Increased neuroinflammatory signaling and memory deficits caused by early-life ethanol exposure and the potential benefits of anti-inflammatory treatment

Undergraduate Research Thesis

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Abstract

Alcohol consumption during pregnancy can lead to physical irregularities and deficits in cognition collectively known as fetal alcohol spectrum disorders (FASD). Impairments in cognition, particularly hippocampus-dependent memory, may be due to ethanol-induced neuroinflammation resulting from third trimester alcohol consumption, a period of development in which the brain undergoes rapid growth and synaptogenesis. This study utilized a third trimester binge-drinking rodent model of FASD, in which rats were intubated and given ethanol (5g/kg/day; 5E) or sham intubated (SI) over postnatal days (PD)4-9. The ability of two anti-inflammatory drugs, ibuprofen and glycyrrhizin, to normalize neuroinflammation and cognition were investigated. Animals in the ibuprofen group received subcutaneous injections 2h following ethanol treatment (100mg/kg on PD4, 50mg/kg/day following, IBU) or an equivalent volume of PBS (vehicle; VEH). Glycyrrhizin animals received the drug (20mg/kg/day; GLY) or an equivalent volume of VEH via intraperitoneal injections 20-30min prior to ethanol administration. On PD10 the rats were euthanized and hippocampi removed for quantification of inflammation-related gene expression. In the IBU experiment, but not the GLY experiment, the 5E-VEH rats showed significant increases in hippocampal IL-1β and TNF-α, but not CD11B or GFAP, expression compared to SI-VEH and 5E-IBU. Next, we assessed in juvenile 5E rats whether ibuprofen or glycyrrhizin could rescue previously documented deficits in Trace Fear Conditioning (TFC), a challenging hippocampus-dependent associative memory task. Results indicate IBU but not GLY rescued TFC test performance in 5E rats. Taken together, postnatal ethanol upregulated pro-inflammatory cytokine expression on PD10 and impaired TFC over PD31-33. Ibuprofen, but not glycyrrhizin, ameliorated both effects, suggesting the potential benefits of anti-inflammatory drug treatment in improving cognition in FASD individuals.

Introduction

Fetal Alcohol Spectrum Disorders (FASD), resulting from alcohol exposure during gestation, is estimated to affect 2-7 children in every 1000 births. The disorder is characterized by deficits in behavior and cognition, as well as physical irregularities, such as facial dysmorphology (Hoyme et al., 2005). Impairments in behavior and cognition include difficulties in executive function, and learning and memory (Kodituwakku, 2009). However, there is a lack of established diagnostic methods and measurements, leading to difficulties in making definitive diagnoses, particularly in the absence of overt dysmorphology (May et al., 2009).

FASD is a term that covers a variety of distinct diagnoses resulting from alcohol consumption during pregnancy. Among these are Fetal Alcohol Syndrome (FAS), Partial Fetal Alcohol Syndrome (PFAS), Alcohol Related Birth Defects (ARBD), and Alcohol Related Neurodevelopmental Disorder (ARND). FAS is the most easily recognized of these diagnoses, and is characterized by growth deficiency, facial dysmorphology including small eyes and a smooth philtrum, and severe central nervous system abnormalities (Banakar et al., 2009). This disorder is known to be the most preventable cause of intellectual disabilities in the world, and is estimated to cost $1.4 million per individual for lifetime care (American Academy of Pediatrics). The PFAS diagnosis is given to individuals who have most, but not all, of the growth deficiencies and facial abnormalities characteristic of FAS. ARBD is identified by facial dysmorphology and a confirmation of prenatal alcohol exposure, but no abnormalities in growth or central nervous system features. Lastly, ARND is diagnosed in cases of central nervous system dysfunction and confirmation of maternal alcohol consumption (Banakar et al., 2009); the lack of obvious physical abnormalities makes ARND difficult to diagnose. The experiments below model ARND in rats.

Impairments in the central nervous system can result in difficulties in cognitive functioning in individuals with FASD. Facial abnormalities do not need to be present for an FASD diagnosis – in fact, the majority of FASD individuals do not exhibit physical anomalies. In contrast, evidence suggests that neurocognitive deficits present similarly in individuals diagnosed across the FASD spectrum (Nash et al., 2006). Children with FASD have average IQs in the borderline to low average range, as well as deficits in various areas of cognitive function, including executive functions like planning, attentional set shifting, concept formation, and error correction. Children with FASD had greater difficulty with executive control tasks requiring high levels of manipulation and regulation, and slowed information processing was seen in both children and toddlers. As the neurocognitive demands of tasks in memory and language were increased, the performance of FASD children declined at a more rapid rate than that of control groups. Particularly with tests of memory, children with FASD showed deficits when the tasks involved conscious effort, such as free recall and organization (Kodituwakku, 2009).

The mechanism by which early life ethanol produces cognitive dysfunction is still unclear. Recent studies have focused on ethanol causing damage to brain structures by activating the neuroinflammatory system (Drew et al., 2015). In the brain, ethanol may directly activate toll-like receptor 4 (TLR4) on microglia, or indirectly activate it via the release of high-mobility group box 1 (HMGB1) protein, an endogenous TLR4 ligand (Zou and Crews, 2014). To do so, ethanol inhibits histone deacetylases (HDACs), leading to increased production and acetylation of HMGB1, allowing movement of the protein into the cytoplasm and the extracellular environment. Among the downstream effects of ethanol or HMGB1 binding to TLR4, nuclear factor kappa B (NFκB) activates cyclooxygenase (COX)-2, leading to the release of pro-inflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α (Alfonso-

Loeches et al., 2010) (Figure 1). Postnatal ethanol exposure can cause neuroinflammation in the hippocampus, among other regions (Drew et al., 2015; Tiwari and Chopra, 2011; Topper et al., 2015), which may be particularly harmful during early development due to low levels of antioxidants in the brain (Brocardo et al., 2011). Increased neuroinflammation and decreased antioxidant properties may interfere with normal processes of neural development, including synaptic pruning (Paolicelli et al., 2011), neurogenesis (Bland et al., 2010b), and synaptic plasticity (Bilbo and Schwarz, 2009). This damage is thought to contribute to impairments in executive function, and learning and memory seen in FASD individuals and rodent models.

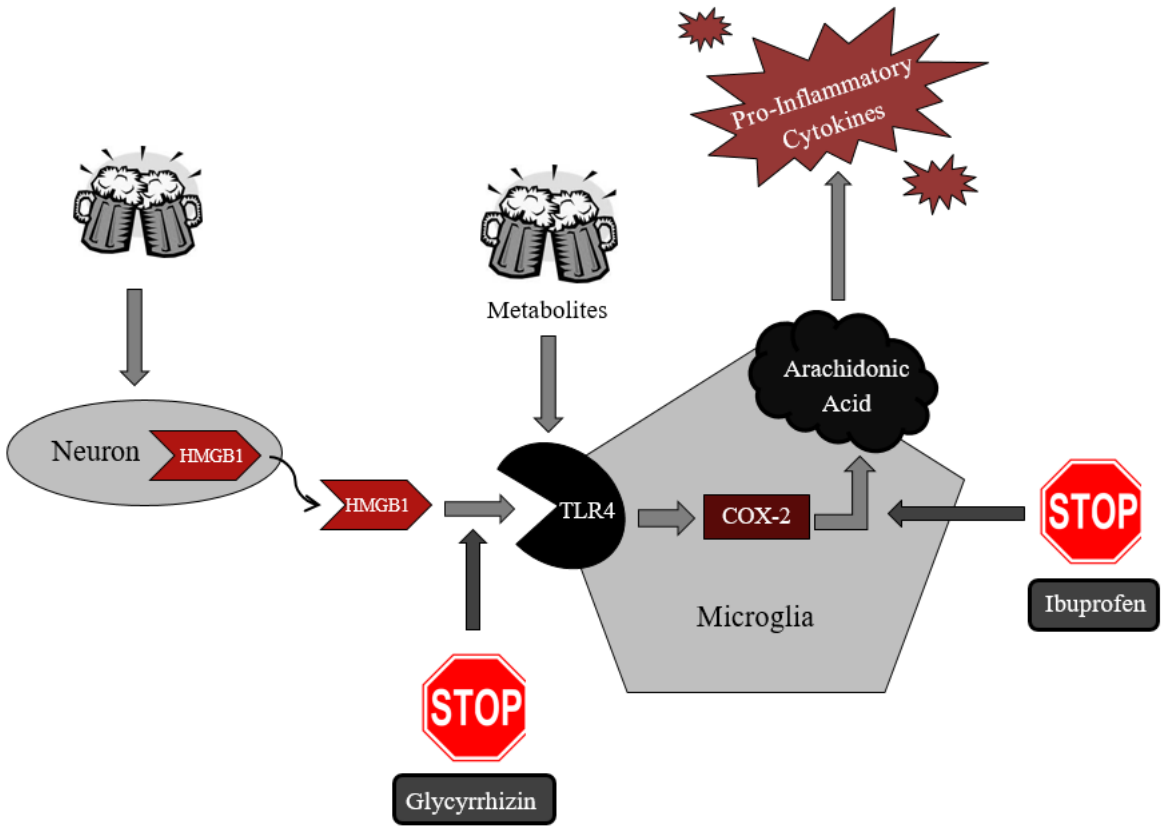

Figure 1. A schematic of the neuroinflammatory process by which ethanol induces an elevated inflammatory profile and mechanisms of the anti-inflammatory drugs, IBU and GLY.

Modulation of normal development by early life inflammation results in differences in the brain and behavior in adulthood. Cytokines have been implicated in normal brain development, including neuronal and glial cell migration and differentiation, and synaptic pruning (Nawa and Takei, 2006). For example, expression of cluster of differentiation protein 11B (CD11B) and resulting reactive oxygen species have been shown to be important for normal developmental neuronal death (Wakselman et al., 2008). However, over-expression of cytokines such as IL-1β, IL-6, and TNF in early life, for example due to perinatal complications (Miller et al., 1990) or bacterial meningitis, have been correlated with neurological disorders (Mustafa et al., 1989). The early activation of inflammation was linked to later-life cognitive dysfunction, including deficits in learning and memory and attention disorders (Bauman et al., 1997). Ethanol has been shown to have region-specific effects in the brain, rather than causing a global depression of all brain activity (White et al., 2000). In particular, the hippocampus was found to be especially vulnerable to inflammatory stress compared to other brain regions. Over-activation of the inflammatory response early in life caused by ethanol exposure is proposed to induce damage to the developing hippocampus and cause cognitive deficits later in life (Bilbo & Schwarz, 2009).

Similar to children with FASD who struggle with Pavlovian associative memory (Jacobson et al., 2008), rats exposed to ethanol demonstrate deficits in hippocampus-dependent memory, which can be tested using Trace Fear Conditioning (TFC). TFC is a cognitively challenging Pavlovian task that depends on a distributed neural circuit, including the hippocampus and prefrontal cortex (Gilmartin et al,. 2013). TFC is an important FASD modeling task as it can be used test many of the executive function deficits seen in FASD individuals, including attention, working memory, and long term memory (Kodituwakku, 2009). In TFC, a conditioned stimulus (CS), a tone, is separated in time from an unconditioned stimulus (US), a footshock, by a 15s stimulus-free trace

interval (TI) (Figure 2B). Although it is unclear the exact role of the hippocampus in TFC, it is thought to be important for the integration of multi-modal information – in this case, the temporally separated CS and US (Quinn et al., 2002). Interestingly, the dorsal hippocampus is only required when the TI is longer than approximately 5s (Chowdhurty et al., 2005). Prior work also demonstrated that FASD model rats were impaired in TFC memory tests when the TI was approximately 5s or longer (DuPont et al., 2014; Wagner and Hunt, 2006), suggesting impaired TFC CS-US associative memory is likely due to hippocampal dysfunction.

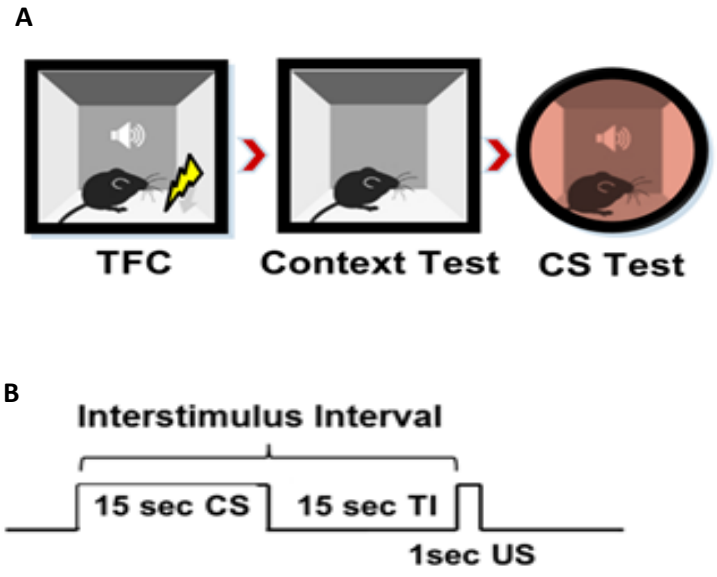

Figure 2. A schematic of trace fear conditioning. Trace fear conditioning acquisition, context test, and in a novel context, CS-alone test **(A)**. An example of a single conditioning session trial, showing 15s trace interval and total 30s interstimulus interval (ISI) consisting of 15s CS and 15s TI **(B)**.

Previous studies have found that rats exposed to ethanol during the third trimester equivalent of human pregnancy exhibited significant reductions in CS-evoked conditioned fear (DuPont et al., 2014, Hunt et al., 2009). This reduced freezing to the CS is proposed to reflect impairments in synaptic plasticity and memory consolidation, not encoding (Runyan et al., 2004). Memory encoding and consolidation are thought to be facilitated in part by *N*-methyl-*D*-aspartate receptor (NMDAR), a glutamatergic Ca^{2+} channel, which can be found in the hippocampus (Quinn et al., 2005). Postnatal ethanol can cause long lasting changes to NMDAR subunits and NMDAR-dependent signaling, such as decreased ERK1/2 phosphorylation, both of which underlie long-term potentiation (LTP) maintenance and CS-US associative memory consolidation (Goodfellow et al., 2016, DuPont et al., 2014). In addition, IL-1β release also contributes to LTP maintenance and hippocampus-dependent memory consolidation in a dose-dependent, bell-curve-like manner in which either too little or too much impairs consolidation (Williamson et al., 2011, Yirmiya et al., 2002, Goshen et al., 2007). Neonatal elevations in inflammatory cytokines in response to postnatal ethanol may lead, in juvenile 5E rats submitted to TFC, to experience-dependent discrete changes in IL-1β release and synaptic plasticity, contributing to a weaker (less consolidated) TFC memory and diminished CS-evoked freezing behavior.

In the experiments below, rats were intubated with ethanol (5g/kg/day; 5E), or sham intubated (SI) over postnatal days (PD) 4-9. This time period is comparable to brain development during the third trimester of pregnancy in humans (Bayer et al., 1993), when the brain is extremely susceptible to neurotoxic damage as it undergoes rapid growth and synaptogenesis (Gil-Mohapel et al., 2010). Exposing rats to ethanol during this period has been shown to result in neural degeneration in the hippocampus (Ikonomidou et al., 2000) and hippocampus-dependent TFC memory deficits (DuPont et al., 2014; Hunt et al., 2009).

This study investigated the effectiveness of two anti-inflammatory drugs, ibuprofen (IBU) and glycyrrhizin (GLY), on normalizing acute inflammatory expression on PD10 and ameliorating TFC memory deficits in juvenile (PD31-33) 5E rats. IBU is a non-steroidal anti-inflammatory drug that works through the inhibition of COX-2, which was injected 2h following PD4-9 ethanol administration. GLY is an anti-inflammatory drug that works by preventing the binding of HMGB1 and TLR4 (Mollica et al., 2007), which was injected 20-30m prior to ethanol treatment on PD4-9. IBU was administered following ethanol administration, as it blocks the effects of the metabolites of ethanol. GLY, on the other hand, inhibits the actions of ethanol and not its metabolites, therefore GLY treatment occurred prior to ethanol administration. On PD10, mRNA quantification of IL-1β, TNF-α, microglial marker (CD11B), and astrocyte marker (glial fibrillary acidic protein; GFAP) were used to quantify neuroimmune activation. Separate groups of IBU and GLY treated juvenile 5E rats underwent TFC for investigation of hippocampal-dependent memory consolidation. Both anti-inflammatory treatments were hypothesized to normalize ethanol-induced neuroinflammation in the hippocampus, and alleviate associative memory deficits in 5E juvenile rats.

Materials & Methods

Subjects & Neonatal Treatment

All procedures followed the guidelines of The Ohio State University Institutional Animal Care and Use Committee (IACUC). All appropriate measures were taken to minimize discomfort and pain in the animals. Long-Evans rats purchased from Envigo Laboratories (Indianapolis, IN) were housed and bred with *ad libitum* food and water on a 12h light/dark cycle in The Ohio State

University psychology vivarium, an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facility. One male and one female were pair-housed for one week, then females were checked daily beginning 3 weeks later for pups. After birth, pups were kept with their mother until weaning at PD21, when they were housed with 1-2 same-sex littermates. On PD3, the litters were culled to a maximum 12 animals per litter. The pups were subcutaneously injected a 30 gauge needle with non-toxic black ink for identification and separated into treatment groups.

On PD4-9, male and female rats received binged-like doses of ethanol (5g/kg/day; 5E) in milk solution (11.33% vol/vol) via intragastric intubation, in which polyethylene tubing was lubricated with corn oil and lowered into the stomach through the esophagus. Animals in the ibuprofen (100mg/kg on PD4, 50mg/kg PD5-9; IBU) group received the drug or an equivalent volume of phosphate buffered saline vehicle (VEH) via subcutaneous (SC) injections 2 h after ethanol administration (Carty et al., 2011). Rats in the glycyrrhizin (20mg/kg/day; GLY) received intraperitoneal (IP) injections of the drug of an equivalent volume of saline (VEH) with 30 gauge needles 20-30min prior to ethanol treatment (Yamamura et al., 1995; Wang et al., 2015). The rats received milk only intubations 2h later, with a third milk alone dose only on PD4. The milk alone intubations supplemented the caloric intake of 5E animals that may nurse less due to inebriation. Sham intubated (SI) rats received intragasteric intubations but no solution. Prior to the first milk alone dose on PD4, 5E and SI pups were tail clipped and approximately 40μL of blood collected for blood alcohol concentration (BAC) analysis. Following tail clipping, blood clotting was promoted by dipping the rats' tails in Kwik-Stop® styptic powder with benzocaine (ABC Laboratories, Dayton, OH). Blood collected from SI rats were discarded. Blood from 5E rats collected in capillary tubes were transferred into

micropipette tubes and centrifuged for 10min to separate plasma. The plasma was separately stored at -80°C until analysis via an Analox GL5 Analyzer (Analox Instruments, Lunenberg, MA). The Analox GL5 Analyzer measured the rate of oxygen consumption via the oxidation of ethanol in the plasma samples, which was compared to a known ethanol standard used for instrument calibration. Rats were weighed each day of intubations, then again on PD10, PD15, PD21 and PD30 (in groups undergoing TFC) to track development.

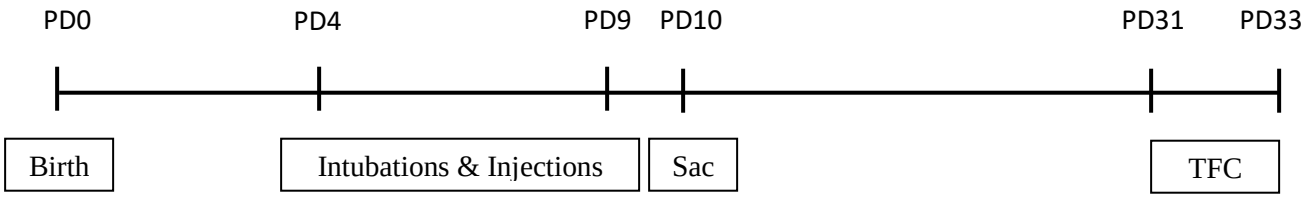

Figure 3. Timeline of Treatment and Behavioral Testing

Polymerase Chain Reaction

Rats were euthanized with isoflurane and decapitated on PD10 and hippocampi collected. A Total RNA Tissue Kit (IBI, Peosta, IA) and DNase1 (Qiagen, Valencia, CA) was used to isolate RNA from the dorsal hippocampus. First Strand cDNA Kit from Origene (Rockville, MD) was used to prepare cDNA, which was then diluted (1:5). A mastermix of Bullseye EvaGreen Supermix (MidSci, St. Louis, MO), primers (PrimeTime® GAPDH and IL-1β, or custom GFAP, TNF-α, and CD11B), and water were added to the cDNA. Assays were run in triplicates (CD11B, GFAP) or quadruplicates (IL-1β and TNF-α) using GAPDH as a normalizing comparison (citation). The assays were run with a CFX96, C1000 Thermal Cycler (Bio-Rad, Richmond, CA) and quantified via the comparative cycle threshold ($2\text{-}\Delta\Delta C_t$) method. Primer sequences used (5'-3') were as follows: GAPDH (forward: AACCCATCACCATCTTCCAG,

reverse: CCAGTAGACTCCACGACATAC), CD11B (forward: CTGGGAGATGTGAATGGAG, reverse: ACTGATGCTGGCTACTGATG) (Bland et al., 2010a), GFAP (forward: AGATCCGAGAAACCAGCCTG, reverse: CCTTAATGACCTCGCCATCC) (Frank et al., 2012), IL-1β (forward: GTGCTGTCTGACCCATGT, reverse: TTGTCGTTGCTTGTCTCTCC), and TNF-α (forward: GACCCTCACACTCAGATCATCTTCT, reverse: TGCTACGACGTGGGCTACG) (Fernandez-Lopez et al., 2012).

Trace Fear Conditioning

Rats were weaned on PD21 and housed with 2-3 littermates of the same sex. Body weight was recorded on PD10, PD15, PD21, and PD30. Conditioning and tests occurred on PD31-33 in 5E and SI rats placed inside standard operant boxes (Coulbourn Instruments, Allentown, PA). These boxes were made up of two stainless steel and two Plexiglas® walls with a floor of .5cm stainless steel bars 1.5cm apart. Footshocks (US) were delivered via a shock generator (Lafayette Instruments, Lafayette, IN) connected to the grid floor. An infrared camera (Circuit Specialists, Inc., Mesa, AZ) at the top of the box recorded freezing behavior (a species-specific fear response), which was transmitted to FreezeScan (CleverSys, Inc., Reston, VA) to quantify freezing behavior according to experimenter-defined time bins.

Rats were separated into clean cages in the vivarium and carried to a well-lit testing room. The operant boxes, illuminated by a 15W bulb, were scented with a vinegar/water (1:5) solution before the rats were placed inside. Following a baseline of 180 ± 30s, rats were presented with a 15s, 2.8kHz, 80dB tone CS, then 15s later (trace interval; TI) a 1s, 0.8mA footshock (US). The inter-stimulus interval (ISI), therefore, was 30s from CS onset to US onset. The rats received a total of 10 CS-US presentations, separated by an inter-trial interval of 180 ± 30s. Rats

underwent context and CS-alone testing at 24 and 48 h later, respectively (Figure 2), with no foot shocks in either test. The context test was run under the same conditions as the acquisition, and freezing was measured for 10 minutes following a 2min baseline. CS-alone tone testing was conducted in a novel context, to which the rats were wheeled into on metal carts in darkened cages. The chambers were unilluminated, scented with Windex®, and gray plexiglass covered the grid bars. In addition, white noise was produced by a fan, and a magnet and pink geometric Figure were placed on separate walls. Freezing behavior was measured across ISI for the acquisition and tone test sessions.

Statistical Analyses

Data was analyzed via single-factor analyses of variance (ANOVA) and, when appropriate, Fisher's LSD post-hoc tests (significance implies $p<0.05$). PCR was performed in 57 rats total, 27 the IBU experiment: SI-VEH (n=9), 5E-VEH (n=8), and 5E-IBU (n=10). The remaining 30 were in the GLY experiment: SI-VEH (n=10), 5E-VEH (n=10), and 5E-GLY (n=10). A total of 57 rats underwent TFC, with 27 in the IBU experiment, SI-VEH (n=9), 5E-VEH (n=8), and 5E-IBU (n=10). Thirty rats were in the GLY experiment, SI-VEH (n=10), 5E-VEH (n=10), and 5E-GLY (n=10).

Results

Blood Alcohol Concentration

The mean (±SE) BAC for the IBU experiment was 348.0 ± 7.21mg/dL and 360.6 ± 6.56mg/dL for 5E-VEH and 5E-IBU treatment groups, respectively. Mean (±SE) BAC in the GLY

experiment was 360.8 ± 6.22mg/dL for 5E-VEH rats and 358.4 ± 5.02mg/dL for 5E-GLY rats. Single-factor (Drug) ANOVA found no significant differences in BAC levels between groups.

Body Weights

Body weights were analyzed on the first day of treatment (PD4) and one day after the end of treatment (PD10) via single-factor (Treatment) ANOVAs for rats undergoing PCR. In the IBU experiment, significant differences were found at PD10 $F(2,27) = 3.02$, $p<0.05$, but not at PD4 ($p=0.37$) (Table 1). Post-hoc tests showed that 5E-IBU rats weighed significantly less than SI-VEH and 5E-VEH rats. Significant differences in body weight were also found in the GLY experiment at PD10, $F(2,24) = 9.915$, $p<0.05$, but not at PD4 ($p=0.65$) (Table 2). Post-hoc tests indicated that SI-VEH rats weighed significantly more than 5E-VEH and 5E-GLY rats. Rats undergoing TFC were weighed again at PD15, PD21, and PD30 to track development. As shown in Table 1, no significant treatment group differences were found at these times ($p=0.44$, $p=0.68$, $p=0.55$ at PD15, PD21, and PD30, respectively). Similarly, Table 2 shows no significant differences in body weight between treatment groups at these times in development ($p=0.26$, $p=0.37$, and $p=0.64$ for PD15, PD21, and PD30, respectively).

IBU	**PD4**	**PD10***	**PD15**	**PD21**	**PD30**
SI-VEH (n=9)	9.87 ± 0.69	20.68 ± 1.35	33.91 ± 1.67	52.41 ± 2.76	95.87 ± 5.89
5E-VEH (n=8)	10.88 ± 0.35	20.19 ± 1.02	35.00 ± 0.78	54.08 ± 1.15	100.32 ± 2.15
5E-IBU (n=10)	10.69 ± 0.49	19.3 ± 1.07	32.16 ± 1.70	50.83 ± 2.84	93.20 ± 4.16

Table 1. Body Weight (g) across development of rats undergoing TFC in the IBU experiment. At PD10 5E-IBU rats weighed significantly less than SI-VEH and 5E-VEH rats. Significance is denoted by *.

GLY	**PD4**	**PD10***	**PD15**	**PD21**	**PD30**
SI-VEH (n=10)	10.22 ± 0.51	20.89 ± 0.94	30.96 ± 1.27	47.84 ± 2.18	85.98 ± 6.11
5E-VEH (n=10)	9.49 ± 0.46	18.08 ± 0.86	28.49 ± 1.02	45.02 ± 1.32	82.69 ± 2.97
5E-GLY (n=10)	11.1 ± 0.30	19.71 ± 0.72	30.08 ± 0.73	48.71 ± 1.92	88.67 ± 3.12

Table 2. Body Weight (g) across development of rats undergoing TFC in GLY experiment. At PD10 SI-VEH rats weighted significantly greater than 5E-VEH and 5E-GLY rats. Significance is denoted by *.

Polymerase Chain Reaction

Gene expression data for pro-inflammatory cytokines were normalized to SI-VEH animals for both IBU and GLY experiments. For each gene, expression was normalized to the expression of the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which did not vary between groups. Figure 4A-B shows that neonatal ethanol treatment in the IBU experiment had no significant effect on the expression of CD11B ($p=0.32$) or GFAP ($p=0.61$). In the IBU experiment, postnatal ethanol significantly altered the expression of IL-1β, $F(2,24) = 4.32$, $p<0.05$, and TNF-α, $F(2,24) = 6.74$, $p<0.01$, as seen in Figures 4C-D. Gene expression of both inflammatory cytokines were significantly elevated in the 5E-VEH rats compared to SI-VEH and 5E-IBU rats. In contrast, rats in the 5E-VEH treatment groups did not show significant elevations in the expression of CD11B ($p=0.08$), GFAP ($p=0.49$), IL-1β ($p=0.34$), or TNF-α ($p=0.94$), compared to 5E-GLY or SI-VEH animals (Figure 5A-D).

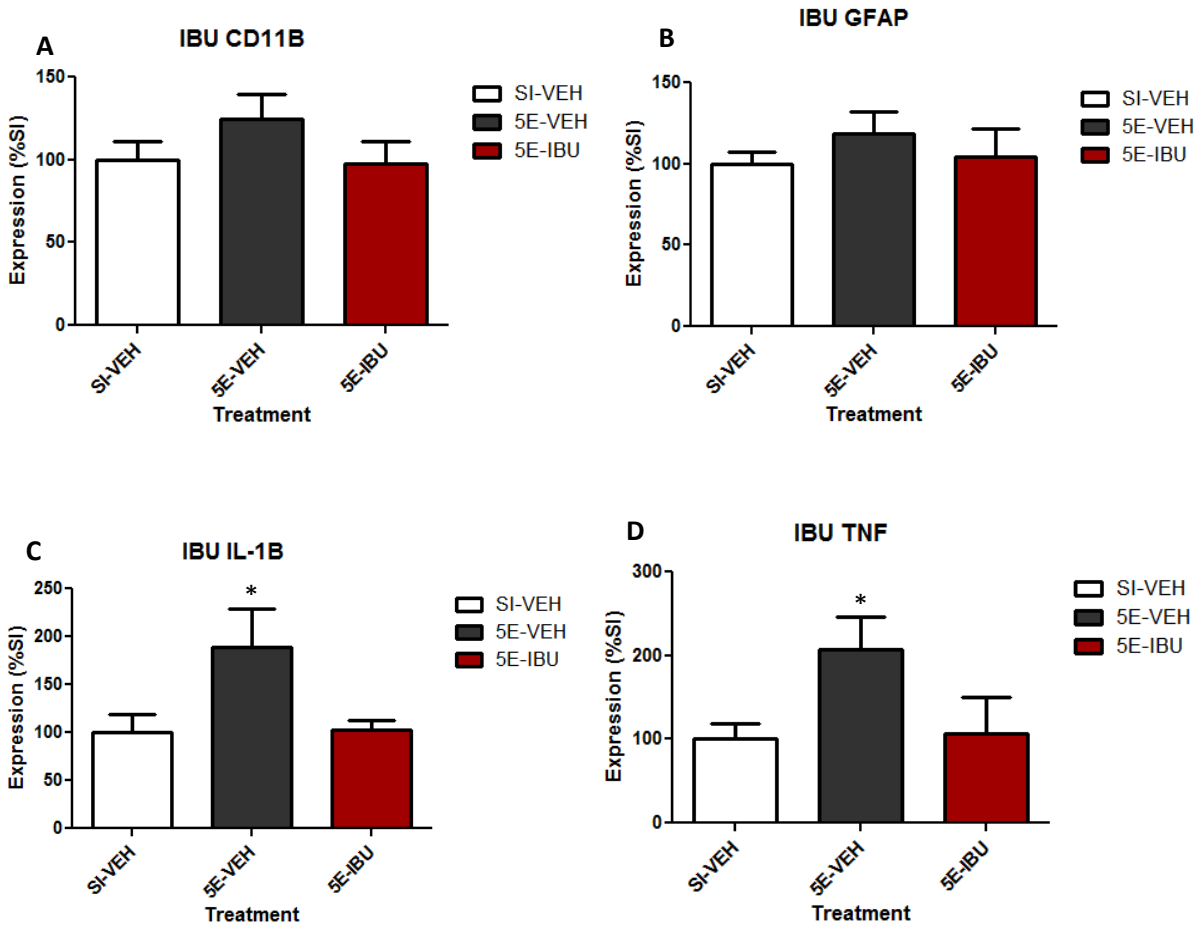

Figure 4. PCR: Gene expression of pro-inflammatory cytokines in IBU treatment groups at PD10. Gene expression was normalized to GAPDH as a percentage of group SI-VEH expression. CD11B **(A)** and GFAP **(B)** were not significantly affected by neonatal ethanol treatment. Ethanol treatment significantly elevated expression of IL-1β **(C)** and TNFα **(D)**, as seen in the 5E-VEH treatment group compared to all other groups. Significance is denoted by *.

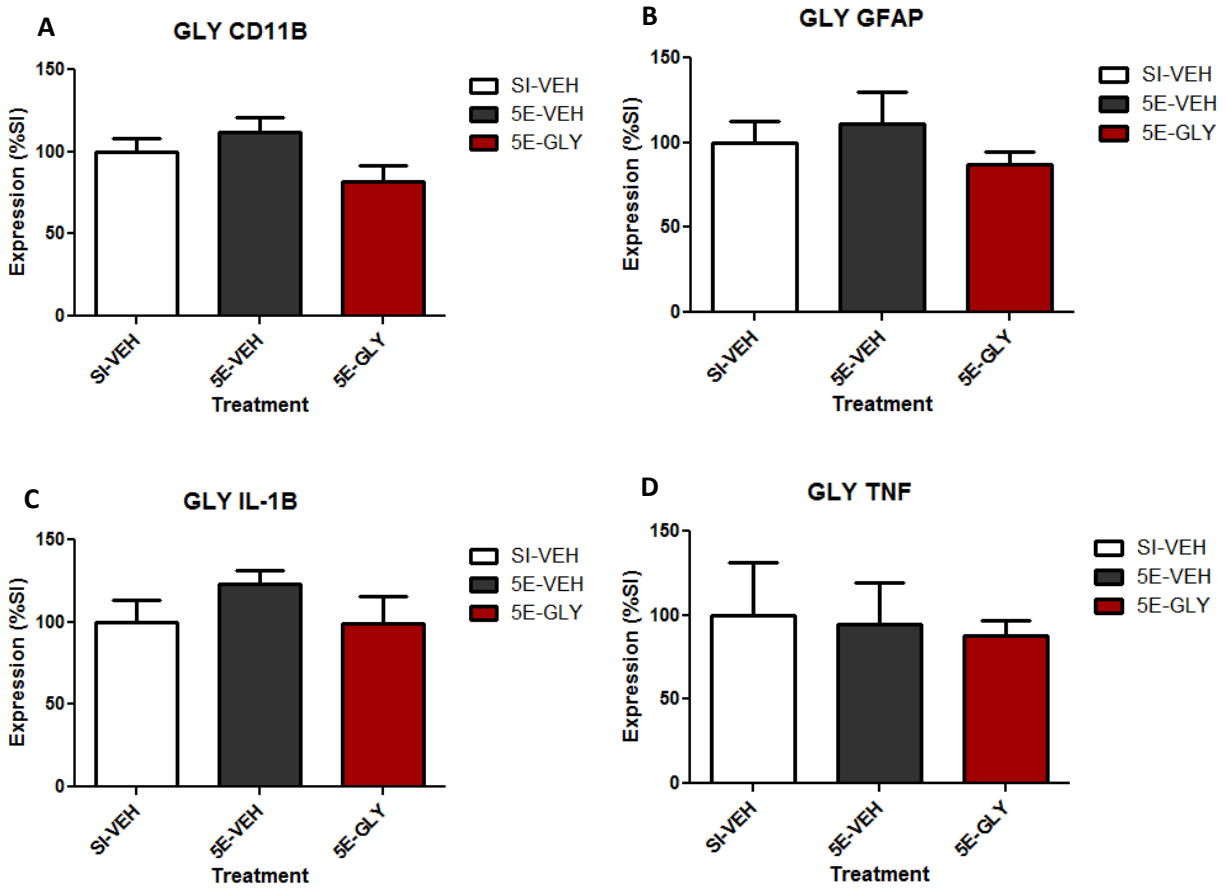

Figure 5. PCR: Gene expression of pro-inflammatory cytokines in GLY treatment groups at PD10. Gene expression was normalized to GAPDH as a percentage of group SI-VEH expression. Neonatal ethanol treatment had no significant effect on the expression of CD11B **(A)**, GFAP **(B)**, IL-1β **(C)**, or TNFα **(D)**.

Trace Fear Conditioning

Consistent with results seen in DuPont et al. (2014) in adult rats and in Hunt et al. (2009) in adolescent rats, no group differences were seen in the mean freezing across the ISI during TFC acquisition in either the IBU or GLY experiments, p=0.71 (data not shown) and p=0.94 (data not shown), respectively. There were also no differences in mean freezing during the 10min context test in either experiment, p=0.89 (Figure 6A) and p=0.87 (Figure 7A). In the novel context, there were no differences in mean freezing between treatment groups during the 2min

baseline in either the IBU or GLY experiments (p=0.69, Figure 6B; p=0.99, Figure 7B), signifying a successful context switch. Significant differences were found in mean freezing during the ISI by treatment group in the IBU experiment, $F(2,24) = 5.61$, $p<.01$ (Figure 6B), but not in the GLY experiment, p=0.99 (Figure 7B). Post-hoc test indicated that mean freezing was significantly less in 5E-VEH rats compared to SI-VEH and 5E-IBU rats (Figure 6B).

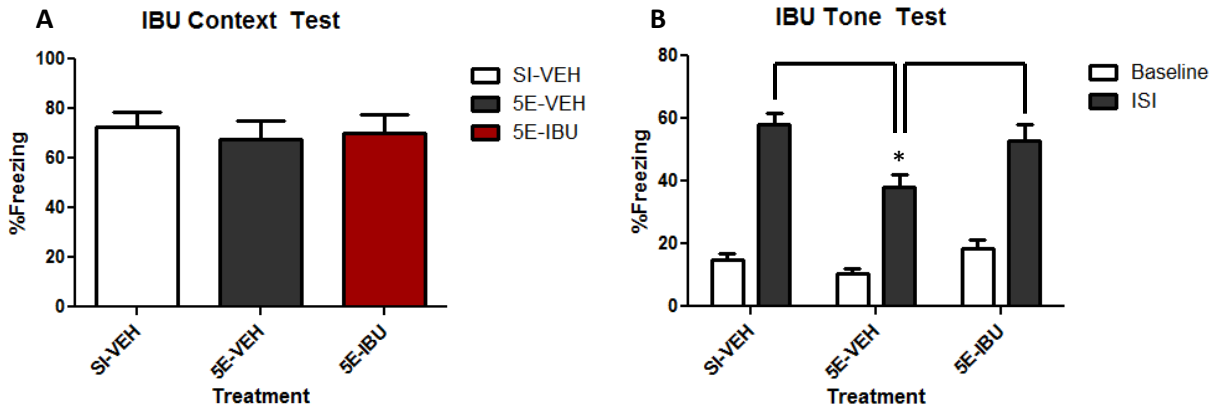

Figure 6. TFC Freezing behavior in IBU treatment groups at adolescence (PD31-33). Mean (±SE) freezing did not differ between treatment groups during the context test **(A)** or the 2 min baseline in a novel context **(B)**. Freezing during the ISI (CS+TI) was significantly enhanced in 5E-VEH rats compared to SI-VEH and 5E-IBU rats **(B)**. Significance is denoted by *.

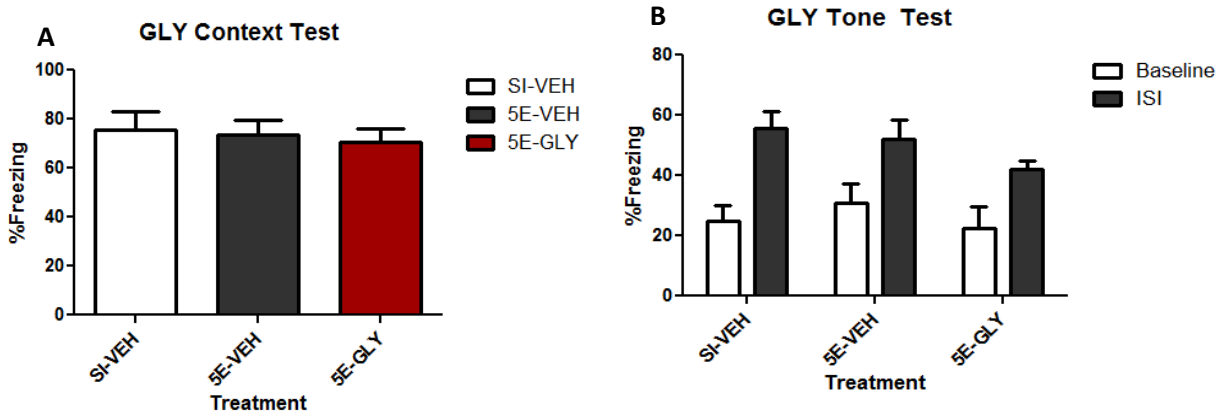

Figure 7. TFC freezing behavior in GLY treatment groups at adolescence (PD31-33). Mean (±SE) freezing did not differ between treatment groups during acquisition (not shown), the context test **(A)**, the 2 min baseline in a novel context, or during the ISI (CS+TI) **(B)**.

Discussion

This thesis investigated the ability of two anti-inflammatory drugs, IBU and GLY, to attenuate ethanol-induced neuroinflammation and later-life executive function and memory deficits in later life. Results from the IBU experiment indicated a significant upregulation of pro-inflammatory cytokines IL-1β and TNF-α, but not of CD11B or GFAP, at PD10 in 5E-VEH rats as a result of early-life ethanol exposure. Elevated cytokine gene expression was not found in 5E-VEH rats in the GLY study, and no significant differences between treatment groups were seen. TFC test performance results indicated that PD4-9 ethanol exposure significantly decreased CS-evoked freezing behavior in 5E-VEH rats in the IBU experiment. As seen with elevations in neuroinflammation, IBU treatment increased freezing during the ISI in 5E-IBU rats relative to 5E-VEH rats. Significant treatment group differences in neuroinflammatory markers on PD10 or later-life TFC test performance were not found in the GLY experiment.

Neonatal Neuroinflammation

Ethanol is predicted to initiate neuroinflammation in one of two ways. It may directly activate TLR4 on microglia (Pascual et al., 2011), leading to release of pro-inflammatory cytokines such as IL-1β and TNF-α (Drew et al., 2015, Topper et al., 2015). Alternatively, ethanol may indirectly activate TLR4 through HMGB1 release (Zou and Crews, 2014). Based on these possible mechanisms of inflammation, our study used two anti-inflammatory drugs: IBU and GLY (Figure 1). IBU is a non-steroidal anti-inflammatory drug that works through the inhibition of COX-2. GLY inhibits the binding of HMGB1 and TLR4 to block the downstream enhancement in inflammation (Zou and Crews, 2014). CD11B is a protein found on the surface of microglia and can be used as a preliminary indicator of microglial activation (Crain et al., 2013). Astrocytes may also contribute to neuroinflammatory response, as IL-1β has been shown

to downregulate glutamate transporter 1 (GLT1) on astrocytes to increase synaptic glutamate concentrations (Liu et al., 2013). The implications of these molecules in ethanol-induced inflammation were investigated in this thesis by looking at PD10 gene expression of CD11B, GFAP, and pro-inflammatory cytokines IL-1β, and TNF-α.

Past studies investigating FASD have used CD11B and GFAP expression as indicators of microglia and astrocytes. Alfonso-Loeches et al. (2010) found significant upregulation in CD11B and GFAP, as well as IL-1β and TNF-α with chronic ethanol exposure. In a study of ethanol exposure over PD4-9, CD11B was investigated as a pro-inflammatory cytokine and found to be elevated in the hippocampi of both ethanol-exposed and SI rats (Boschen et al., 2016). Topper et al. found increased expression of GFAP as a marker for astrocytes at PD10 (2015). Also, Topper et al. (2015) and Drew et al. (2015) both reported an increased expression of pro-inflammatory cytokines IL-1β and TNF-α at PD10, during the ethanol withdrawal period (Drew et al., 2015; Topper et al., 2015). Consistent with these finding, results from the IBU experiment showed increased hippocampal gene expression of IL-1β and TNF-α, but not of CD11B (microglia) or GFAP (astrocytes) at PD10 in 5E-VEH rats (Figure 4A-D). Previous studies have also shown a reduction of hippocampal IL-1β and TNF-α expression when treated with an immunosuppressant, pioglitazone (Drew et al., 2015). Similarly, this study demonstrated IBU was able to attenuate the elevated inflammatory cytokine response in 5E-IBU rats at PD10.

In contrast, the expected elevations in pro-inflammatory gene expression were not found in 5E-VEH rats of the GLY experiment. Previous studies have found that GLY treatment reduces ethanol-induced gene expression of IL-1β and TNF-α (Zou & Crews, 2014). However, results from this study indicated that rats in the GLY experiment did not show significant changes in the expression of CD11B, GFAP, IL-1β, or TNF-α between treatment groups (Figure 5A-D). The

lack of significant elevations in IL-1β and TNF-α expression in 5E-VEH contradicted previous studies that found neonatal ethanol exposure increases hippocampal inflammatory cytokine expression at PD10 (Topper et al., 2015). Additionally, the lack of significant differences between 5E-VEH and 5E-GLY rats contradicted studies by Zou & Crews (2014), which found that GLY treatment attenuated ethanol-induced elevation of IL-1β and TNF-α expression in the hippocampal-entorhinal cortex. A similar study showed that GLY treatment mitigated elevated hippocampal IL-1β and TNF-α expression to normalize memory deficits (Wang et al., 2015). However, the protocol used by Zou & Crews utilized GLY exposure to brain slices, rather than exposure via injections in live rats (2014). Additionally, the lack of significant differences in neuroinflammation between treatment groups may have been due to the stress caused by IP injections. Studies such as Ryabinin et al. (1999) have utilized IP injections to induce stress and stress-related immunoreactivity in the brain. Additional stress caused by the injections may have led to increased pro-inflammatory cytokine expression, so that no significant differences between treatment groups were found.

In addition, GLY has been found to inhibit TLR4 internalization, so that once the receptor has been stimulated it cannot move intracellularly into the microglia (Schröfelbauer et al.,2009). The GLY injections 20-30 min prior to ethanol administration may have assisted in the induction of the inflammatory cascade by maintaining TLR4 on the extracellular surface of microglia and maintaining microglial activation. If GLY is broken down quickly, it is possible that the neuroprotective effects of the drug are ineffective compared to the lasting membrane-stabilization effects. However, GLY has a half-life of 2.5-2.9h, and reaches maximum plasma levels within 30m of injections (Yamamura, et al., 1995), so it is unlikely that the membrane sustaining effect would last longer than its inhibition of HMGB1 and TLR4 binding. In addition,

data from this study contradicted this theory, as there were no significant elevations in microglial activation or pro-inflammatory cytokines in 5E-GLY rats (Figure 5B).

Lastly, the expected increase in inflammation in 5E-VEH rats was absent in the GLY experiment, suggesting the effect may be due too little information in 5E-VEH rats rather than the inability of GLY to mitigate neuroinflammation. The reason behind the absence of replication of increased neuroinflammation at PD10 in 5E-VEH rats as found by other studies such as Drew et al. (2015) and Topper et al. (2015), as well as the IBU experiment is unknown. It is possible the lack of ethanol-induced inflammation could have been due to a sex effect, as males and females have different neuroinflammatory profiles throughout development (Schwarz et al., 2012). As such, it is possible there would be sex-specific results of immune activation. Many studies, such as Wang et al. (2015), use only male rats, therefore, the ethanol-induced inflammatory profile of groups containing both males and females may be different.

Trace Fear Conditioning

Among treatment groups in both the IBU and GLY experiments, no significant differences were found during training, suggesting CS-US associative memory acquisition is intact following postnatal ethanol exposure. No treatment group differences were seen for context-elicited fear (Figures 6A and 7A). In the IBU experiment, the 5E-VEH rats did show diminished CS-evoked freezing behavior compared to SI-VEH and 5E-IBU rats (Figure 6B). There were no differences, however, between treatment groups in CS-evoked freezing in the GLY experiment (Figure 7B).

Jacobson et al. previously showed that children with FASD are impaired in eyeblink conditioning, a Pavlovian task that asked the children to associate a CS with an aversive US (2008). Similarly, in this study, cognition in rats was tested using TFC, a Pavlovian paradigm

that relies on a distributed forebrain circuit (Raybuck and Lattal, 2014) and engages the dorsal hippocampus when the TI is longer than approximately 5s (Guimarãis et al., 2011). This is the same length of TI at which significant deficits have been seen previously reported in juvenile and adult rats exposed to PD4-9 ethanol (DuPont et al., 2014, Hunt et al., 2009), indicating dorsal hippocampal dysfunction is primarily responsible for TFC deficits in 5E rats. Hippocampus dysfunction in TFC is thought to be related to deficits in LTP maintenance and memory consolidation (DuPont et al., 2014; Goodfellow et al., 2016). Specifically, evidence suggests that ethanol disrupts hippocampus-related cognition by disrupting the subunit composition and function of NMDA receptors, which are abundant in the hippocampus and involved in LTP induction and maintenance. The NMDAR consists of either NR2A or NR2B subunits, along with consistent NR1 subunits. The NR2 subunits play a role in determining the channel kinetics of NMDARs, leading to downstream signaling transduction cascades and LTP. The expression of NR2 subunits differ across development, with NR2B subunits normally being expressed early in development (Monyer et al., 1994). The NR2B subunits have a greater affinity for glutamate, as well as lower open channel probability and slower kinetics, and play a critical role in TFC (Gao et al., 2010). Reduced NR2B expression compared to NR2A is proposed to impede LTP and learning and memory. Goodfellow et al. (2016) found that postnatal ethanol exposure shifts the balance of NR2 subunits so that there is upregulation of NR2A and/or downregulation of NR2B, contributing to impaired synaptic plasticity and TFC memory deficits.

Abnormal levels of IL-1β during or following TFC could also contribute to disruptions in LTP maintenance and memory consolidation (Yirmiya, et al., 2002, Williamson et al., 2011). Early immune activation has been associated with increases in basal and experience-dependent inflammatory cytokine expression, specifically IL-1β (Wang et al., 2015). In addition to basal

increases in inflammation due to early life ethanol exposure, neuroinflammatory profile may be elevated in response to the experience of TFC. IL-1β also plays a role in LTP maintenance (Lynch, 2015; Williamson et al., 2011) and hippocampus-dependent context memory consolidation (Goshen et al., 2007). Elevated basal levels of IL-1β from early-life ethanol exposure, and/or increased experience-dependent levels following TFC could contribute to deficits in LTP maintenance and memory consolidation seen in 5E-VEH rats in the IBU experiment, similar to the effects in juvenile and adult rats from previous studies (DuPonet et al., 2014, Hung et al., 2009).

Just as inflammation-related gene expression did not vary between groups in the GLY experiment, TFC results showed no significant differences in CS-evoked freezing (Figure 7A-B). As seen in other studies, freezing during the acquisition and to the context did not differ between treatment groups (Figure 4A-B) (Hunt et al., 2009). However, the expected significant impairments tone-elicited conditioned fear in 5E-VEH rats compared to SI-VEH and 5E-GLY rats were not found (Figure 7B). As discussed above, the lack of differences in CS-evoked freezing between treatment groups may be due to increased stress from ethanol or IP injections, as early life stress can affect later life cognition by causing changes in the developing hippocampus (Lupien et al., 2009). In addition, proposed increased activation of microglia by GLY inhibition of the internalization of TLR4 (Schröfelbauer et al., 2009) may increase inflammation and damage the hippocampus. However, gene expression data from GLY treatment animals at PD10 contradicted this, as there were no significant differences in pro-inflammatory gene expression between treatment groups (Figure 5A-D).

This current study contributed to prior research findings that postnatal ethanol results in cognitive deficits through, we propose, increased neuroinflammation in early life. In the IBU

experiment, the 5E-VEH rats showed significant elevation in pro-inflammatory cytokines, which was significantly mitigated in the 5E-IBU rats. However, the elevations of immune-related gene expression were not replicated in the GLY experiment as no significant differences in pro-inflammatory cytokines were seen between treatment groups, possibly due to confounds in the research design. In addition, 5E-VEH rats in the IBU experiment had impaired TFC memory deficits, an effect that was not seen in 5E-VEH rats of the GLY experiment. The disruption in normal inflammatory profile and differences in synaptic plasticity are suggested to contribute to the deficits in memory consolidation. Hippocampus-dependent TFC test performance was significantly enhanced in 5E-IBU but not 5E-GLY rats. The lack of significant effects in the GLY experiment may be due to the stress response to the IP injections and/or the absence of a significant deficit in the 5E-VEH rats. The disruption in normal inflammatory profile and differences in synaptic plasticity are suggested to contribute to the deficits in memory consolidation. Although IBU is contraindicated for use during pregnancy, results from the IBU experiment suggest that anti-inflammatory drugs could be an effective treatment for improving cognition in FASD individuals. Although GLY did not have the same effect, other anti-inflammatory drugs may still work to normalize inflammatory profile and cognitive function.

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