Original Research *Highlight Article*

Conditioning the neuroimmune response to ethanol using taste and environmental cues in adolescent and adult rats

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Impact statement

A combined odor and taste cue was paired with a binge-like ethanol exposure (4 g/kg intraperitoneal) using a single-trial learning paradigm. Re-exposure to the CS alone was sufficient to evoke a conditioned Interleukin (IL)-6 elevation in the amygdala in adolescents, an effect that was not observed in young adults. This demonstrates a particular sensitivity of adolescents to alcohol-associated cues and neuroimmune learning, whereas prior work indicated that adults require multiple pairings of ethanol to the CS in order to achieve a conditioned amygdala IL-6 response. While the role of immune conditioning has been studied in other drugs of abuse, these findings highlight a previously unknown aspect of alcohol-related learning. Given the emergent importance of the neuroimmune system in alcohol abuse, these findings may be important for understanding cue-induced reinstatement of alcohol intake among problem drinkers.

Abstract

Our work in adult Sprague-Dawley rats has shown elevation of the cytokine Interleukin (IL)-6 in the hippocampus and amygdala following acute and repeated binge-like doses of ethanol during intoxication. Previously, we have shown that in adults, the central IL-6 response to a sub-threshold dose of ethanol was sensitized by repeated pairings of ethanol as an unconditioned stimulus (US) with an odor conditioned stimulus (CS).In the present studies, acute ethanol exposure (4 g/kg intraperitoneal) was paired with a combined odor and taste cue using a single trial learning procedure, after which rats were tested for conditioned effects of the CS on neuroimmune gene expression. We found that IL-6 was significantly elevated in the amygdala based on exposure to the CS after just one CS–US pairing in young adolescent rats (age P32–40), an effect that was more modest in young adults (P72–80). These data indicate that, despite a normal disposition toward a blunted neuroimmune response to ethanol, adolescents were more sensitive than adults to forming learned associations between ethanol's neuroimmune effects and conditioned stimuli. Given the emergent role of the immune system in alcoholism, such as regulating ethanol intake, these ethanol-induced conditioned effects on cytokine levels may contribute to our understanding of the unique attributes that make adolescence a time period of vulnerability in the development of later alcohol abuse behaviors.

Keywords: Ethanol, conditioning, neuroimmune, cytokine, IL-6, hippocampus, amygdala, adolescent, development

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Introduction

Pre-clinical and clinical studies indicate that alcohol and other drugs of abuse modulate the immune system across the central nervous system (CNS) and in the periphery. Individuals diagnosed with alcohol use disorders exhibit microglial activation and chemokine expression in the brain,[1] as well as increased circulating levels of cytokines such as tumor necrosis factor alpha (TNF-α), Interleukin-(IL)-1, and IL-6.[2] In animal models, there is evidence that chronic ethanol administration alters the immune response to a later challenge with ethanol or lipopolysaccharide (LPS).[3,4]

Acute ethanol also affects neuroimmune signaling. An acute ethanol challenge (100 mg/dL) elevated the levels of TNFα, IL-1, and IL-6 in the hypothalamus 48 h after exposure,[5] and withdrawal from an acute binge-like ethanol

administration (6.0 g/kg intragastric) dose-dependently altered microglial cytokine expression *in vivo*.[6] We have shown that an acute dose of ethanol resulting in blood ethanol concentrations of ~200 mg/dL or higher increased IL-6 gene expression widely across the CNS, with effects especially evident in the hippocampus (HPC) 3 h after ethanol administration at the time of intoxication.[7] Follow-up work replicated this effect and extended it to other brain regions (i.e. amygdala), repeated ethanol exposures, and other rat strains,[8–10] suggesting that ethanol-induced IL-6 expression is highly reproducible and much more widespread in the CNS than originally thought.

Several exciting studies indicate that perturbations in neuroimmune function, such as alterations in cytokine signaling, may influence ethanol intake. Subjects withdrawing from chronic alcohol exhibited increased levels of plasma cytokines (which were positively associated with ethanol craving) and heightened LPS plasma levels.[11] In rats, a single LPS administration resulted in a long-lasting increase in voluntary ethanol consumption.[12] Experimental manipulation of immune signaling has also yielded some findings that further support the involvement of cytokines in ethanol consumption. The deletion of chemokines and chemokine receptors from the mouse brain altered ethanol consumption,[13] and the deletion of IL-6 specifically reduced ethanol intake.[14] Thus, experiences which increase IL-6 expression may contribute to heightened ethanol intake, reinforcement and potentially cue-induced reinstatement among alcohol users.

The adaptive properties of the immune system have been harnessed by Pavlovian paradigms,[15] in which gustatory stimulation has been largely chosen as the conditioned stimulus (CS), probably due to the natural evolutionary connection between ingestion and illness.[16] For instance, in classic studies with humans and rodents, cyclosporine A endowed gustatory CSs with conditioned, immunosuppressive qualities.[17,18] As more connections are found between ethanol and the immune system, it is important to analyze the mutual influence between neuroimmune signaling and ethanol-induced learning. Yet, to our knowledge, very few studies have assessed if immunomodulation induced by drugs of abuse can also be subject to conditioning.[19] Studies have reported heroin-induced conditioned immunosuppression, which has been shown to depend on central cytokine signaling.[20,21] Data from our laboratory have also suggested an ethanol-induced, conditioned neuroimmune response. Specifically, rats received four pairings of a lemon odor CS with an ethanol (2.0 g/kg, intraperitoneal [i.p.]) Unconditioned stimulus (US) on an every-other-day schedule. When re-exposure to the lemon CS occurred in combination with a subthreshold dose of ethanol (0.5 g/kg), enhanced IL-6 mRNA expression was observed in the HPC and amygdala (AMG).[22] While this conditioned effect only emerged when accompanied by a small dose of ethanol, it is important to note that the 0.5 g/kg dose of ethanol alone did not significantly evoke cytokine expression. Despite some of these caveats, our findings can be considered a valuable proof-of-concept model and worthy of further exploration for the identification of situations that favor the acquisition or expression of ethanol-mediated conditioned neuroimmune responses, which may have important implications for cue-induced reinstatement of ethanol intake.

In the present study, we investigated whether an odor–taste cue would support the expression of neuroimmune conditioned responses in adolescent and adult rats, after just one pairing between the odor–taste CS and the effects of a relatively high, binge-like, ethanol dose (4.0 g/kg i.p.). We expected that cues associated with ethanol would elicit a conditioned increase in IL-6 and IκBα two neuroimmune genes known to increase during ethanol intoxication.[7,8,10] As an added benefit, this procedure allowed us to examine behavioral conditioning (i.e. avoidance of the taste CS) in tandem with the potential neuroimmune adaptations. We chose to examine areas previously shown to be sensitive to conditioning (HPC, AMG, see Gano *et al*.[22]), as well as those (nucleus of the solitary tract [NTS], parabrachial nucleus [PbN]) implicated in the circuitry underlying conditioned taste aversion (CTA) responses,[23] predicting that these sites would be most likely to display conditioned changes in neuroimmune gene expression.

Another important goal of the current study was to assess potential age-related differences in the conditioned neuroimmune response to ethanol exposure. Typically, the initiation of alcohol consumption occurs during adolescence.[24,25] Hence, it is relevant to examine the first contact with ethanol and how environmental factors may facilitate escalation of alcohol intake patterns. A plethora of studies have indicated that the effects of ethanol are dramatically different in adolescents compared to adults. For example, whereas adolescent and juvenile rats exhibit conditioned preference for ethanol,[26,27] this effect is not observed in their adult counterparts. While these results demonstrate that adolescents are more sensitive to the conditioned rewarding effects of ethanol, other data suggest that they might also be less sensitive to the conditioned aversive properties of ethanol. Using the CTA paradigm, studies have shown that adolescent rats require higher doses of ethanol and more CS-ethanol pairings in order to evince a conditioned aversion to an ethanol US.[28,29] Moreover, it is important to consider that adolescents have been found to exhibit altered sensitivity to ethanol intoxication-related increases in central cytokine expression.[9] When administered doses of ethanol that result in significant elevations in central cytokines across multiple brain regions in adults, adolescents exhibited attenuated levels of these immune factors. Given age-related differences in the conditioned effects of ethanol, as well as in response to acute ethanol-induced alterations in brain cytokines, it was hypothesized that adolescents would also exhibit altered sensitivity to conditioned ethanol-induced immune responses. To the extent that adolescents may demonstrate a unique sensitivity to the conditioned effects of ethanol on the immune system, such an effect may contribute to an overall pattern of responses that may put adolescents at increased risk for initiation and escalation of alcohol intake.[30]

General methods

Subjects

These experiments utilized adolescent (postnatal day [P] 32 through P40) and adult (P72 through P80) male Sprague-Dawley rats reared in our Binghamton University, AAALAC-accredited facility colony (for details, see Vore *et al.*[4]). At weaning (P21), the rats were pair-housed with a non-littermate with *ad libitum* access to food and water, unless specifically noted otherwise. The vivarium was kept at 22 ± 1°C with a 12:12 light cycle (lights on at 07:00). Cage mates were assigned to the same experimental conditions and, to avoid litter effects,[31] no more than 1–2 animals from the same litter were assigned to a given experimental group. The procedures were approved by the Institutional Animal Care and Use Committee at Binghamton University and were in accordance with Public Health Service policy.

Drug administration

Ethanol (20% v/v) for i.p. administration was diluted fresh daily using sterile saline (0.9%, Sigma-Aldrich, St. Louis, MO). This saline was used for all vehicle injections.

CTA procedures

During training and testing, the rats were placed in a context with a distinct combination of taste and odor cues, which signaled (or not) the post-absorptive effects of ethanol. Training and test procedures and the characteristics of the distinctive context, which are common across experiments, are detailed in the next sections. Experiment 1 and 2 exhibit some procedural differences, which will be specified later (for experimental timelines, see Figure 1).

Ad libitum water intake per cage was measured for 24 h prior to training day (Experimental Day 1). The night before training (Experimental Day 2), each cage was provided 50% of the water consumed the previous day. On training day (Experimental Day 3), at 09:00, the rats were transferred to a room adjacent to the colony room, individually placed into novel cages containing lavender-scented bedding (Kaytee, Chilton, WI), and given 30 min access to 5% sucrose solution (w/v in tap water). The sucrose solution was then removed, and the animals were administered vehicle or 4.0 g/kg ethanol, a dose that reliably induces IL-6 gene expression.[7,32] Rats remained in the lavender context for three additional hours (for a total of 3.5 h of exposure) and were subsequently returned to the home cage in the colony room. All rats then had at least 48 h of undisturbed rest before testing. On the night before test day (test day was on Experimental Day 6 for Experiment 1, Experimental Day 7 for Experiment 2) water access was again restricted to 50% of the water consumed the previous day. On test day, the rats were given 30 min access to 5% sucrose solution in the lavender-scented context, after which they were either injected with 0.5 g/kg i.p. ethanol, injected with equivolumetric vehicle, or remained un-injected. Thereafter, rats remained in the context for an additional 3 h until the time of tissue harvesting.

Sucrose intake was expressed and analyzed as mL consumed per kilogram of body weight (mL/kg). Failure to drink on training day (i.e. <1 mL consumed, as in Saalfield and Spear[33]) resulted in exclusion from the study. This resulted in the exclusion of seven rats (two

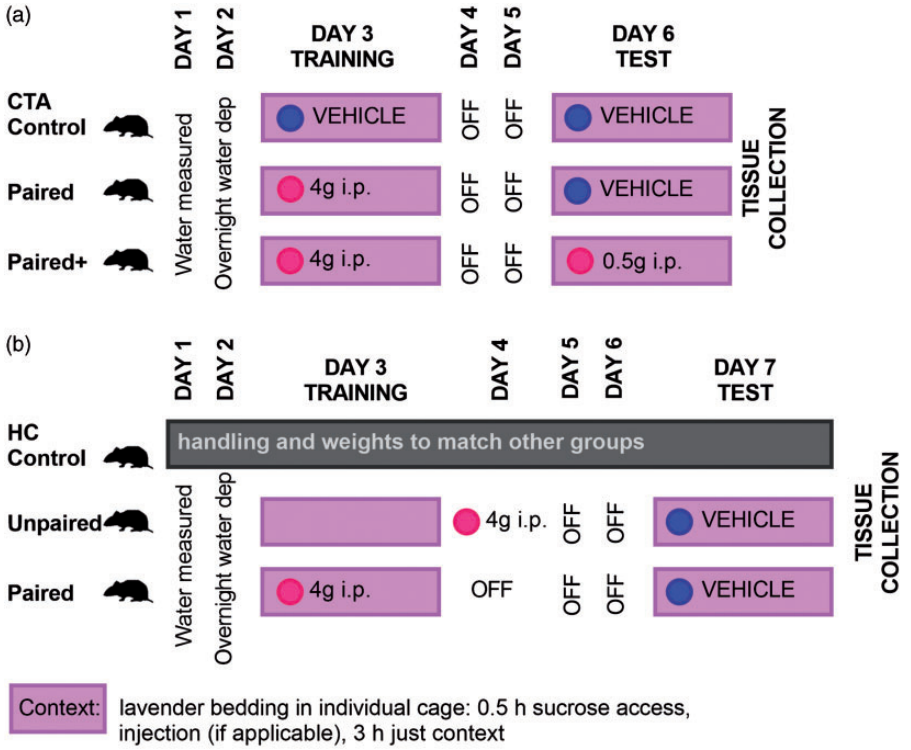

Figure 1. This figure depicts experimental timelines for (a) Experiment 1 and (b) Experiment 2. (A color version of this figure is available in the online journal.)

rats from the CTA Control group, two rats from the Paired group, and three rats from the Paired+ group) from Experiment 1, whereas in Experiment 2, three Adult/Paired animals and three Adolescent/Unpaired animals were removed.

Tissue collection

Trunk blood and brain tissue were harvested using rapid, unanesthetized decapitation. Trunk blood was collected in EDTA-coated Vacutainers (BD Vacutainers, VWR Cat. No. VT6450, Radnor, PA), and plasma was separated via refrigerated centrifugation and stored at –20°C until time of use. Brains were extracted and stored at –80°C until subsequent tissue dissection using a core-sampling punch technique (as described in Gano *et al.*[10]) for the extraction of the HPC, AMG, NTS, and PbN.

Plasma hormones

Plasma concentrations of CORT were determined using a commercially available EIA kit (Cat. No. ADI-901-907, Enzo Life Sciences, Farmingdale, NY, USA) in accordance with the manufacturers' instructions, with the exception that samples were heat-inactivated by immersion in 75°C water for 1 h in order to denature endogenous corticosteroid-binding globulin.[34,35] The assay had a sensitivity of 27.0 pg/mL, an inter-assay variability of 6.0%, and an intra-assay variability of 1.9%.

Real time RT-PCR

Gene expression in the brain was analyzed using real time reverse transcription polymerase chain reaction (RT-PCR), as described elsewhere.[9] Briefly, tissue was placed into a 2.0 mL Eppendorf tube containing 500 μL Trizol RNA reagent and a 5 mm stainless steel bead, and then homogenized using a Qiagen Tissue Lyser II. Following homogenization, RNA was extracted using Qiagen's RNeasy mini kit (catalog #74106), according to manufacturer's instructions. Synthesis and storage of cDNA included a DNAse treatment step, and probed cDNA amplification was performed and captured in real time using the CFX384 Real-Time PCR Detection System (Bio-Rad, #185-5485). All PCR data were adjusted using the $2^{-\Delta\Delta C(T)}$ method[36] and are shown relative to a stable reference gene and expressed as percent of control. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the housekeeping reference gene in Experiment 1. However, as experimental manipulations in Experiment 2 significantly affected GAPDH expression, cyclophilinA was instead used as the control housekeeping gene for Experiment 2.

Experiment 1 specific methods

This experiment assessed, in adult male rats, if the cytokine response to a single ethanol challenge (4.0 g/kg i.p.) would endow a context, featuring distinctive odor and taste cues, with conditioned properties. To do this, a three-group design was used: CTA Control, Paired, and Paired+ (n = 12/group; see Figure 1(a) for experimental outline). During the training phase, both the Paired and Paired+ groups received an i.p. ethanol injection (4 g/kg) in the lavender context. The control group (hereinafter referred to as CTA control group) was composed of animals administered vehicle during conditioning. On test day, the 30-min sucrose intake period was followed by i.p. administration of saline (CTA Control and Paired group) or 0.5 g/kg i.p. ethanol (Paired+ group). The aim of this procedure was—similar to our prior published work[22]—to promote the expression of conditioned neuroimmune responses by recreating some of the procedural and interoceptive cues experienced during training. After injection, rats remained in the context for an additional 3 h until tissue collection. There was no untreated (i.e. home cage) control group and all gene expression data were adjusted to the CTA control group.

Experiment 2 specific methods

This study further explored ethanol-induced conditioned immune responses and investigated potential age differences in these conditioned responses. Thus, this experiment employed a 2 (Age: Adolescent vs. Adult) × 3 (Group: home cage control [HCC]; Unpaired, Paired) factorial design (n = 8–12/group; see Figure 1(b)), Because rats from the Paired group in Experiment 1 exhibited a slight increase in IκBα expression in the HPC, as HCC group (i.e. completely untreated) was added to the design of Experiment 2 in order to better understand the influence of procedural manipulations on gene expression (see Figure 1(c)). During training, Paired rats received an injection of ethanol (4.0 g/kg i.p.) immediately after the sucrose solution was removed, and rats remained in the lavender-scented context for 3 h. In contrast, animals in the Unpaired group were exposed to the lavender context and to the sucrose solution but received ethanol 24 h later while in the home cage. Because of the inclusion of this group, we added an additional day of "rest" between training and testing so that all rats were given at least 48 h to allow any withdrawal (i.e. "hangover") symptoms to fully subside.

On the test day, intake of the sucrose solution was first measured in Unpaired and Paired animals. Following this intake test, and to keep consistency with the procedures of Experiment 1, rats were administered vehicle (saline equivolumetric to 0.5 g/kg i.p. ethanol) and remained in the context for 3 h until the point of tissue collection. Rats in the HCC group experienced handling and weighing parallel to that of the other groups, but received no water deprivation, context exposure, or injections (i.e. tissue collection was done at baseline conditions). Consequently, HCC rats were used as the ultimate controls for gene expression, with each age group adjusted to its own individual HCC and analyzed separately.

Data analysis

All data were analyzed using the appropriate ANOVAs as specified in the results section. *Post hoc* analyses were performed using Fisher's LSD test, with the alpha criterion set to $p < 0.05$ for all comparisons. Group sizes were determined based on power analyses performed on a

comparable data set in which conditioned effects of ethanol were previously reported.[22] Specifically, power analysis of IL-6 gene expression data in a context conditioning procedure with n = 6–8 per group and $\alpha = 0.05$, power was 0.67 with an effect size (ηp2) of 0.20, suggesting use of 8–10 rats per group would provide adequate power. Thus, studies presented here were performed with starting n = 12 per group to account for occasional attrition due, for instance, to inadequate sampling of the CS (sucrose) on the day of training as per our a priori exclusion criteria described above.

Results

Experiment 1

CTA. The Paired and Paired+ groups were similarly trained and tested for taste aversion; thus, these groups were combined into an "Ethanol" group for the analysis of CTA scores (Figure 2(a)). Sucrose intake at test was analyzed using a 2 (Group: Control vs. Ethanol) × 2 (Day of assessment: Training vs. Test) repeated measures ANOVA. The analysis yielded a significant interaction ($F_{1, 27} = 28.36$, $p < 0.0001$), with *post hoc* tests revealing similar levels of intake between training and testing in Control rats, but not in Ethanol rats. The latter exhibited, according to the *post hoc* tests, a significant decrease in sucrose acceptance when compared to the levels exhibited at training, and also when compared to their Control counterparts. The *post hoc* tests also revealed a slight, yet significant, difference in consumption of sucrose at training, between the Control and the Ethanol groups (21.90 mL/kg and 16.82 mL/kg, respectively, $p = 0.048$). A one-way ANOVA indicated that CORT scores were statistically similar across Control (Mean: 10.52 ± SEM: 3.17), Paired (18.24 ± 4.12), and Paired+ (8.02 ± 1.91) rats.

Gene expression. One-way ANOVAs were performed on all gene expression data. In the HPC, there was a significant main effect of Group on IκBα expression ($F_{2, 24} = 3.43$, $p < 0.05$; Figure 2(b)), with Paired animals exhibiting significantly increased gene expression compared to the Control and Paired+ groups (both $p < 0.05$). There were no significant effects of Group on gene expression in the NTS, PbN, or AMG (Table 1). In the HPC, NTS, and PbN, there were trends for conditioned TNFα suppression ($F_{2, 21} = 3.22$, $p = 0.060$; $F_{2, 23} = 2.72$, $p = 0.087$; and $F_{2, 27} = 3.04$, $p = 0.06$, respectively). In the amygdala, there was a conditioning effect on c-Fos ($F_{2, 27} = 3.25$, $p < 0.05$) with the Paired+

Table 1. Gene expression (% of control) results for Experiment 1, shown as mean ± SEM.

	CTA Control	Paired	Paired+
Hippocampus			
IL-6	113.65±12.38	128.07±41.85	95.53±29.37
IκBα	**106.15±12.41a**	**159.13±24.30b**	**98.86±13.05a**
IL-1β	116.65±18.29	191.58±26.45	138.62±14.23
TNFα	103.07±9.32	109.67±14.05	80.326.07
cFOS	109.62±14.11	120.17±9.79	100.29±10.76
Amygdala			
IL-6	106±12.54	125.47±26.33	120.10±11.72
IκBα	102.03±6.81	114.05±9.28	110.64±13.46
IL-1β	107.15±12.89	99.04±12.53	96.47±10.60
TNFα	104.02±9.37	103.52±7.30	83.43±3.82
cFOS	**110.97±20.52a**	**80.61±9.59ab**	**60.90±7.10b**
Nucleus of the solitary tract			
IL-6	108.79±15.27	189.75±49.09	172.74±35.57
IκBα	107.87±15.73	239.16±65.79	173.32±38.92
IL-1β	113.41±18.14	106.26±17.85	117.60±26.69
TNFα	103.23±7.82	85.20±23.43	70.73±9.81
cFOS	107.71±13.36	123.03±29.00	99.24±12.66
Parabrachial nucleus			
IL-6	109.43±13.79	124.70±17.97	115.33±18.05
IκBα	102.15±6.43	123.99±11.77	113.03±19.03
IL-1β	111.24±15.18	143.46±16.38	126.19±23.61
TNFα	103.27±8.00	107.09±13.13	77.66±8.63
cFOS	101.13±4.90	101.48±7.93	91.34±5.82

Note: Target genes displaying significant main effects are **bolded**, with individual group differences determined by *post hoc* analyses indicated using letters (i.e. groups sharing a letter were statistically similar, whereas significantly different groups are identified by unique letters). Note that the Paired+ group showed a highly consistent, yet marginally significant (all *p*-values < 0.10) reduction in TNFα in the hippocampus, NTS, and PbN, which are indicated by underlining. This tendency for reduced TNFα expression during ethanol intoxication is consistent with our prior published studies, an effect that reverses during acute ethanol withdrawal.[7,8]
TNFα: tumor necrosis factor alpha; IL-6: Interleukin-6.

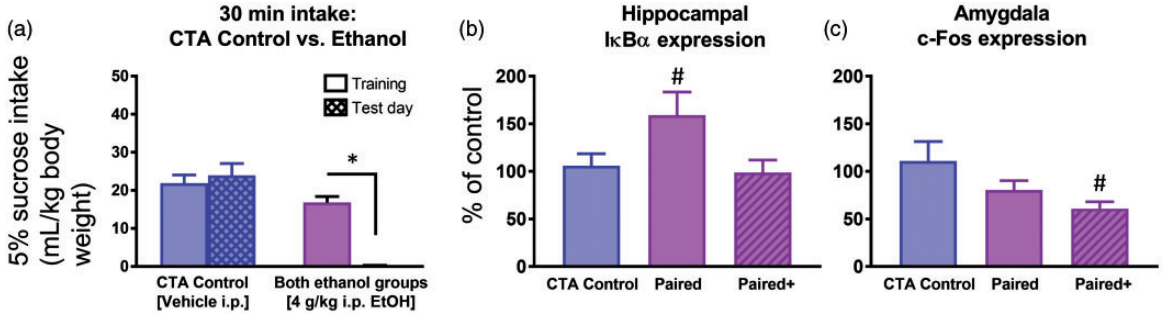

Figure 2. This figure depicts the key outcomes of Experiment 1. (a) The outcome of the conditioned taste aversion (CTA) test, as measured by mL sucrose consumption per kg of rat weight during the 30-min test day trial as compared to sucrose solution consumed during training. An asterisk (*) denotes a significant difference between training and test day (significant interaction; all *post hoc* tests $p < 0.05$). The ethanol groups (Paired and Paired+) are shown as a combined ethanol group as, until the conclusion of CTA testing, these groups were identically manipulated. (b) Hippocampal gene expression of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα), shown as % control (CTA Control group). (c) Reduced expression of the neuronal activation marker c-Fos in the amygdala in the Paired+ group. An octothorpe (#) indicates significant difference from all other groups (significant main effect of Group; all *post hoc* tests $p < 0.05$). (A color version of this figure is available in the online journal.)

group showing significant suppression compared to CTA Controls (Figure 2(c)).

Experiment 2

CTA. When assessing sucrose intake, a mixed 2 (Age: Adolescent vs. Adult) × 2 (Group: Unpaired vs. Paired) × 2 (Day of assessment: Training vs. Test) ANOVA was used. Results of this analysis revealed significantly greater overall sucrose intake in adolescents than in adults (significant main effect of Age: $F_{1,34} = 27.44$, $p < 0.001$). This baseline difference in the level of sucrose acceptance prompted separate analyses for each age. Both ANOVAs revealed significant Group × Day interactions ($F_{1,\ 15} = 21.06$, $p < 0.001$ and $F_{1,\ 19} = 12.98$, $p < 0.01$; adults and adolescents, respectively). The *post hoc* tests revealed, at both ages, that the Unpaired group consumed equal amounts of sucrose on Training and Test days. In contrast, the Paired group significantly decreased their consumption on Test day compared to Training, therefore demonstrating a robust and successful CTA (Figure 3(a) and (b)). The ANOVA for plasma CORT concentrations revealed no significant main effects of Age or Group, nor a significant Age × Group interaction (Adults: HCC 1.77 ± 0.79, Unpaired 8.95 ± 2.27, Paired 9.10 ± 4.25; Adolescents: HCC 4.86 ± 1.68, Unpaired 7.95 ± 2.80, Paired 8.39 ± 2.27).

Gene expression. At each age, gene expression data for each group were adjusted to the age-specific HCC control and analyzed separately using one-way ANOVAs (Group: HCC vs. Unpaired vs. Paired).

Adults. In the HPC, there was a significant main effect of Group on TNFα expression ($F_{2,\ 21} = 4.29$, $p < 0.05$), with reduced gene expression in the Unpaired group as compared to the HCC ($p < 0.01$). There was also a significant main effect of Group on c-Fos expression in the AMG ($F_{2,\ 20} = 16.31$, $p < 0.0001$); c-Fos in Unpaired and Paired groups was elevated significantly ($p < 0.001$) above HCC. In the NTS, there was a trend for a main effect of Group on IκBα expression, with higher magnitude levels observed in Unpaired and Paired rats ($F_{2,\ 21} = 2.76$, $p = 0.086$). In the PbN, there was a significant main effect of Group on c-Fos expression ($F_{2,\ 21} = 8.18$, $p < 0.01$), with the Paired group showing significantly greater neural activation than either HCC ($p < 0.001$) or Unpaired controls ($p < 0.05$). In this structure, there was an additional trend for an effect of Group on TNFα expression ($F_{2,\ 21} = 2.58$, $p = 0.099$; all outcomes for Adults can be found in Table 2).

Adolescents. In the HPC, there was a trend for an effect of Group on IL-6 expression ($F_{2,\ 26} = 3.11$, $p = 0.062$; Figure 4(a)) and IκBα expression ($F_{2,\ 26} = 2.53$, $p = 0.099$; Figure 4(a)), with highest magnitude levels observed in the Paired group. In the AMG, the Adolescents showed a significant main effect of Group ($F_{2,\ 25} = 4.02$, $p < 0.05$; Figure 4(c)) on IL-6 expression, with the Paired group showing significantly greater levels of the transcript than the Unpaired group ($p < 0.05$), but no effect on IκBα (Figure 4(d)). In the AMG, there was a main effect of Group on c-Fos, with the Unpaired group showing significant c-FOS elevation, compared to HCC rats, as well as the Paired ($p < 0.05$) group (Table 2). In the NTS the ANOVA yielded no significant main effect nor significant interaction on IL-6 expression levels (Figure 4(e)), yet there was a significant main effect of Group on IκBα expression ($F_{2,\ 25} = 6.68$, $p < 0.01$; Figure 4(f)), with a significant elevation in the Paired group as compared to HCC controls ($p < 0.01$). There were no significant main effects nor significant interactions in the PbN (for this and all other non-significant effects see Table 2).

Discussion

Previous work[10] has shown that ethanol-induced neuroimmune responses can come under the control of environmental cues. Specifically, four every-other-day pairings of 2.0 g/kg i.p. ethanol to a lemon-scented context resulted in a sensitized IL-6 response to a 0.5 g/kg i.p. ethanol priming dose when given in the same environment. This was seen in both the HPC and AMG of adult rats. In the present study, we sought to extend these findings to a single-trial conditioning paradigm that, in addition to the odor cues, employed a procedure akin to a CTA paradigm and applied it to adolescent and adult rats to describe the ontogeny of this phenomenon. Rats were given a pairing of sucrose

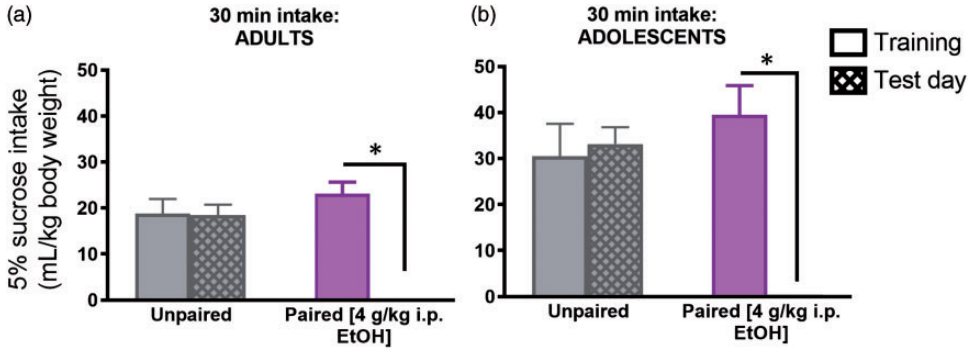

Figure 3. This figure depicts the CTA outcomes of Experiment 2 for (a) adults and (b) adolescents. The outcome of the conditioned taste aversion (CTA) test is shown in mL sucrose consumption per kg of rat weight during the 30-min test day trial as compared to sucrose solution consumed during training. An asterisk (*) denotes a significant difference between training and test day for the Paired group (significant interaction; all *post hoc* tests $p < 0.05$). (A color version of this figure is available in the online journal.)

Table 2. Gene expression (% of control) results for Experiment 2, shown as mean ± SEM.

	Adults			Adolescents		
	HCC	Unpaired	Paired	HCC	Unpaired	Paired
Hippocampus						
IL-6	109.08±17.44	132.08 ± 25.68	113.07±17.59	*104.87±11.05*	*118.61±13.59*	*141.53±18.89*
IκBα	102.83±3.96	124.97±8.89	109.90±11.23	*101.62±6.83*	*102.58±6.41*	*122.70±8.59*
IL-1β	105.84±9.37	94.93±8.81	85.95±10.57	103.82±11.14	100.47±18.59	77.84±9.11
TNFα	**106.49±12.87a**	**69.76±6.09b**	**88.97±9.12ab**	103.24±11.08	104.16±7.93	111.74±11.44
cFOS	104.41±9.50	115.93±9.02	126.15±17.70	102.08±8.04	118.44±9.75	102.34±11.87
Amygdala						
IL-6	110.20±16.45	162.29±18.74	133.75±17.44	**104.90±13.15ab**	**88.69±12.53a**	**137.20±12.20b**
IκBα	100.16±2.15	129.76±11.48	119.15±11.68	100.92±5.20	101.19±6.66	111.85±6.54
IL-1β	103.04±9.39	123.77±10.48	102.16±10.27	103.46±9.60	114.77±8.17	102.26±9.41
TNFα	111.62±10.60	97.47±5.76	92.18±5.28	101.33±6.22	95.06±11.25	106.51±6.58
cFOS	**104.74±12.19a**	**240.30±26.58b**	**210.01±12.26b**	**110.87±20.34a**	**184.46±22.91b**	**128.78±16.31a**
Nucleus of the solitary tract						
IL-6	102.60±12.74	89.34±7.27	96.02±6.37	103.39±10.66	95.03±10.51	129.05±13.05
IκBα	*96.61±9.66*	*130.22±7.52*	*117.11±14.38*	**101.74±7.64a**	**130.96±13.58ab**	**156.62±8.49b**
IL-1β	122.01±25.76	98.75±11.53	91.15±12.71	105.02±13.66	170.77±31.60	125.59±18.91
TNFα	107.99±12.96	84.20±7.19	81.57±7.57	106.34±15.32	118.60±23.27	124.544±10.50
cFOS	99.22±11.12	94.71±10.07	107.65±4.60	104.06±4.60	99.08±8.76	91.37±7.95
Parabrachial nucleus						
IL-6	112.23±12.10	136.52±11.90	128.28±9.01	108.69±18.53	113.18±17.12	133.35±9.75
IκBα	99.53±5.90	114.85±9.30	104.92±7.82	103.68±12.16	103.42±8.61	119.86±6.44
IL-1β	102.49±13.91	78.23±9.47	79.01±12.91	107.86±19.26	113.50±9.70	128.20±13.60
TNFα	*105.06±13.83*	*77.57±7.85*	*99.05±5.24*	101.78±7.31	100.27±13.38	106.44±12.73
cFOS	**98.70±11.47a**	**128.35±9.17a**	**168.12±14.43b**	105.87±13.41	120.78±5.50	101.55±4.21

Note: Trends are *italicized and underlined* and main effects of Group are **bolded**, with *post hoc* differences designated with letters; groups that share a letter in common are not statistically different, whereas groups that have different letters differ statistically.
TNFα: tumor necrosis factor alpha; IL-6: Interleukin-6.

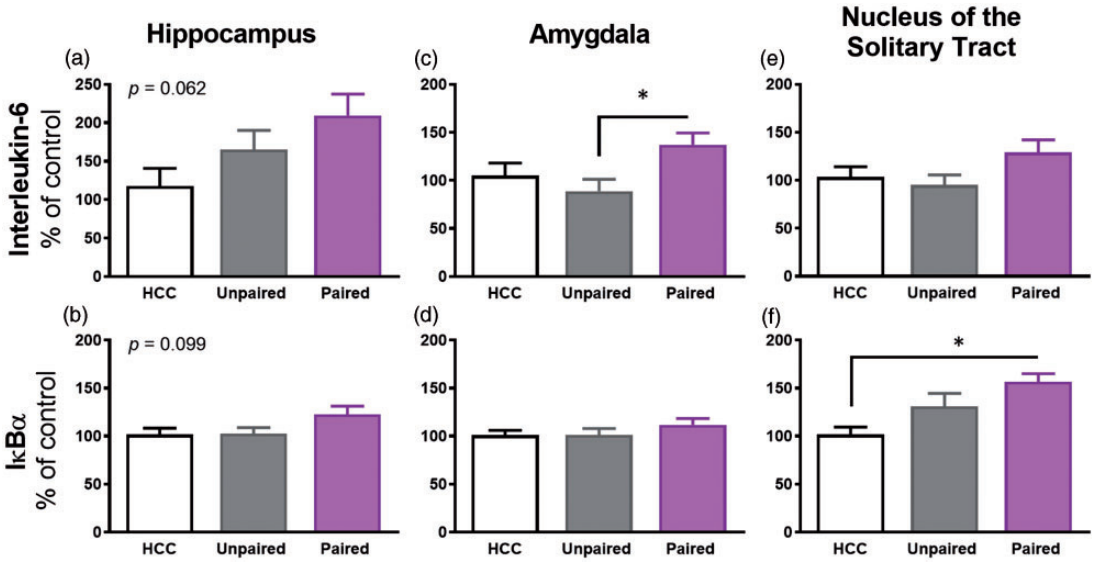

Figure 4. This figure depicts the gene expression outcomes of Experiment 2 for the adolescent group in the hippocampus, amygdala, and nucleus of the solitary tract for Interleukin-6 ((a), (c), (e)) and nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα; (b), (d), (f)). Data are shown as % of control (HCC group). Asterisks (*) denote a significant difference between groups (main effect of Group, all *post hoc* tests $p < 0.05$). Trends ($p < 0.1$) are denoted in the graphs. (A color version of this figure is available in the online journal.)

water, delivered in a lavender-scented context, and the pharmacological effects of ethanol (4 g/kg i.p.). This allowed us to examine the role of chemosensory cues (both taste and smell) in neuroimmune conditioning, while also registering a behavioral correlate to the potential cytokine effects. We also expected the combination of odor and taste to potentiate the salience of the conditioned stimulus, thus the likelihood of finding a reliable neuroimmune conditioned response.

As expected, both experiments yielded a reliable CTA. This behavioral response was associated with a complex pattern of neuroimmune responses, which exhibited

regional and ontogenetic variability. In Experiment 1, we examined the adult HPC, a site in which we previously noted cytokine conditioning by ethanol,[22] as well as the NTS and PbN, sites involved in CTA conditioning and in brain-to-immune system communication.[37] IL-6 was not affected by the conditioning, although paired animals exhibited a significant increase of IκBα—a reporter of NfκB activity that, in our previous work, was consistently associated with IL-6 expression.[8,10] The Paired and Paired+ groups showed higher levels of IL-6 and IκBα than controls, yet this difference did not reach statistical significance.

We can only speculate as to the reasons underlying the relative lack of significant, conditioned immune, effects in Experiment 1. It should be noted, however, that a similar lack of significant results was observed in our prior[22] study after using one or two-conditioning trials. Differences in the experimental designs and procedures hinder a direct comparison, yet it seems that ethanol-conditioned immune responses in adults may require repeated CS-US pairings to be acquired. It is also possible that the high dose of ethanol utilized, necessary to induce reliable cytokine gene expression,[7] exerts unconditional, dampening effects upon cognitive function that detract from finding reliable conditioned responses. Consistent with this, ethanol dose-dependently impaired HPC-dependent context conditioning.[38] It is interesting to note that, while the HPC showed no signs of IL-6 conditioning, there were some consistent trends for IL-6 and IκBα at the level of the NTS. It is possible that ethanol impaired information processing at cortical, high-order, structures (such as the HPC) yet spared the phylogenetically more primitive circuitry of the basal forebrain. This is, of course, just a hypothesis that needs to be tested in future work. Consistent with this possibility, however, it has been shown that an acute ethanol administration (4 g/kg i.p.) activated norepinephrine neurons in the NTS.[39]

Experiment 1 yielded subtle, yet encouraging, signs of ethanol-induced conditioned neuroimmune responses. Experiment 2 aimed at better addressing the potential of IL-6 and IκBα conditioning and pursued possible age-differences in these responses. A potential complicating factor to the initial experiment was the use of a vehicle-only control. This group controlled for the chance that the large volume of saline injected as a control could have, in and of itself, produced a CTA or nonspecific conditioned immune responses. While this was not the case, this group's large i.p. injection in combination with extended exposure to the context could have produced a higher baseline against which more delicate conditioned effects on cytokine expression in the paired groups were less likely to be observed. Also, the lack of unpaired controls made it difficult to differentiate the conditioned and the lingering, unconditioned effects, of ethanol upon immune function. Therefore, in Experiment 2, a drug-naïve HCC was used as a baseline for cytokine gene expression, in conjunction with an explicitly unpaired ethanol group.

Adolescents and adults exhibited strong and reliable CTA in Experiment 2, with adults replicating levels observed in Experiment 1, and adolescents showing overall greater sucrose solution consumption, as reported previously.[40] In the adults, the unpaired rats showed TNFα suppression in the HPC, and adolescents showed conditioned IL-6 increase in the amygdala as well as a trend for this effect in the HPC. This is consistent with our prior work demonstrating suppressed TNFα during ethanol intoxication, yet it is notable that a conditioned suppression in TNFα was not observed. In our previous study[22] c-Fos was sensitive to conditioning paradigms when cytokines were not, a dissociation also found in Experiment 2. Adult rats exhibited conditioned c-Fos increases in the PbN, and unconditioned (i.e. seen in paired and unpaired groups alike) c-Fos increase in the amygdala. In adolescents, the latter response was only observed in the unpaired group. Neuronal activation is frequently inferred by c-Fos activation, and the role of the amygdala in aversive conditioning has been well documented.[41] More importantly, it has been shown that inactivation of projections from the basolateral amygdala to the nucleus accumbens disrupts cue-driven ethanol consumption.[42] In this work, and as an initial approach, we analyzed the amygdala in its entirety and have now twice reported ethanol-induced conditioning in amygdala c-Fos and IL-6 expression. Future studies should dissect the role of specific amygdala regions (e.g. basolateral vs central or medial amygdala) and cell types (i.e. glutamatergic vs. GABA-ergic neurons) in the emergence of ethanol-induced neuroimmune conditioning. For instance, the reduced c-fos expression in the amygdala of Experiment 1 could have distinctly different implications if this effect occurred in GABA-ergic neurons, which might suggest enhanced BLA output due to reduced neural inhibition under these conditions. In contrast, reduced c-fos expression in glutamatergic neurons in the BLA could reflect the opposite. These details will be the subject of future studies in due course.

One limitation of the present studies is that several effects reported here approached but did not achieve statistical significance with $p < 0.05$. We defined marginally significant effects as those whose probability values were between 0.05 and 0.10 and only cautiously interpret such effects and in particular where these effects were predicted a priori. Based on prior power analyses, our studies were adequately powered to detect moderate-sized effects, and we have no reason to believe these marginal effects reflect low power, as all groups included 8–12 subjects per group. Consistent with this, behavioral data (sucrose consumption) were robustly significant in both experiments, supporting sufficient power for that component of the studies. Although we do not have a definitive explanation for marginal effects, conditioned physiological effects such as changes in neuroimmune function tend to be smaller in magnitude than unconditioned responses, presumably reflecting the moderating influence of conditioned cues relative to unconditioned stimuli, which tend to be direct mediators of physiological outcomes. In addition, the marginal significance of some effects does not likely reflect high variability across groups, as variance was verifiably homogeneous within all specific datasets. With that said, one potential explanation for these trends may be that certain effects may reflect a "priming" that makes the system more

sensitive to subsequent, direct immune challenges. Such priming effects are common in cytokine signaling systems and are often associated with broad-reaching biological effects.[43,44] With that in mind, we leave it to the reader to interpret effects reported here that achieved conventional guidance for statistical significance ($p < 0.05$) versus what we interpret as trends ($p < 0.10$).

It is interesting to note that the effects observed, while mild, differed between adolescents and adults. In our previous work, repeated pairings of moderate doses of ethanol resulted in a sensitized IL-6 response in adulthood. In this work, a single bout of ethanol paired to a context was enough to elicit a conditioned IL-6 response in the adolescent amygdala, an effect that did not occur in the adults. It is of particular note that our prior work has shown that adolescents displayed a blunted neuroimmune response to acute ethanol (as a US) relative to adults,[9] together with these data indicating that the cues associated with ethanol exposure in this age group are particularly salient. It is too early yet to speculate whether this may relate to the vulnerability of adolescence in the development of alcohol addiction, or to the mechanisms that drive the attenuation of sensitivity to aversive stimuli in this age group.[28] However, this work posits exciting questions and forms a jumping-off point at which we may examine adolescent sensitivity to alcohol from a neuroimmune conditioning perspective. Additionally, it should be noted that the immune system is intimately tied with stress and anxiety response systems such as the hypothalamic–pituitary–adrenal axis and glucocorticoid signaling.[45] As the role of these systems is also of great interest in the study of addiction, and most notably because adult–adolescent differences in stress- and anxiety-related ethanol behaviors are well-documented.[46,47] further inquiry into the developmental aspects of immune conditioning is of great value. Finally, given the emergent role of neuroimmune genes in regulating ethanol intake, the observation of cue-elicited alterations in neuroimmune genes may have important implications for understanding cue-induced reinstatement of ethanol intake among individuals with recurrent, problematic drinking.

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DISCLAIMER

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