Characterization of the Hippocampal Neuroimmune Response to Binge-Like Ethanol Consumption in the Drinking in the Dark Model

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Keywords

Alcohol abuse · Cytokine · Astrocyte activation · Neuroimmune · Hippocampus · Binge drinking

Abstract

Objectives: Alcohol dependence leads to dysregulation of the neuroimmune system, but the effects of excessive alcohol consumption on key players of the neuroimmune response after episodic binge drinking in nondependence has not been readily assessed. These studies seek to determine how the neuroimmune system within the hippocampus responds to binge-like consumption prior to dependence or evidence of brain damage. **Methods:** C57BL/6J mice underwent the drinking in the dark (DID) paradigm to recapitulate binge consumption. Immunohistochemical techniques were employed to determine the effects of ethanol on cytokine and astrocyte responses within the hippocampus. Astrocyte activation was also assessed using qRT-PCR. **Results:** Our results indicated that binge-like ethanol consumption resulted in a 3.6-fold increase in the proinflammatory cytokine interleukin (IL)-1 β immunoreactivity in various regions of the hippocampus. The opposite effect was seen in the anti-inflammatory cytokine IL-10. Binge-like consumption re-

sulted in a 67% decrease in IL-10 immunoreactivity but had no effect on IL-4 or IL-6 compared with the water-drinking control group. Moreover, astrocyte activation occurred following ethanol exposure as GFAP immunoreactivity was increased over 120% in mice that experienced 3 cycles of ethanol binges. PCR analyses indicated that the mRNA increased by almost 4-fold after one cycle of DID, but this effect did not persist in abstinence. **Conclusions:** Altogether, these findings suggest that binge-like ethanol drinking prior to dependence causes dysregulation to the neuroimmune system. This altered neuroimmune state may have an impact on behavior but could also result in a heightened neuroimmune response that is exacerbated from further ethanol exposure or other immune-modulating events.

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Introduction

Alcohol use disorders (AUDs) are a prevalent epidemiological issue both in the USA and abroad [1, 2]. Problems associated with alcohol misuse affect various societal constructs, including the economy, health care systems, and interpersonal relationships [3, 4]. The majority of these

problems occur as a result of binge drinking, making it particularly problematic [5]. Binge drinking is defined as consuming enough alcohol to reach a blood ethanol concentration (BEC) of 80 mg/dL in a short period of time [6]. It is well established that repetitive binge drinking can induce neuroplastic events that alter the physiologic responses to alcohol and are thought to be one of the underlying mechanisms leading to the development of an AUD [7–10]. Although alcohol abuse can elicit maladaptations in various biologic systems, this study focuses on alcohol-induced neuroimmune responses as the neuroimmune system has the capacity to contribute to both alcohol-induced neurodegeneration and alcohol's pharmacodynamic properties [11, 12]. For example, increases in the proinflammatory cytokine tumor necrosis factor- α after repeated ethanol consumption has been proposed as an underlying factor in alcohol-induced brain damage in the peri-entorhinal cortex [13, 14]. Moreover, alcohol's effects on the proinflammatory cytokine interleukin-1 β (IL-1 β) in the amygdala alters neurotransmission and has behavioral implications [15–17]. Elucidating the impact of alcohol on the neuroimmune system during various periods of alcohol abuse is critical to determining how it contributes to the cycle of alcohol misuse and the development of AUDs.

Preclinical studies examining alcohol-induced neuroimmune responses have predominantly focused on models of alcohol dependence. Alcohol-dependence studies suggest that the neuroimmune response can exacerbate neuronal cell death through both hyper- and hyporeactivity [13, 18–21]. Although alcohol dependence and binge-like consumption share many neurobiological underpinnings, determining the neurochemical maladaptations that occur specifically after binge drinking in nondependent individuals merits separate attention as the effects of dependence and binge drinking can be distinct [22]. Koob [9] has published multiple reports on the differences between the neurobiological contributions of positive reinforcement during initial alcohol abuse (e.g., binge drinking) and the negative reinforcement associated with withdrawal and physical dependence from alcoholism. In fact, most individuals who binge drink do so sporadically and will never be diagnosed with or reach the criteria for alcohol dependence [23, 24]. When one also considers the “quasi” social acceptability of bingeing [25, 26], it becomes clear why understanding the neurobiological consequences of binge drinking in nondependent states is crucial. Discerning the neurologic effects of alcohol in nondependent individuals is especially relevant to the neuroimmune system because neuroimmune re-

sponses can be primed by an initial insult leading to an exacerbated response in the presence of secondary immunomodulators [27–29]. These secondary immunomodulators could be more ethanol use in dependence or an infectious pathogen. For example, in a model of alcohol dependence, the Crews lab was able to show that ethanol experience exacerbates the effects of a peripheral infection on the neuroimmune system and neurodegeneration [29, 30]. Because binge drinking often occurs as multiple events and overlaps with other pathological problems, it is necessary to elucidate the neuroimmune responses across the AUD spectrum, including more acute binge exposures [20]. This study specifically focuses on the contribution of binge drinking to neuroimmune responses independent of alcohol dependence to highlight differences between ethanol's effects on the neuronal environment during alcohol abuse compared with dependence.

To distinguish between the neuroimmune effects specifically associated with binge drinking and those in dependence, these studies utilize the drinking in the dark (DID) paradigm. The DID model is uniquely suited for an examination of the neuroimmune profile as a result of binge-like consumption prior to alcohol dependence [31, 32]. This study extends our previous findings on alcohol's effects on amygdala cytokines by determining the impact of alcohol on cytokines and astrocytes in the hippocampus [15, 33]. The hippocampus has been chosen as the region of interest because both clinical and preclinical studies have repeatedly shown it to be susceptible to the detrimental effects of excessive alcohol consumption [34–37]. This study examines the neuroimmune response to binge-like alcohol consumption using immunohistochemical (IHC) analysis of the anti-inflammatory cytokines IL-10 and IL-4, and the pro-inflammatory cytokines IL-1 β and IL-6, in the dentate gyrus (DG), cornu ammonis 1 (CA1), and cornu ammonis 2/3 (CA2/3) hippocampal subfields. Astrocyte activation was assessed using both IHC procedures and qRT-PCR.

Materials and Methods

Animals

Male C57BL/6J mice (6–8 weeks; $n = 121$) from Jackson Laboratories (Bar Harbor, ME, USA) were individually housed in a reversed 12:12-h light:dark cycle vivarium maintained at 22°C. During the experiments, animals had ad libitum access to water and chow unless otherwise indicated. The mice used to assess cytokines were given a Teklad Diet® 2920X (Harlan Laboratories Inc., Indianapolis, IN, USA), but the mice used to assess astrocyte activation received Prolab® RMH 3000 (Purina LabDiet®, St. Louis, MO, USA) due to a change in the standard diet provided by our

Table 1. DID binge data for immunohistochemistry

Study	Treatment	Consumption		BEC, mg/dL	
		1 week	3 weeks	1 week	3 weeks
IL-10, IL-4, and IL-1 β : Teklad Diet [®] 2920X	Ethanol, g/kg	4.2 $\pm$ 0.4 (<i>n</i> = 10) ^a	4.7 $\pm$ 0.4 (<i>n</i> = 9)	54.1 $\pm$ 13.3 ^a	91.3 $\pm$ 16.3
	Sucrose, g/kg	4.4 $\pm$ 0.2 (<i>n</i> = 10)	4.5 $\pm$ 0.6 (<i>n</i> = 9)	–	–
	Water, mL/kg	–	42.8 $\pm$ 4.8 (<i>n</i> = 8)	–	–
IL-6: Prolab [®] RMH 3000	Ethanol, g/kg	4.9 $\pm$ 0.2 (<i>n</i> = 6)	5.1 $\pm$ 0.5 (<i>n</i> = 8)	117.1 $\pm$ 13.7	113.2 $\pm$ 20.9
	Sucrose, g/kg	5.6 $\pm$ 0.4 (<i>n</i> = 9)	5.9 $\pm$ 0.4 (<i>n</i> = 7)	–	–
	Water, mL/kg	–	53.36 $\pm$ 8.3 (<i>n</i> = 10)	–	–
GFAP: Prolab [®] RMH 3000	Ethanol, g/kg	4.7 $\pm$ 0.4 (<i>n</i> = 6)	4.8 $\pm$ 0.4 (<i>n</i> = 6)	102.5 $\pm$ 10.3	100.4 $\pm$ 13.8
	Sucrose, g/kg	4.6 $\pm$ 0.5 (<i>n</i> = 6)	4.6 $\pm$ 0.5 (<i>n</i> = 8)	–	–
	Water, mL/kg	–	45.6 $\pm$ 5.9 (<i>n</i> = 9)	–	–

BEC, blood ethanol concentration.

^a The Teklad diet significantly reduced ethanol consumption and BECs during week 1.

university [38]. All animals were given at least a week to acclimatize to the environment before experimentation. The procedures used in this study were all approved by both High Point University and the University of North Carolina's Institutional Animal Care and Use Committees following the Guidelines for the Care and Use of Laboratory Animals.

DID Procedures

Nondependent binge drinking was modeled using a 4-day DID paradigm as previously described [31, 39]. Briefly, home cage water bottles were removed 3 h into the dark cycle, and the mice received access to a single bottle of 20% (v/v) ethanol solution or a control solution of either 3% (w/v) sucrose or tap water. For the first 3 days of each cycle mice had 2 h of access, but on the fourth day consumption (g/kg) was measured over 4 h. The mice used in IHC experiments went through the DID procedure for 1 or 3 cycles with each 4-day DID cycle separated by 3 days [32]. Tail blood samples (approx. 60 μ L) were taken from tail nicks immediately following the final DID procedure to determine BEC. Control animals were also subjected to the tail nick procedure to mitigate the effects of the stress associated with the procedure on neuroimmune measurements. Serum was obtained through centrifugation and run in duplicates using the AM1 Alcohol Analyzer (Analox, London, UK) for BEC determination (mg/dL). The mice were euthanized by transcardial perfusion following the final DID, similar to the procedure of previous studies [15, 33]. Briefly, an overdose of a mixture of ketamine and xylazine (66.67 and 6.67 mg/mL, respectively; 0.9% saline) was administered before perfusion with 0.1 M phosphate-buffered saline (PBS; pH = 7.4) and 4% paraformaldehyde in PBS (pH = 7.4). Brains post-fixed in paraformaldehyde were sliced using a vibratome (Leica VT1000S; Wetzlar, Germany) to obtain 40- μ m coronal sections.

For PCR studies, however, the mice were subjected to a single cycle of DID and euthanized immediately, 1 day, or 10 days after ethanol consumption. The brains were removed following rapid decapitation and the hippocampus was dissected out and flash frozen on dry ice. To avoid changes in mRNA associated with re-

Table 2. Methods for antigen detection

Antibody	Concentration	Incubation time, h
Rb anti-IL-1 β (R&D Systems)	1:1,000	48
Rb anti-IL-10 (R&D Systems)	1:1,000	48
Gt anti-IL-4 (Lifespan BioSciences)	1:2,000	72
Rb anti-IL-6 (Rockland)	1:500	48
Rb anti-GFAP (Sigma Aldrich)	1:500	72

straint stress, blood was only taken for animals euthanized immediately by collecting trunk blood [40].

IHC and Immunoreactivity Quantification

Three cohorts of animals were used for IHC procedures targeting the cytokines IL-1 β , IL-4, IL-6, and IL-10 as well as glial fibrillary acidic protein (GFAP; see Table 1 for group details). GFAP was used as a marker of morphological change and activation in astrocytes. IHC techniques were similar to previous studies [15, 33, 41]. Briefly, cryoprotectant was removed in a series of rinses in 0.1 M of PBS before endogenous peroxidases were quenched with 0.6% H₂O₂. Sections used for IL-10 IHC analysis then underwent an additional step of antigen retrieval in citrate buffer at 65 °C for 1 h. After serial PBS washes, all nonspecific binding was blocked (PBS/0.1% triton-X/3% rabbit or goat serum; Vector Laboratories, Burlingame, CA, USA). Sections were then incubated in primary antibody (see Table 2 for additional details). After the primary antibody was removed by serial washes in block, the sections were incubated in the primary appropriate biotinylated secondary antibody (1:2,000) for 1 h, followed by signal amplification with an avidin-biotin-peroxidase complex (ABC elite kit, Vector Labs) for 1 h. Finally, the chromagen, 3,3'-diaminobenzidine tetrahydrochloride (Polysciences; Warrington, PA, USA) was used for detec-

Table 3. DID binge data for PCR

Study	Treatment	Consumption, g/kg	BEC, mg/dL
PCR:	Ethanol + 0 days ($n = 10$)	5.1 ± 0.3	102.0 ± 12.5
Prolab®	Ethanol + 1 day ($n = 10$)	4.8 ± 0.4	–
RMH 3000	Ethanol + 10 days ($n = 12$)	5.0 ± 0.4	–
	Water ($n = 8$)	37.3 ± 3.1	–

BEC, blood ethanol concentration.

tion of the antigen of interest. Mounted sections were coverslipped with SHUR/Mount™ (Triangle Biomedical Sciences, Durham, NC, USA).

Either a Zeiss Axio Zoom V16 microscope (Zeiss, Jena, Germany) or Nikon Eclipse 80i with an Olympus DP73 digital camera was used to image the sections at 100× magnification. For all images, experimental bias was avoided by coding the slide images prior to quantification. The subregions of the hippocampus (DG, CA1, and CA2/3) were individually traced on sections between Bregma -1.06 and -2.54 mm [42]. Approximately 6–8 sections were analyzed per animal. Animals that did not have at least 6 analyzable sections within the area of interest were excluded from the study. Immunoreactivity was measured with Zen Pro 2012 (Zeiss) or CellSens (Olympus) using experimenter-determined optical density thresholds. Immunoreactivity is expressed as the percentage area (immunoreactive positive area/total area of region of interest).

qRT-PCR

RNA was extracted in a fourth cohort of animals ($n = 40$; see Table 3 for details) from hippocampal tissue homogenized in 200 μ L of Trizol® (Invitrogen Corporation, Carlsbad, CA, USA) using a PowerGen tissue homogenizer, identical to previous studies [43, 44]. The total RNA was quantified using a Qubit® 3.0 Fluorometer, and 1 μ g of RNA was reversed transcribed to cDNA. Standard Taq-Man® assays were purchased from ThermoFisher Scientific for the measurement of GFAP (Mm01253033_m1) and an endogenous control PPIA (Mm02342430_g1). Each sample was run in triplicate using the Quant Studio 6 Flex (Invitrogen). Measurements were normalized to the water groups and are expressed as the fold change, similar to previous reports [15, 45].

Statistical Analysis

Prism version 7.02 (GraphPad Software Inc., La Jolla, CA, USA) was used to analyze and graph all data reported herein. ANOVAs were used to assess ethanol consumption, immunoreactivity, and fold change of mRNA. Post hoc tests were only utilized if a main effect of treatment was observed. Dunnett's post hoc tests were used for all post-analyses with the water group serving as the control group except BECs, where Bonferroni tests were used because there was no true control group. All data are reported as the mean $\pm$ standard error of the mean and considered significant with two-tailed $p < 0.05$.

Results

Ethanol Consumption and BECs Impacted by Diet

A two-way ANOVA (cohort $\times$ number of cycles) of ethanol consumption ($\mu = 4.98 \pm 0.31$) indicated a main effect of cohort ($F_{(2, 40)} = 3.31$, $p = 0.047$) but not number of cycles ($F_{(1, 40)} = 0.29$, $p = 0.59$) or an interaction ($F_{(2, 40)} = 0.52$, $p = 0.59$; Table 1). Importantly, the average BEC ($\mu = 98.1 \pm 11.9$ mg/dL) was above the threshold of binge-like consumption. A two-way ANOVA indicated that the BECs were significantly different between the groups associated with a main effect of cohort group ($F_{(2, 40)} = 4.36$, $p = 0.02$). However, no interaction ($F_{(2, 40)} = 1.15$, $p = 0.32$) or effect of the number of cycles ($F_{(1, 40)} = 0.64$, $p = 0.43$) were found for the BECs. Post hoc analyses indicated that cohort 1 animals receiving the Teklad diet had lower consumption and BECs compared with the other animal cohorts during the first week of DID exposure, but this effect did not continue after 3 weeks of exposure. This finding reinforces previous studies that suggest changes in standard protocols like diet can impact behavioral outcomes [38, 46].

Due to differences in the number of DID cycle exposures, animals used in the PCR study could not be included in a two-way ANOVA and were analyzed separately (Table 3). The average BEC ($\mu = 102.0 \pm 12.5$ mg/dL) was also above the threshold of binge-like consumption in this cohort. No differences were observed in consumption among the groups according to a one-way ANOVA ($F_{(2, 29)} = 0.31$, $p = 0.73$).

Binge-Like Ethanol Consumption Promotes a Proinflammatory Cytokine Environment

Photomicrographs indicate that there is basal expression of IL-1 β , IL-6, IL-4, and IL-10 in the hippocampus, as shown in the control group for each cytokine assessed. One-way ANOVAs revealed that binge-like ethanol drinking resulted in increased IL-1 β immunoreactivity

Fig. 1. Representative photomicrographs at $\times 100$ magnification of IL-1 β within the hippocampus of mice exposed to water (a), 3 sucrose DID cycles (b), 1 ethanol DID cycle (c), and 3 ethanol DID cycles (d). **Inset** A cell expressing IL-1 β at $\times 400$ magnification. Our analyses indicated that 3 cycles of ethanol (EtOH) exposure resulted in an increase in IL-1 β immunoreactivity within the DG (h) and CA1 (i), but not the CA2/3 (j). Moreover, no statistically significant differences were observed between the water and sucrose (Suc) drinking groups in any subregion of the hippocampus (e–g). **c** Scale bar, 10 μ m. Each photomicrograph has traces outlining the hippocampal fissure. All data are presented as the mean $\pm$ SEM. * $p < 0.05$ compared to the water group.

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1

2

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compared with the water-drinking control group in the DG ($F_{(2, 24)} = 4.04, p = 0.03$) and CA1 ($F_{(2, 24)} = 4.05, p = 0.03$), but not the CA2/3 ($F_{(2, 24)} = 0.09, p = 0.91$) subregions of the hippocampus (Fig. 1). Post hoc Dunnett's multiple comparison tests indicated a significant increase of IL-1 β immunoreactivity in mice that experienced 3 cycles of DID in both the DG and the CA1 compared with water-drinking control mice ($p < 0.05$). However, the DG appeared to be more susceptible to ethanol as even one cycle of the DID paradigm resulted in significant upregulation of IL-1 β . Sucrose consumption did not alter IL-1 β immunoreactivity in any region assessed (DG: [$F_{(2, 24)} = 1.34, p = 0.28$]; CA1: [$F_{(2, 24)} = 1.45, p = 0.25$]; CA2/3: [$F_{(2, 24)} = 0.10, p = 0.91$]). Moreover, no changes in IL-6 immunoreactivity were associated with either ethanol (DG: [$F_{(2, 20)} = 0.21, p = 0.81$]; CA1: [$F_{(2, 20)} = 0.24, p = 0.78$]; CA2/3: [$F_{(2, 20)} = 0.48, p = 0.63$]) or sucrose (DG: [$F_{(2, 23)} = 0.16, p = 0.85$]; CA1: [$F_{(2, 23)} = 0.75, p = 0.48$]; CA2/3: [$F_{(2, 23)} = 0.07, p = 0.93$]) consumption in the hippocampus (Fig. 2).

IL-10 immunoreactivity was only depressed in the CA1 ($F_{(2, 24)} = 4.13, p = 0.03$) of animals that experienced binge-like ethanol with no significant effects in the DG ($F_{(2, 24)} = 0.02, p = 0.98$) or CA2/3 ($F_{(2, 24)} = 0.07, p = 0.93$) subregions compared to the water group. Post hoc Dunnett's multiple comparison tests indicated a significant decrease in IL-10 immunoreactivity in the CA1 of mice that experienced 3 cycles of DID, but not mice that experienced 1 cycle, compared to the water-drinking control mice ($p < 0.05$). Importantly, no difference was indicated by one-way ANOVAs between animals drinking water versus sucrose in any of the hippocampal subregions (DG: [$F_{(2, 24)} = 0.55, p = 0.58$]; CA1: [$F_{(2, 24)} = 0.03, p = 0.97$]; CA2/3: [$F_{(2, 24)} = 0.27, p = 0.76$]), suggesting that decreased IL-10 concentrations were specific to ethanol consumption and no other rewarding solutions (Fig. 3). Moreover, no changes in IL-4 immunoreactivity were associated with either ethanol (DG: [$F_{(2, 24)} = 0.03, p = 0.97$]; CA1: [$F_{(2, 24)} = 2.43, p = 0.11$]; CA2/3: [$F_{(2, 24)} = 0.79, p = 0.47$]) or sucrose (DG: [$F_{(2, 24)} = 0.03, p = 0.97$]; CA1: [$F_{(2, 24)} = 1.61, p = 0.22$]; CA2/3: [$F_{(2, 24)} = 1.17, p = 0.33$]) consumption in the hippocampus (Fig. 4).

Fig. 2. Representative photomicrographs of IL-6 within the hippocampus of mice exposed to water (a), 3 sucrose DID cycles (b), 1 ethanol DID cycle (c), and 3 ethanol DID cycles (d). *Inset* A cell expressing IL-6 at $\times 400$ magnification. Analyses indicated that neither ethanol (EtOH; h–j) nor sucrose (Suc; e–g) had any effect on IL-6 expression within the regions of the hippocampus compared with the water control. **c** Scale bar, 10 μ m. Each photomicrograph has traces outlining the hippocampal fissure.

Astrocyte Activation Associated with Repeated Binge-Like Consumption

Astrocytes were assessed using GFAP immunoreactivity. Although GFAP is a marker for all astrocytes, upregulation of GFAP immunoreactivity is a common tool to examine astrocyte activation [41, 47]. One-way ANOVAs indicated that ethanol significantly increased GFAP immunoreactivity in the DG ($F_{(2, 18)} = 15.77, p = 0.0001$) and CA1 ($F_{(2, 18)} = 6.79, p = 0.006$) but not the CA2/3 region ($F_{(2, 18)} = 2.99, p = 0.08$). Post hoc Dunnett's multiple comparison tests indicated a significant increase in GFAP immunoreactivity in the DG and CA1 of mice that experienced 3 cycles of DID but not 1 cycle compared to the water-drinking control mice ($p < 0.05$; Fig. 5). No effect of sucrose consumption was seen in any subregions of the hippocampus (DG [$F_{(2, 20)} = 1.68, p = 0.21$]; CA1 [$F_{(2, 20)} = 2.08, p = 0.15$]; CA2/3 [$F_{(2, 20)} = 2.19, p = 0.14$]).

Increases in GFAP mRNA during Intoxication from Binge-Like Consumption

To determine if changes in GFAP persist during abstinence, mRNA was measured immediately, 1 day, and 10 days after ethanol consumption. A one-way ANOVA indicated a significant impact of treatment ($F_{(3, 36)} = 4.67, p = 0.007$) on GFAP mRNA expression. Dunnett's post hoc multiple comparison test revealed that immediately after ethanol consumption there was a significant increase in GFAP mRNA compared with the water controls. However, no changes were observed in either of the abstinent groups (1 or 10 days) compared with the water-only group (Fig. 6).

Fig. 3. Representative photomicrographs of IL-10 within the hippocampus of mice exposed to water (a), 3 sucrose DID cycles (b), 1 ethanol DID cycle (c), and 3 ethanol DID cycles (d). *Inset* A cluster of cells expressing IL-10 at $\times 400$ magnification. Analyses indicated that 3 cycles of ethanol (EtOH) reduced IL-10 within the CA1 (i), but this effect of ethanol was not present in either the DG (h) or the CA2/3 (j). Moreover, no statically significant differences were observed between the water and sucrose (Suc) drinking groups in any subregion of the hippocampus (e–g). **c** Scale bar, 10 μ m. Each photomicrograph has traces outlining the hippocampal fissure. All data are presented as the mean $\pm$ SEM. * $p < 0.05$ compared to the water group.

Fig. 4. Representative photomicrographs of IL-4 within the hippocampus of mice exposed to water (a), 3 sucrose DID cycles (b), 1 ethanol DID cycle (c), and 3 ethanol DID cycles (d). *Inset* A cell expressing IL-4 at $\times 400$ magnification. Analyses indicated that neither ethanol (EtOH; h–j) nor sucrose (Suc; e–g) had any effect on IL-4 expression within the regions of the hippocampus. **c** Scale bar, 10 μ m. Each photomicrograph has traces outlining the hippocampal fissure.

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3

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Discussion

The reciprocal and reinforcing relationship between alcohol misuse and the various actors of the neuroimmune response remains of distinct interest in developing therapies to curb alcohol abuse. This report identified key neuroimmune responses in the hippocampus following binge-like consumption in a nondependent model. More specifically, these data indicate that mice with DID ethanol experience had: (a) an induction of IL-1 β , (b) a decrease in IL-10, and (c) an initiation of astrocyte activation in the hippocampus. Importantly, these signs of a neuroimmune response were seen prior to evidence of either dependence-like phenotypes or evidence of neurodegeneration [15, 31, 48].

The alcohol-induced cytokine milieu results described herein align with our previous studies examining the neuroimmune response to binge-like consumption in the DID model. Our previous reports indicate that there was a similar depression of IL-10 and an increase in IL-1 β in the basolateral amygdala [15, 33]. However, this is the first report to specifically examine these cytokines within the hippocampus in this AUD model. It is important to denote that the effect of alcohol on cytokines were not universal, as no changes were observed in either IL-4 or IL-6 immunoreactivity, suggesting that the ethanol effects are somewhat selective. Moreover, regional differences were observed within the hippocampus depending on the cytokine of interest. These results are similar to those seen in models of dependence as well as fetal alcohol spectrum disorders, which have shown that the cytokines in the hippocampus are changed following chronic consumption, or in models with BECs well over 300 mg/dL [13, 19, 37, 49, 50]. However, the altered cytokine milieu observed herein reflects maladaptations that occur just above the legal limit of 80 mg/dL. Considering IL-6 and IL-4 were not impacted during this nondependent binge-

like model, IL-1 β and IL-10 may represent more vulnerable systems to binge drinking. For example, in a preclinical study of alcohol dependence increases in IL-4 and IL-6 in the central nervous system were observed following alcohol withdrawal [51]. The response observed herein after such acute exposure is troubling, but future studies should determine whether these changes in cytokine expression persist.

The functional implications of this cytokine dysregulation were not specifically examined in the current study, but our previous reports indicate that there is no neurodegeneration following the DID model, suggesting that this initial induction of cytokines is not necessarily engaged in the “classical” immune response associated with cell damage [15, 48, 52]. It is much more likely given the lack of neurodegeneration that these cytokines are involved with synaptic transmission and would lead to changes in behavior [53, 54]. Various reports have shown that cytokine dysregulation in the hippocampus can cause memory impairments [55], including IL-1 β [56, 57] and IL-10 [58]. Given that lapses in memory are common following binge drinking [59, 60], it is perceivable that the dysregulation in cytokines observed herein play a role in these “gray-outs.” Preclinical studies suggest that these lapses in memory occur even in the absence of neurodegeneration [61, 62]. The cytokine-specific changes observed herein may mediate many of the complex behaviors caused by acute alcohol exposure and deserve further exploration.

Astrocyte activation has previously been shown to be induced by acute binge alcohol exposure in both in vitro and in vivo studies [63–65]. In fact, Mulligan et al. [66] found a cluster of astrocyte genes that were upregulated following just 1 day of binge-like alcohol consumption in a modified version of the DID paradigm. These results agree with those previous studies showing an increase in GFAP immunoreactivity induced by binge-like consumption levels. These studies only observed astrocyte activation after 3 cycles of DID using immunohistochemistry while others have reported activation after just a day of exposure [63, 66]. These dissonant findings could be due to differences in the sensitivity of assays when examining gene changes in a microarray compared to proteins using IHC analysis [66]. In fact, our results showed binge-like alcohol consumption caused an increase in GFAP mRNA following just 1 week of DID, suggesting that qRT-PCR may be a more sensitive tool than IHC techniques in assessing ethanol’s impact on astrocytes. Moreover, the Nixon lab has shown that higher BEC thresholds (>300 mg/dL) can

Fig. 5. Representative photomicrographs of astrocytes within the dentate gyrus of mice exposed to water (**a**), 3 sucrose DID cycles (**b**), 1 ethanol DID cycle (**c**), and 3 ethanol DID cycles (**d**) indicate an effect of ethanol on GFAP immunoreactivity. **Inset** GFAP+ cells at $\times 400$ magnification. Our analyses showed that 3 cycles of ethanol (EtOH) resulted in an increase in GFAP immunoreactivity within the DG (**h**) and CA1 (**i**), but not the CA2/3 (**j**). Moreover, no statically significant differences were observed between the water and sucrose (Suc) drinking group in any subregion of the hippocampus (**e–g**). **c** Scale bar, 10 μ m. Each photomicrograph has traces outlining the hippocampal fissure. All data are presented as the mean $\pm$ SEM. * $p < 0.05$ compared to the water group.

Fig. 6. One cycle of DID exposure caused increased GFAP mRNA expression in the hippocampus in the ethanol (EtOH) group compared with the water controls. This effect was only seen during alcohol intoxication and did not persist days after abstinence. All data are presented as the mean $\pm$ SEM. * $p < 0.05$ compared to the water group.

cause changes in GFAP protein revealed by immunohistochemistry after a single day of exposure [63]. It is important to denote that increased GFAP immunoreactivity could be indicative of an increased astrocyte number or morphological changes in astrocytes, but both are characteristic of astrogliosis [47]. Regardless of the temporal and alcohol exposure differences used in these models, the data converge to suggest that astrocytes within the hippocampus are particularly vulnerable to alcohol misuse. The role of astrocytes in alcohol consumption and related behaviors is an emerging field [67], and the current characterization of the astrocytic response after binge-like consumption is a step in determining the reciprocal relationship between astrocyte activation and alcohol abuse.

Together these findings indicate, even in the absence of neurodegeneration or dependence phenotypes, that binge alcohol consumption has a significant impact on neuroimmune functions in the hippocampus. One of the limitations of the current study is that it was not feasible to characterize other cytokines or the microglial response. Future studies should continue to examine cytokines and determine whether microglia are also dysregulated following binge-like consumption in the DID paradigm. A neuroimmune response in this model's more moderate, albeit problematic, level of binge-like alcohol consump-

tion has serious implications for binge drinkers. Binge alcohol consumption often occurs in conjunction with other neuroimmune-modulating events, such as peripheral infections or traumatic brain injury [68–70]. These data suggest that more care should be taken to consider how alcohol misuse may confound neuroimmune function during such episodes, especially in light of the fact that binge drinking often goes unreported in clinical visits [71]. The social acceptability and prevalence of binge drinking makes considering how alcohol affects the neuroimmune system in nondependent individuals of critical importance. Binge-drinking effects on the neuroimmune system, as observed herein, have the potential to exacerbate neurodegeneration, but may also contribute to changes in behavioral comorbidities like memory deficits.

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Statement of Ethics

The procedures used in this study were all approved by both High Point University and the University of North Carolina's Institutional Animal Care and Use Committees following the Guidelines for the Care and Use of Laboratory Animals.

Disclosure Statement

The authors declare no conflicts of interest.

Author Contributions

Alex Marshall, Todd Thiele, and Donald Lysle conceived and designed the experiments. Alex Marshall, Isabella Grifasi, and Scott McIntosh performed the experiments. Alex Marshall, Isabella Grifasi, and Rhiannon Thomas analyzed the data. Alex Marshall and Todd Thiele contributed reagents/materials/analysis tools. The report was written by all of the authors listed.

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