



Short communication

Mitigation of postnatal ethanol-induced neuroinflammation ameliorates trace fear memory deficits in juvenile rats

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ABSTRACT

Impairments in behavior and cognition are common in individuals diagnosed with fetal alcohol spectrum disorders (FASD). In this study, FASD model rats were intragastrically intubated with ethanol (5 g/kg/day; 5E), sham-intubated (SI), or maintained as naïve controls (NC) over postnatal days (PD) 4–9. Ethanol exposure during this human third trimester-equivalent period induces persistent impairments in hippocampus-dependent learning and memory. The ability of ibuprofen (IBU), a non-steroidal anti-inflammatory drug, to diminish ethanol-induced neuroinflammation and rescue deficits in hippocampus-dependent trace fear conditioning (TFC) was investigated in 5E rats. Phosphate buffered saline vehicle (VEH) or IBU was injected 2 h following ethanol exposure over PD4-9, followed by quantification of inflammation-related genes in the dorsal hippocampus of PD10 rats. The 5E-VEH rats exhibited significant increases in *Il1b* and *Tnf*, but not *Itgam* or *Gfap*, relative to NC, SI-VEH, and 5E-IBU rats. In separate groups of PD31-33 rats, conditioned fear (freezing) was significantly reduced in 5E-VEH rats during TFC testing, but not acquisition, compared to SI-VEH and, critically, 5E-IBU rats. Results suggest neuroimmune activation in response to ethanol within the neonate hippocampus contributes to later-life cognitive dysfunction.

Fetal alcohol spectrum disorders (FASD) encompass a range of cognitive impairments, including aberrant executive function, learning, and memory [1]. The current study modeled FASD by administering ethanol to rat pups over postnatal days (PD) 4–9, a period comparable to the human third trimester [2]. Juvenile and adult FASD model rats are consistently impaired in multiple hippocampus-dependent learning and memory tasks (e.g., [3]), including trace fear conditioning (TFC). Intriguingly, the dorsal hippocampus is required for TFC but only when the trace interval—separating the offset and onset of a tone conditioned stimulus (CS) and electrical footshock unconditioned stimulus (US), respectively—exceeds 5–10 s [4]. This is the same trace interval at which significant impairments emerge in PD4-9 ethanol-exposed rats [5,6], signifying hippocampal dysfunction is largely responsible for observed TFC test performance deficits.

Ethanol promotes the release of high-mobility group box 1 protein (HMGB1), an endogenous ligand of toll-like receptor 4 (TLR4) which, in turn, stimulates the activation of microglia [7]. Downstream of TLR4, nuclear factor kappa B (NF κ B) and activator protein 1 (AP1) induce

expression of cyclooxygenase (COX)-2, an enzyme that facilitates pro-inflammatory cytokine and chemokine release [8]. The neonate brain may be especially vulnerable to neuroimmune activation due to low antioxidant levels [9] and potential interference with normal developmental processes, such as synaptic pruning [10]. Indeed, postnatal ethanol exposure increases microglia activation and pro-inflammatory cytokine release, most prominently interleukin [IL]-1 β and tumor necrosis factor (TNF)- α , in the hippocampus and other brain regions [11–13]. Crucially, recent findings in rats indicate neuroinflammation in response to various agents (e.g., lipopolysaccharide) during early postnatal life can impair later-life hippocampus-dependent learning and memory; while anti-inflammatory treatment can rescue such impairments [12,14]. It is currently unknown if ethanol-induced neuroinflammation contributes to hippocampus-dependent cognitive deficits in FASD rats. In the experiment below, ibuprofen (IBU), a blood-brain barrier permeable non-steroidal anti-inflammatory drug that inhibits COX-2, was administered concurrent with ethanol over PD4-9. Hippocampus-dependent TFC was assessed in separate rats over PD31-33. The

Abbreviations: 5E, 5 g/kg/day ethanol; SI, sham-intubated; NC, naïve control; PD, postnatal days; IBU, ibuprofen; VEH, PBS vehicle; TFC, trace fear conditioning; TI, trace interval; ISI, interstimulus interval; CPFE, context pre-exposure facilitation effect

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mitigation of postnatal ethanol-induced neuroinflammation by IBU was hypothesized to normalize hippocampal development and ameliorate TFC impairments in juvenile FASD rats.

Long-Evans rats were housed and bred at The Ohio State University in an AAALAC-accredited facility, as described in Goodfellow et al. [3]. All procedures were performed in accordance with The Ohio State University Institutional Animal Care and Use Committee (IACUC) and all necessary measures were taken to minimize pain and discomfort. On PD3, litters were culled to 10–12 pups and paw-marked for identification, retaining equal numbers of males and females when possible. From PD4 to PD9, binge-like doses of ethanol (5 g/kg/day; 5E) were administered in milk solution (22.67% vol/vol) via intragastric intubation (0.0278 ml/g). Milk-alone was administered 2 h later and, on PD4 only, after another 2 h. Sham-intubated (SI) rats underwent identical treatment but received no ethanol or milk. Naïve control (NC) rats were weighed daily. On PD4, just before the second intubation, blood from SI and 5E rats was taken via tail clip. Samples from 5E rats were centrifuged, plasma separated, and blood alcohol concentration (BAC) measured via a GL5 Analyzer (Analox Instruments, Lunenburg, MA). IBU (Sigma-Aldrich, St. Louis, MO) or phosphate buffered saline vehicle (VEH; pH 8.0) was injected (S.C.) daily, immediately after the second (milk-alone) intubation. The 5E-IBU rats received a 100 mg/kg IBU bolus on PD4, followed by 50 mg/kg daily through PD9 (see [15]). An equivalent volume of phosphate buffered saline was administered to the SI-VEH and 5E-VEH rats. A total of 61 rats were used, with no more than one rat per sex per treatment group per litter.

On PD10, 24 h after the last ethanol exposure, rats were anesthetized with isoflurane, decapitated, and the hippocampus excised. RNA was isolated with a Total RNA Tissue Kit (IBI, Peosta, IA) and DNaseI (Qiagen, Valenica, CA). cDNA was prepared with a First Strand cDNA Kit (OriGene, Rockville, MD) and diluted (1:5). PrimeTime[®] (*Gapdh* and *Il1b*) or custom primers for *Gfap* [16], *Tnf* [17], and *Itgam* [18] (IDT, Coralville, IA) and Bullseye EvaGreen Supermix (MidSci, St. Louis, MO) were added to the cDNA and assayed in triplicate, followed by melt curve, with a CFX96 C1000 Thermal Cycler (Bio-Rad, Richmond, CA). Data were normalized to a standard sample across plates and then analyzed by the comparative cycle threshold (2- $\Delta\Delta C_t$) method. Reaction efficiencies fell between 90 and 110%. Final results were normalized to *Gapdh*—which did not significantly vary between treatment groups—as a percentage of group NC expression [19]. See Table 1 for primer sequences.

Rats were weaned on PD21 and same-sex group housed. For training and testing, conditioned fear was inferred from freezing behavior, recorded and analyzed with FreezeScan (CleverSys, Inc., Reston, VA), based on experimenter-defined time bins. Beginning on PD31, rats were placed inside a standard operant conditioning chamber containing a shock generator and neon grid scrambler connected to the floor (Coulbourn Instruments, Holliston, MA). After a 180 \pm 30 s baseline, rats received 10 trials of a 15 s, 2.8 kHz, 80 dB tone conditioned stimulus (CS) and, 15 s later (the trace interval or TI), a 1 s, 0.8 mA footshock unconditioned stimulus (US), resulting in a 30 s interstimulus

interval (ISI; see Fig. 1A). The intertrial interval was 240 \pm 30 s. Context and CS-alone testing occurred 24 and 48 h later, respectively. Rats were returned to the conditioning context and freezing measured during a 120 s baseline period and the ensuing 600 s (context test). In a novel context, freezing was again measured during a 120 s baseline period and, over 10 trials (separated by 120 \pm 30 s), during the 15 s tone CS and 15 s TI.

Data were analyzed via single-factor analyses of variance (ANOVA) tests and, when appropriate, Fisher's LSD post-hoc tests (significance implies $p < 0.05$). PCR was performed in 34 rats: NC ($n = 8$), SI-VEH ($n = 9$), 5E-VEH ($n = 8$), and 5E-IBU ($n = 9$). TFC was conducted on 27 rats: SI-VEH ($n = 9$), 5E-VEH ($n = 8$), and 5E-IBU ($n = 10$). Sex did not significantly affect results—thus, the data below are based on treatment group differences only.

The mean (\pm SE) BAC in 5E-VEH and 5E-IBU rats was 350.5 \pm 6.9 and 359.7 \pm 6.3 mg/dl, respectively. A single-factor (Drug) ANOVA confirmed BAC levels did not significantly differ between groups ($p = 0.33$). Body weights were analyzed with single-factor (Treatment) ANOVAs on PD4 and PD10 (one day after ethanol termination) in NC, SI-VEH, 5E-VEH, and 5E-IBU rats. Significant differences were seen on PD10, $F(3, 58) = 3.02$, $p < 0.05$, but not PD4 ($p = 0.37$). Post-hoc testing indicates 5E-IBU rats weighed significantly less than NC and SI-VEH, but not 5E-VEH, rats on PD10. Body weights were again measured on PD30 in SI-VEH, 5E-VEH, and 5E-IBU rats. No significant treatment group differences were detected ($p = 0.63$), verifying body weight was not a confounding factor in TFC.

Gene expression data, normalized to the NC group mean, were analyzed via single-factor (Treatment) ANOVAs. Consistent with results in ethanol-exposed mice [11], postnatal treatment significantly altered the expression of *Il1b*, $F(3, 30) = 3.64$, $p < 0.05$, and *Tnf*, $F(3, 30) = 6.74$, $p < 0.01$. Both genes were significantly elevated in 5E-VEH rats relative to all other groups on PD10. The lack of treatment group differences between NC and SI-VEH rats signifies the elevated cytokine production in 5E-VEH rats is primarily driven by ethanol and not the intubation or injection procedures. Alternatively, Boschen et al. [19] observed elevated *Il1b* and *Tnf* expression on PD10 in both PD4-9 ethanol-exposed and SI rats, relative to NC rats. The discrepant SI results might be attributable to variations in the number of stress-inducing daily intubations between studies: two in the current study and Drew et al. [11], as opposed to three in Boschen et al. [19]. The PD10 results—i.e., during the ethanol withdrawal period—are also consistent with observed increases in pro-inflammatory (but not anti-inflammatory) cytokine production by Topper et al. [13]. Finally, Drew et al. [11] reported—comparable to the suppressive effects of IBU in Table 1—a reduction in *Il1b* and *Tnf* following administration of pioglitazone, an immunosuppressant drug that modulates glucose and lipid metabolism. Attenuating pro-inflammatory signaling in early postnatal life is proposed to limit the long-term detrimental effects of neuroinflammation in the developing brain [20].

Postnatal treatment had no significant effects on the astroglial marker, *Gfap* ($p = 0.25$) or, more surprisingly, the microglia marker,

Table 1

PCR primer sequences and PD10 hippocampal gene expression (mean \pm SE), normalized to *Gapdh* as a percentage of group NC expression. Postnatal treatment had no significant effect on *Itgam* or *Gfap* expression. It did alter *Il1b* and *Tnf* expression, with both genes significantly increased in 5E-VEH rats relative to all other groups (asterisks).

Gene	Sequences (5'–3')	NC	SI-VEH	5E-VEH	5E-IBU
<i>Gapdh</i>	F: AACCCATCACCATCTTCCAG R: CCAGTAGACTCCAGCATAC				
<i>Il1b</i>	F: GTGCTGTCTGACCCATGT R: TTGTCTGTCTGTCTCTCC	100.0 \pm 8.5	93.0 \pm 17.4	176.2 \pm 36.7*	96.0 \pm 8.9
<i>Gfap</i>	F: AGATCCGAGAAACAGCCTG R: CCTAATGACCTCGCCATCC	100 \pm 15.5	79.1 \pm 5.5	93.9 \pm 10.0	82.6 \pm 12.9
<i>Tnf</i>	F: GACCCACACTCAGATCATCTTCT R: TGCTACGACGTGGCTACG	100 \pm 10.9	116.3 \pm 22.0	245.4 \pm 46.8*	86.5 \pm 26.2
<i>Itgam</i>	F: CTGGGAGATGTGAATGGAG R: ACTGATGCTGGCTACTGATG	100 \pm 15.0	85.8 \pm 9.7	106.8 \pm 12.9	84.0 \pm 11.4

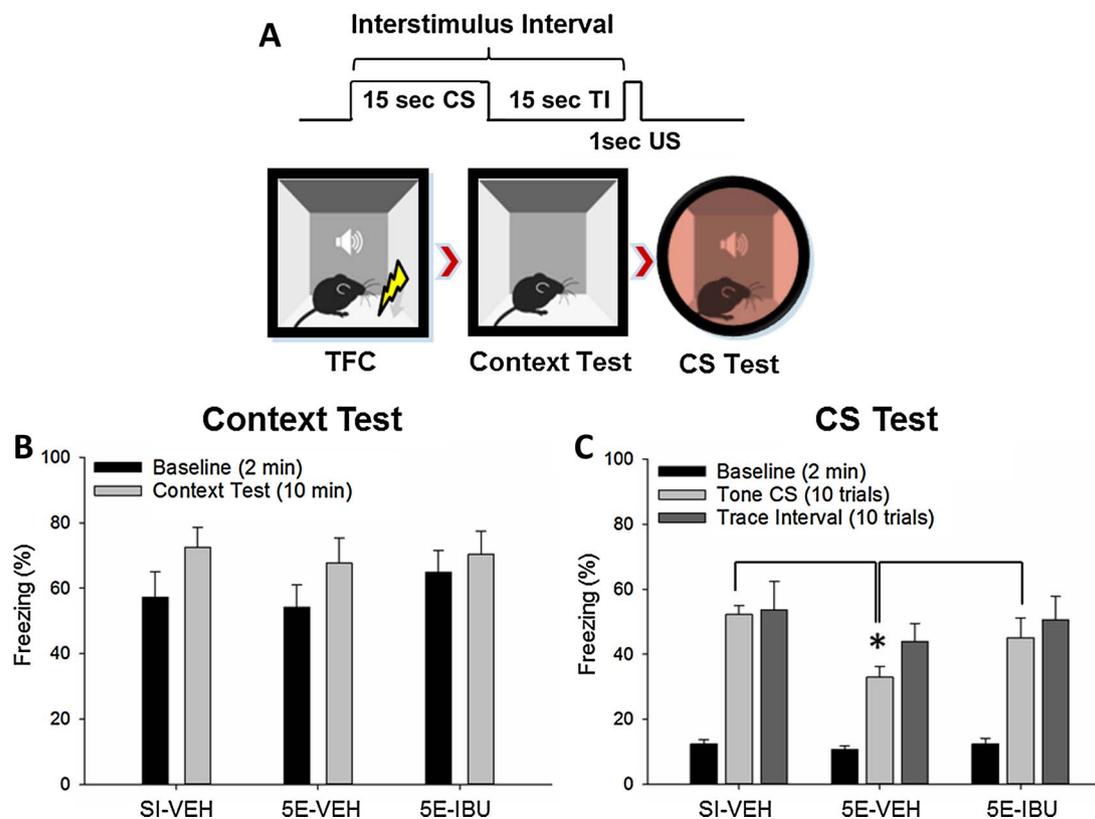


Fig. 1. Trace fear conditioning (TFC) paradigm. (A) Top: the 15 s tone conditioned stimulus (CS) is followed 15 s later (the trace interval or TI) by a 1 s footshock unconditioned stimulus (US), resulting in 30 s interstimulus interval (ISI). Bottom: TFC is followed by context and CS-alone retention tests 24 and 48 h later, respectively. (B) Mean (\pm SE) freezing did not differ between treatment groups during the 2 min baseline or averaged across the 10 min context test. (C) Mean (\pm SE) freezing did not differ between groups during the 2 min baseline in a novel context or during the 15 s TI, averaged across 10 trials. Freezing was significantly decreased in the 5E-VEH rats, relative to SI-VEH and 5E-IBU rats, during the 15 s tone CS, averaged across 10 trials (asterisk).

Itgam ($p = 0.79$). In the brain, microglia are a primary source of cytokine release—hence, we anticipated *Itgam* to increase commensurate with *Il1b* and *Tnf*. In line with current results, Boschen et al. [19] found no group differences in hippocampal *Itgam* expression between SI and ethanol-exposed rats on PD10. Immunohistochemical analyses, however, revealed decreased microglial cell territory in the hippocampus of ethanol-exposed rats relative to controls. Since ‘activated’ microglia are identified by shorter processes with increased cell body size as compared to quiescent microglia [21], it is possible, despite our current lack of *Itgam* group differences, that microglia may still have adopted an ‘activated’ phenotype in the 5E-VEH rats.

NC and SI-VEH rats had comparable gene expression patterns and, as previously documented, do not differ in TFC test performance [5,6]; thus NC rats did not undergo TFC. During training, no group differences were seen in freezing behavior during the tone CS ($p = 0.71$) or TI ($p = 0.78$) based on single-factor (Treatment) ANOVAs (data not shown). This result is consistent with our prior work in adult rats (e.g., [5]) and suggests all groups acquired the CS-US association at a similar rate. For the context test (Fig. 1B), no group differences were seen in freezing behavior during the initial 2 min baseline period ($p = 0.55$) or when averaged across the 10 min test ($p = 0.89$). While prior work has reported context fear deficits in FASD rats, impairments are typically seen only when task parameters are made especially challenging, such as the context pre-exposure facilitation effect (CPFE) task [22].

Freezing did not differ between treatment groups during the 2 min baseline period in a novel context, prior to the first CS-alone trial ($p = 0.69$). For the 10 CS-alone test trials, freezing did significantly differ between treatment groups when averaged across the 15 s CS, $F(2,24) = 4.68$, $p < 0.05$, but not when averaged across the 15 s TI ($p = 0.67$). As shown in Fig. 1C, CS-evoked freezing was significantly reduced in the 5E-VEH rats, relative to SI-VEH and 5E-IBU rats, based

on Fisher’s post-hoc analyses. The 5E-IBU and SI-VEH rats exhibited comparable levels of CS-evoked freezing (Fig. 1C), suggesting anti-inflammatory treatment provided some degree of neuroprotection in 5E rats, enhancing later-life TFC test performance.

In a related study, anti-oxidative treatment with curcumin rescued Morris water maze performance, another hippocampus-dependent task, in PD7-9 ethanol-exposed juvenile rats [23]. However, rats in this study were chronically treated from PD6 through the end of behavioral testing on PD28, complicating interpretation of the drug’s beneficial cognitive effects—i.e., it might be due to the drug’s acute anti-oxidative and/or anti-inflammatory properties during ethanol exposure or other later-acting effects, such as enhanced neurogenesis (e.g., [24]). To our knowledge, the present study is the first to directly link acute ethanol-induced neuroimmune activation in the neonate hippocampus to later-life cognitive impairment.

Mechanistically, anti-inflammatory treatment could stabilize various processes required for proper neurodevelopment, such as synaptic pruning [10]. It could also normalize putative changes in neuroimmune signaling across development—e.g., postnatal neuroinflammation has been shown to persistently increase IL-1 β expression in the hippocampus, up to PD71 [14]. In addition to its role in immune function, IL-1 β is critically involved in the ‘maintenance’ of hippocampus-dependent synaptic plasticity and long-term memory consolidation [25]. Inasmuch, aberrant release of IL-1 β in 5E-VEH rats during or following TFC could interfere or impede CS-US associative memory consolidation, resulting in a weaker long-term trace fear memory and less CS-evoked freezing at test (cf., [3]).

Collectively, results suggest ethanol-induced neuroinflammation contributes to the cognitive dysfunction seen in 5E rats across maturation. The data supports further research into the pro-cognitive benefits of other anti-inflammatories (e.g., COX-2 selective celecoxib or

anti-TNF α biologics) and antipyretics (e.g., acetaminophen). Such compounds may prove safer for the mother and fetus than IBU, which is contraindicated for use during human pregnancy. Additional research is also needed to investigate brain regions outside the hippocampus that show a similar inflammatory response to postnatal ethanol, such as the prefrontal cortex [11], and are known to play a key role in successful TFC [4]. Finally, it will be important to determine whether anti-inflammatory treatment after (rather than during) the third trimester-equivalent period confers cognitive benefits in order to more closely mimic potential therapeutic applications in humans.

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