = 7–8 animals/group. Male and female are denoted as ▲ and ● respectively. Variance between groups was analyzed by Student's unpaired *t*-test. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

the expression of the G-protein coupled receptor GPR83 which is a potent suppressor of inflammation (Fig. 1i: $t_{14} = 7.854$, $P < 0.0001$, Table 2). In addition, we evaluated the effects of ethanol on other anti-inflammatory molecules including IL-1ra, IL-4, IL-10, IL13, and TGF-β in the developing hippocampus. We demonstrated that ethanol did not alter the expression of the anti-inflammatory molecules IL-4 (Fig. 1j: $t_{14} = 0.1016$, $P = 0.9206$, Table 2) and TGF-β (Fig. 1k: $t_{14} = 0.0119$, $P = 0.9906$, Table 2). Furthermore, the expression of the anti-inflammatory molecules IL-1ra, IL-10, and IL-13 were below the level of adequate detection by qRT-PCR in the developing hippocampus (mean CT > 38, data not shown).

We evaluated the effects of ethanol on molecules known to play critical roles in regulating the function of microglia. P2Y12R (also called P2RY12), Siglec-H, and GPR34 are expressed by microglia, and have been used as markers to distinguish microglia from peripheral macrophages. These molecules have been demonstrated to play important roles in microglial function including homeostasis, cell migration, cell activation, and phagocytosis. In the current study, we demonstrate that ethanol decreased the expression of P2Y12R (Fig. 2a: $t_{14} = 6.422$, $P < 0.0001$, Table 2), Siglec-H (Fig. 2b: $t_{14} = 4.71$, $P = 0.0003$, Table 2), and GPR34 (Fig. 2c: $t_{14} = 2.481$, $P = 0.0264$, Table 2) in the developing hippocampus. Another notable molecule, CD38 metabolizes the purine NAD which activates immune responses in cells including microglial, resulting in neuroinflammation. We demonstrate here that ethanol decreased the expression of CD38 in the developing hippocampus (Fig. 2d: $t_{14} = 6.425$, $P < 0.0001$, Table 2). Collectively, these studies

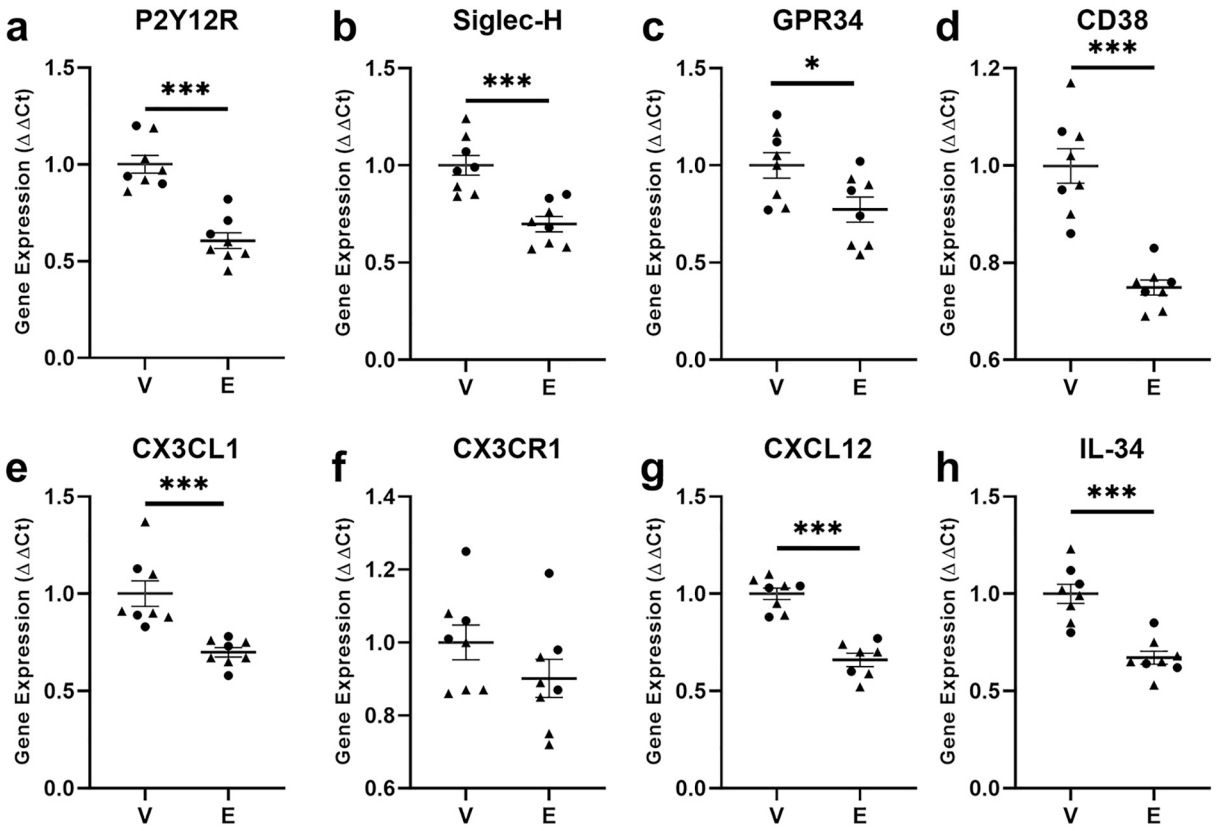

Fig. 2. Effect of ethanol on microglia associated genes. Neonates were gavaged with either 4 g/kg/day ethanol (E) or vehicle (V) from P4-P9 and sacrificed on P10. Expression of (a) P2Y12R, (b) Siglec-H, (c) GPR34, (d) CD38, (e) CX3CL1, (f) CX3CR1, (g) CXCL12, and (h) IL-34 mRNA was measured using qRT-PCR as described in the Methods. Results are expressed as a fold change in the ethanol group relative to the vehicle control group. Values are mean +/− SEM. $N = 7$–8 animals/group. Male and female are denoted as ▲ and ● respectively. Variance between groups was analyzed by Student's unpaired t-test. ***$p < 0.001$, *$p < 0.05$.

suggest that ethanol alters microglial activation and function by suppressing the expression of molecules including P2Y12R, Siglec-H, GPR34, and CD38. Additionally, we investigated the effects of ethanol on neuron-microglia signaling mechanisms. Fractalkine (CX3CL1), CXCL12, and IL-34, for example, are primarily expressed by neurons in the CNS and play critical roles in modulating microglial function. With ethanol exposure, we observed a significant decrease in the expression of CX3CL1 (Fig. 2e: $t_{14} = 4.362$, P = 0.0007, Table 2) but not its receptor CX3CR1 (Fig. 2f: $t_{14} = 1.4$, $P = 0.1832$, Table 2). While CX3CL1 is expressed by neurons, CX3CR1 is expressed exclusively by microglia in the CNS, and interaction between this ligand and its receptor suppresses microglial activation. Thus, ethanol suppression of CX3CL1 expression is expected to result in microglial activation by altering CX3CL1-CX3CR1 signaling. This reveals a novel mechanism by which ethanol induces neuroinflammation in this postnatal mouse model of FASD and highlights the impact of ethanol on neuron-microglial signaling. Our studies further demonstrate that ethanol decreased the expression of CXCL12 (Fig. 2g: $t_{13} = 7.574$, $P < 0.0001$, 1 E outlier excluded, Table 2) and IL-34 (Fig. 2h: $t_{14} = 5.514$, $P < 0.0001$, Table 2) in the developing hippocampus, suggesting additional novel mechanisms by which ethanol alters microglial functions.

Developmental ethanol exposure alters myelination which may contribute to the neurological sequelae of FASD. However, the effects of ethanol on myelin and oligodendrocyte linage cells in the hippocampus are understudied. We demonstrate that ethanol suppressed the expression of molecules associated with mature oligodendrocytes including MAG (Fig. 3a: $t_{13} = 3.877$, $P = 0.0019$, 1 E outlier excluded, Table 2), MBP (Fig. 3b: $t_{13} = 3.874$, P = 0.0019, 1 E outlier excluded, Table 2), MOG (Fig. 3c: $t_{13} = 4.090$, $P = 0.0013$, 1 E outlier excluded, Table 2), and PLP (Fig. 3d: $t_{13} = 3.601$, $P = 0.0032$, 1 E outlier excluded, Table 2). In addition, ethanol suppressed the expression of the OPC markers PDGFR-α (Fig. 3e: $t_{13} = 4.774$, $P = 0.0004$, 1 E outlier excluded, Table 2) and NG2 (also referred to as CSPG4) (Fig. 3f: $t_{14} = 3.727$, $P = 0.0023$, Table 2). We further demonstrate that β-galactosidase activity was not elevated in PLP(+)Z transgenic mice relative to wild-type mice at P10 (Fig. 4a: F (2,23) = 0.7870, $P = 0.4671$, Table 3). Therefore, we evaluated β-galactosidase activity at a later age, following the same period of ethanol exposure at P4–9. We determined that β-galactosidase activity was significantly elevated in PLP(+)Z transgenic relative to wild-type littermates at P15, and that ethanol suppressed the activity of the transgene (Fig. 4b: F (2, 34) = 56.03, P < 0.0001, Table 3). Collectively, these studies suggest ethanol suppresses the expression of genes encoding molecules expressed by mature and progenitor oligodendrocytes, and likely has an adverse effect on the formation of normal myelin in the developing hippocampus.

Gamma-Aminobutyric acid (GABA) is an inhibitory neurotransmitter that binds GABA receptors, which are ligand-gated ion channels that mediate the majority of fast inhibitory neurotransmission in the brain. GABA neurotransmission plays a significant role in synaptic plasticity and hyperexcitability in the hippocampus. In the current study, we demonstrate that ethanol decreased the expression of the GABA Type A receptor subunit delta (GABRD) in the developing hippocampus (Fig. 5a: $t_{14} = 4.415$, $P = 0.0006$, Table 2). Carbonic anhydrase VII (CAR7) is important in the generation of high-frequency stimulation induced GABAergic excitation in the hippocampus. We demonstrate that ethanol decreased the expression of CAR7 in the hippocampus in our mouse model of FASD (Fig. 5b: $t_{14} = 3.611$, $P = 0.0028$, Table 2). The *N*-methyl-D-aspartate (NMDA) receptor is an ionotropic glutamate receptor which plays an important role in synaptic plasticity and memory. We demonstrate that ethanol decreased the expression of the GRIN2c

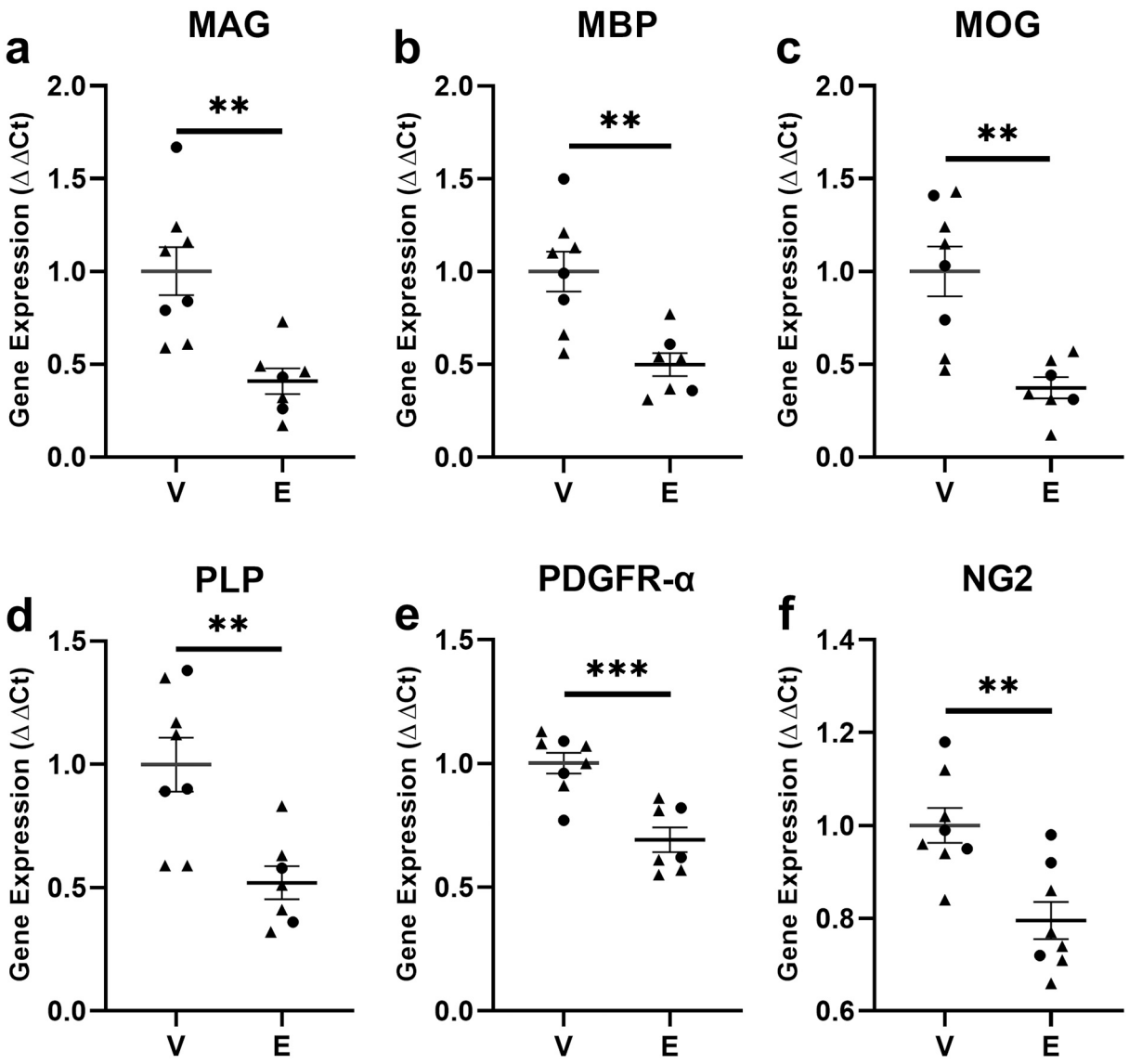

Fig. 3. Effect of ethanol on myelin related genes. Neonates were gavaged with either 4 g/kg/day ethanol (E) or vehicle (V) from P4-P9 and sacrificed on P10. Expression of (a) MAG, (b) MBP, (c) MOG, (d) PLP, (e) PDGFR-α, and (f) NG2 mRNA was measured using qRT-PCR as described in the Methods. Results are expressed as a fold change in the ethanol group relative to the vehicle control group. Values are mean +/− SEM. N = 7–8 animals/group. Male and female are denoted as ▲ and ● respectively. Variance between groups was analyzed by Student's unpaired t-test. ***p < 0.001, **p < 0.01.

subunit of the NMDA receptor (Fig. 5c: $t_{14} = 2.711$, $P = 0.0169$, Table 2). We further demonstrate that ethanol altered the expression of ion channels in the developing hippocampus. For example, ethanol decreased the expression of the potassium channels KCNJ9 (also referred to as Kir3.3 or GIRK3) (Fig. 5d: $t_{14} = 6.577$, P < 0.0001, Table 2) and KCNC2 (also referred to as Kx3.2) (Fig. 5e: $t_{14} = 3.669$, $P = 0.0025$, Table 2) as well as the calcium channel CACNG3 (Fig. 5f: $t_{14} = 5.699$, P < 0.0001, Table 2). Furthermore, ethanol decreased the expression of Connexin 30 (also referred to as GJB6), which is a major component of astrocyte gap junctions and controls hippocampal excitatory synaptic transmission through modulation of astrocyte glutamate transport (Fig. 5g: $t_{14} = 3.372$, $P = 0.0046$, Table 2). We evaluated the effects of ethanol on molecules important in neurogenesis and neuritogenesis and determined that the expression of two of these molecules, TRIM67 and Neu4, were significantly increased and decreased in the hippocampus, respectively (Fig. 5h: $t_{14} = 4.501$, $P = 0.0005$, Fig. 5i: $t_{14} = 6.087$, P < 0.0001, Table 2). We next evaluated the effects of ethanol on a series of molecules important in neuropeptide mediated functions in the brain. We demonstrate that ethanol decreased the expression of vasoactive intestinal protein (VIP) (Fig. 5j: $t_{14} = 3.683$, P = 0.0025, Table 2), increased the expression of the arginine vasopressin receptor 1a (AVPR1a) (Fig. 5k: $t_{14} = 4.762$, $P = 0.0003$, Table 2), and decreased the expression of prodynorphin (PDYN) (Fig. 5l: $t_{14} = 2.555$, $P = 0.0229$, Table 2) in the developing hippocampus. The molecules investigated in the current study may begin to define novel mechanisms by which ethanol modulates neuroinflammation and neuropathology in the developing hippocampus.

4. Discussion

Previously, postnatal ethanol exposure was demonstrated to increase hippocampal expression of pro-inflammatory molecules, most notably during periods of alcohol withdrawal (Boschen et al., 2016; Drew et al., 2015; Ruggiero et al., 2018; Tiwari and Chopra, 2011; Topper et al., 2015). In the current study, we demonstrate that ethanol increased the expression of additional pro-inflammatory molecules including IL-1α and CD24a mRNA (Di Paolo and Shayakhmetov, 2016; Dinarello, 2018; Zhou et al., 2020). It should be noted that the current study evaluated

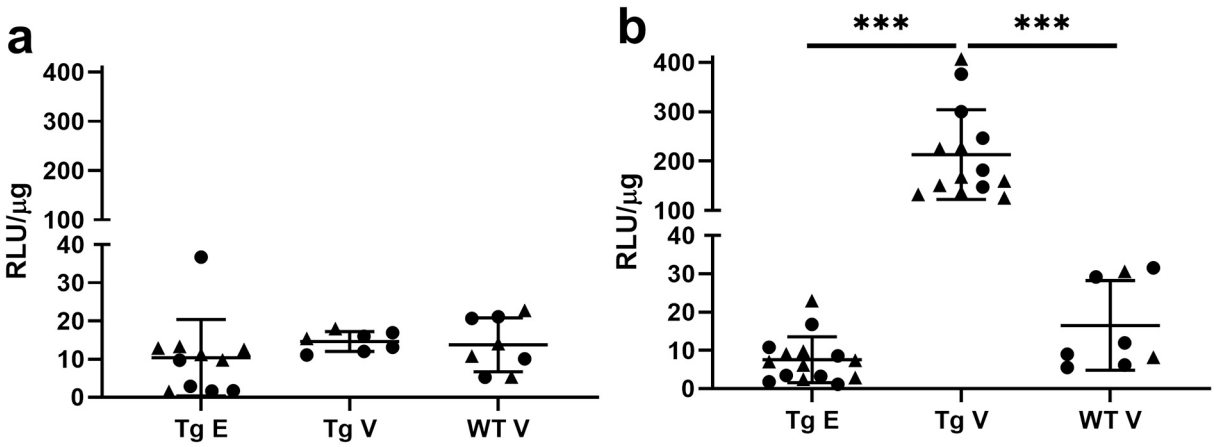

Fig. 4. Effect of ethanol on PLP(+)Z transgene expression as determined by β-galactosidase activity in the hippocampus. Transgenic (Tg) and wild type (WT) neonates were gavaged with either 4 g/kg/day ethanol (E) or vehicle (V) from P4-P9 and sacrificed on either (a) P10 or (b) P15. Expression of β-galactosidase activity denoted as Relative Light Units (RLUs) per microgram total protein was measured as described in Methods. Values are mean +/− SD. (a) P10, $N = 11$ Tg E, 7 Tg V, and 8 WT V. (b) P15, $N = 15$ Tg E, 14 Tg V, and 8 WT V. Male and female are denoted as ▲ and ● respectively. Variance between groups was analyzed using One-Way ANOVA with Tukey's post-hoc multiple comparisons test. $^{***}p < 0.001$.

the effects of ethanol on expression of molecules at the level of mRNA which may not always reflect changes at the level of protein. Future studies are needed to determine the effects of ethanol on the expression of these molecules at the protein level. However, collectively, the current studies suggest that ethanol-induced neuroinflammation in the developing hippocampus may contribute to neuropathology common in FASD.

The effects of ethanol on expression of anti-inflammatory molecules in the developing CNS have not been adequately investigated. This is important, because both pro- and anti-inflammatory molecules can be expressed in inflammatory disorders and the relative abundance of these molecules may vary at different stages of disease (Strachan-Whaley et al., 2014). In addition, cells such as microglia can become polarized toward inflammatory or alternatively protective phenotypes (Jurga et al., 2020). The current study indicates that ethanol has subtle effects on the expression of anti-inflammatory cytokines. We previously demonstrated that the PPAR-γ agonist Pioglitazone suppressed ethanol induced microglial activation and neuroinflammation in the developing hippocampus (Drew et al., 2015). In the current studies, we demonstrate that ethanol decreased the expression of PPAR-γ and the PPAR-γ co-activator molecule PGC-1a which is known to increase the transcriptional activity of PPAR-γ (Puigserver et al., 1998). This suggests that ethanol induces neuroinflammation in the hippocampus through a novel mechanism of suppressing anti-inflammatory PPAR-γ signaling. Collectively, these studies suggest that ethanol may stimulate neuroinflammation by selectively suppressing the expression of specific anti-inflammatory molecules.

Microglia are resident macrophages of the CNS known to play a critical role in immune response, but are also important in brain development and homeostasis. Microglia, however, have a distinct ontogeny from peripheral macrophages (Ginhoux et al., 2010). Transcripts including P2Y12R, Siglec-H, and GPR34 are preferentially expressed in microglia relative to peripheral macrophages (Butovsky et al., 2014; Gautier et al., 2012; Grassivaro et al., 2021; Hickman et al., 2013; Y. Zhang et al., 2014), and it is noteworthy that ethanol decreased the expression of these molecules in the developing hippocampus in the current studies. P2Y12R is a purinergic receptor and ATP is its native ligand. This receptor plays critical roles in microglial functions including motility, microglial-neuron interactions, and activity dependent synaptic plasticity (Cserép et al., 2020; Eyo et al., 2014; Y. U. Liu et al., 2019; Sipe et al., 2016; Whitelaw et al., 2020). Siglecs play important roles in neuroinflammation (Siddiqui et al., 2019). Siglec-H, for example, plays a critical role in microglial phagocytosis (Kopatz et al., 2013). RNA-Seq analysis also identified Siglec-H as a potential hub gene regulating microglial response to chronic alcohol exposure (McCarthy et al., 2018). GPR34 is a member of the P2Y family of receptors and plays an important role in modulating microglial motility, morphology, and phagocytosis (Preissler et al., 2015). Although not a purine receptor, CD38 catabolizes the purine NAD, which is neuroprotective. CD38 knockout mice are protected against neuroinflammatory and neurodegenerative insults (Guerreiro et al., 2020). CD38 is also important in neuronal development evident in CD38 knockout mice that exhibited abnormal neuron numbers and morphology in multiple brain regions including the hippocampus (Nelissen et al., 2018) and impaired hippocampal-dependent learning and memory (Kim et al., 2016). Collectively, these studies suggest that P2Y12R, Siglec-H, and GPR34, molecules preferentially expressed by microglia, and CD38 are down-regulated following ethanol exposure which may contribute to neuropathogenesis, perhaps through modulation of purine signaling pathways.

The chemokines CX3CL1 and CXCL12 and the cytokine IL-34 are expressed primarily by neurons and modulate immune function through neuron-glia interactions. CX3CL1-CX3CR1 signaling, suppresses microglial activation, as mice genetically deficient in either CX3CL1 or CX3CR1 exhibited activated microglia (Cardona et al., 2006). This signaling pathway plays an important role in CNS development, evident in genetically deficient mice that exhibited deficient synaptic pruning (Paolicelli et al., 2011), and associated aberrant synaptic activity and behavioral abnormalities (Zhan et al., 2014). CXCL12 also suppresses microglial activation (de Haas et al., 2007), and appears to be neuroprotective (Trettel et al., 2020). Mice deficient in CXCL12, or its receptor CXCR4, exhibit abnormal cerebellar development (Hagihara et al., 2009). IL-34 is critical for the development and function of mononuclear phagocytes including microglia (Lelios et al., 2020). In the CNS, IL-34 expression is increased under neuroinflammatory conditions and is critical to the maintenance, proliferation, differentiation, and survival of microglia (Frei et al., 1992; Mizuno et al., 2011; Ponomarev et al., 2013). Collectively, our current studies demonstrating ethanol-mediated decreases in CX3CL1, CXCL12, and IL-34, which are principally expressed by neurons, suggest additional mechanisms by which ethanol mediates neuron-microglia interactions, neuroinflammation, and neuropathology in the developing hippocampus.

Oligodendrocytes mature and produce myelin relatively late in CNS development. In rodents, myelin formation occurs most abundantly during the first two weeks of life (El Waly et al., 2014). Thus, our postnatal model of FASD allows us to evaluate the effects of ethanol on

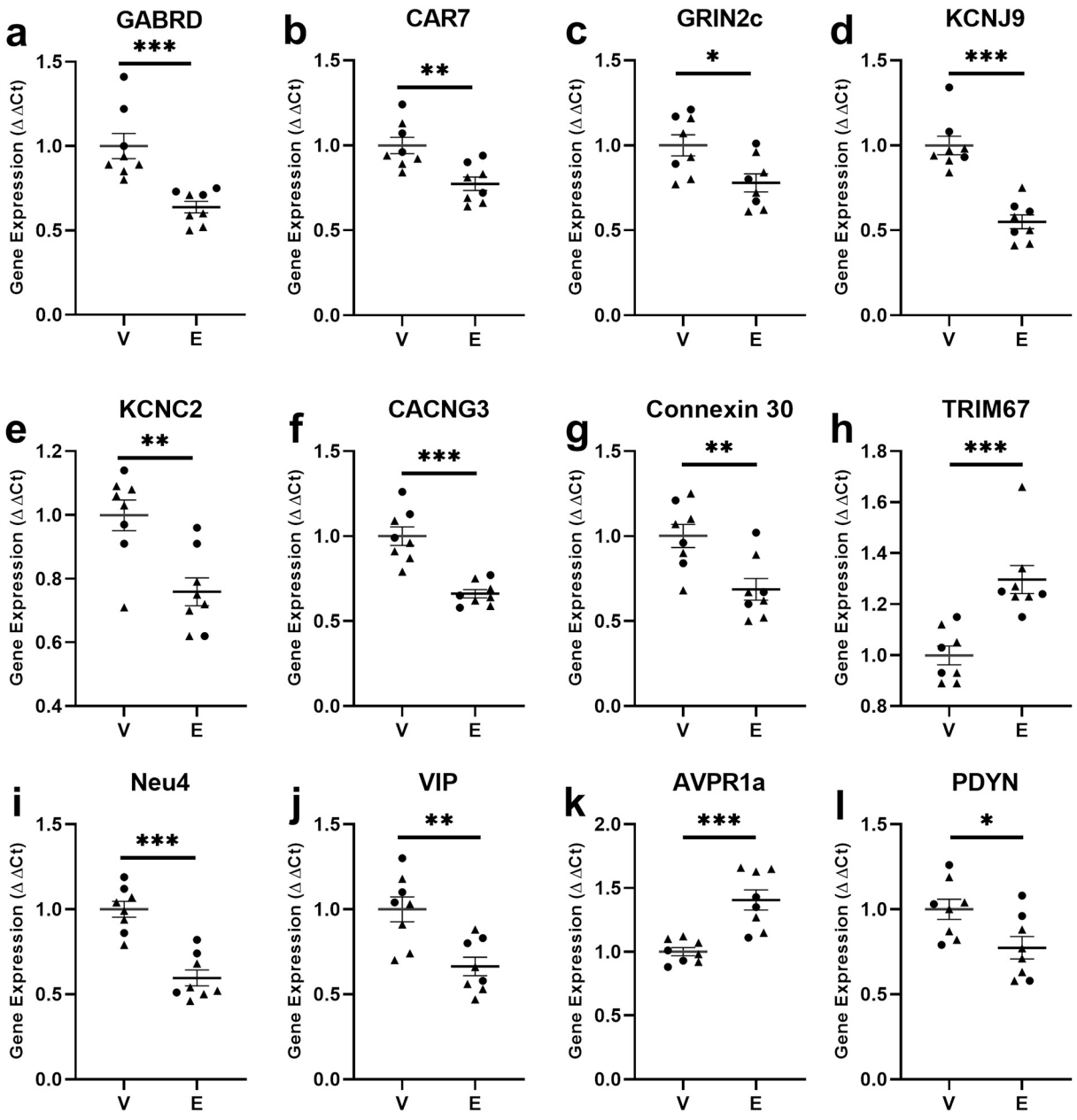

Fig. 5. Effect of ethanol on genes associated with function of the hippocampus. Neonates were gavaged with either 4 g/kg/day ethanol (E) or vehicle (V) from P4-P9 and sacrificed on P10. Expression of (a) GABRD, (b) CAR7, (c) GRIN2c, (d) KCNJ9, (e) KCNC2, (f) CACNG3, (g) Connexin 30, (h) TRIM67, (i) Neu4, (j) VIP, (k) AVPR1a, and (l) PDYN mRNA was measured using qRT-PCR as described in the Methods. Results are expressed as a fold change in the ethanol group relative to the vehicle control group. Values are mean +/− SEM. $N = 8$ animals/group. Male and female are denoted as ▲ and ● respectively. Variance between groups was analyzed by Student's unpaired t-test. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

oligodendrocyte linage cells during this critical stage of development. Relatively little is known concerning the effects of ethanol on myelination in the hippocampus of FASD animal models. In one study, ethanol exposure during gestation and lactation decreased expression of MAG, MBP, MRF, and PLP, which are expressed by mature oligodendrocytes in the developing hippocampus (Cantacorps et al., 2017). Our current study demonstrated that early postnatal ethanol exposure resulted in decreased expression of molecules associated with both mature oligodendrocytes and oligodendrocyte progenitor cells. Outside of the hippocampus, postnatal ethanol exposure resulted in decreased levels of OPCs and mature oligodendrocytes in the corpus callosum. These studies further indicated that OPCs were particularly susceptible to ethanol, although this was dependent on the origin of the OPCs (Newville et al., 2017). In another study, ethanol induced apoptosis of oligodendrocytes in a third trimester macaque model of FASD (Creeley et al., 2013). This suggests that targeting of apoptosis pathways could be warranted for the treatment of FASD.

GABA is a neurotransmitter that binds multi-subunit GABAA receptors that mediate fast inhibitory neurotransmission in the CNS. Ethanol has been shown to alter GABAA receptor neurotransmission in both the cerebellum and hippocampus in postnatal models of FASD (Diaz et al., 2014; Everett et al., 2012; Valenzuela and Jotty, 2015). Down regulation of the GABAA receptor delta subunit GABRD is believed to result in hyperexcitability of hippocampal neurons thereby contributing to epilepsy (T. S. Lee et al., 2021). GABRD knockout mice exhibited altered hippocampal synaptic plasticity (Whissell et al., 2016), and reduced expression of GABRD in the hippocampus was associated with deficits in learning and memory (V. Lee et al., 2016). Furthermore,

GABRD is linked to alcohol use disorder (C. Liu et al., 2018; Mihalek et al., 2001). CAR7 is a carbonic anhydrase which is also associated with GABAergic neurotransmission (Ruusuvuori et al., 2013). It regulates firing of CA1 pyramidal neurons in the hippocampus, and is believed to be an important regulator of long-term plasticity and susceptibility to epileptogenesis (Ruusuvuori et al., 2004). GRIN2c is an ionotropic glutamate NMDA receptor linked to alcohol use disorder (Wang et al., 2016). Furthermore, maternal ethanol consumption results in altered GRIN2c in adult offspring, suggesting potential epigenetic alterations in the expression of these genes (Kleiber et al., 2011). Our current studies demonstrate that ethanol exposure during early postnatal development reduced the expression of GABRD, CAR7, and GRIN2c expression in the hippocampus. This suggests potential mechanisms by which ethanol modulates GABAergic and NMDA glutamate receptor mediated neurotransmission, which may have important implications concerning FASD.

Our current studies demonstrated that ethanol decreased the expression of channels including potassium, calcium, and gap junction channels. KCNC2 knockout mice exhibited increased seizure susceptibility (Lau et al., 2000), and rats treated with ethanol during the early postnatal period also showed decreased KCNC2 expression (Tavian et al., 2011). KCNJ9 is a critical regulator of neuronal hyperexcitability and KCNJ9 knockout mice exhibited increased ethanol binge-like drinking and less severe alcohol withdrawal following ethanol exposure (Herman et al., 2015; Kozell et al., 2018). Connexin 30 controls hippocampal excitatory neurotransmission through modulating glutamate transport at the synapse. Connexin 30 also plays an important role in long-term synaptic plasticity and hippocampal-based contextual memory (Pannasch et al., 2014; Pannasch et al., 2011). Gestational exposure to ethanol resulted in increased connexin 30 expression in the hippocampus of 2–3 week old animals, which may be associated with increased neuronal hyperexcitability observed in these animals (Ramani et al., 2016). Collectively, our studies suggest that ethanol may modulate neuropathology in the developing hippocampus by altering the expression of critical ion and gap junction channels.

TRIM67 and Neu4 are molecules that contribute to neuronal differentiation and neurite formation (Yaguchi et al., 2012). TRIM67 is an E3 ubiquitin ligase and TRIM67 knockout mice exhibit impairments in spatial memory and cognitive flexibility (Boyer et al., 2018). Neu4 is a neuraminidase which hydrolyses the adhesion molecule NCAM and suppresses neurite outgrowth by neurons of the hippocampus (Takahashi et al., 2012). Neu4 knockout mice exhibited increased neuroinflammation and decreased neuritogenesis of hippocampal neurons. These mice also exhibited memory deficits (Pan et al., 2017) and impaired hippocampal-dependent spatial learning (Minami et al., 2016). Our current studies suggest that ethanol may modulate FASD-associated hippocampal pathology by altering the expression of molecules important in neuronal differentiation and neurite formation.

Finally, we evaluated the effects of ethanol on neuropeptides and neuropeptide signaling in the developing hippocampus, and determined that ethanol decreased the expression of VIP, and PDYN and increased the expression of AVPR1a. VIP is important in synaptic plasticity and neurogenesis in the hippocampus (Yang et al., 2010; Yang et al., 2009; Zaben and Gray, 2013). AVPR1a is an arginine vasopressin receptor. AVPR1a mutations have been suggested to affect verbal learning and memory by modulating hippocampal structure and its functional connectivity to the thalamus (Y. Zhang et al., 2020). AVPR1a knockout mice have shown increased alcohol consumption and preference (Sanbe et al., 2008). PDYN plays a role in the kappa opioid receptor system which modulates the development of substance and alcohol use disorders (Anderson and Becker, 2017; Karkhanis & Al-Hasani, 2020). The current studies suggest that altered neuropeptide signaling may contribute to FASD pathogenesis.

Collectively, the current studies demonstrate that ethanol alters the expression of pro-inflammatory and anti-inflammatory molecules in the developing hippocampus and define novel mechanisms of ethanol induced neuroinflammation. The studies also indicate that ethanol alters the expression of molecules associated with OPCs and mature oligodendrocytes. Furthermore, the studies indicate that ethanol alters the expression of molecules critical to neurotransmission. These studies suggest novel mechanisms by which ethanol contributes to neuropathology in the developing hippocampus, which could lead to promising treatments for individuals with FASD.

Author contributions

All authors made substantial contributions, had full access to all of the data in the study, and take responsibility for the integrity of the data and the accuracy of the data analysis.

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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