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Chronic ethanol consumption: role of TLR3/TRIF-dependent signaling

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Abstract

Chronic ethanol consumption stimulates neuroimmune signaling in the brain, and Toll-like receptor (TLR) activation plays a key role in ethanol-induced inflammation. However, it is unknown which of the TLR signaling pathways, the myeloid differentiation primary response gene 88 (MyD88) dependent or the TIR-domain-containing adapter-inducing interferon- β (TRIF) dependent, is activated in response to chronic ethanol. We used voluntary (every-other-day) chronic ethanol consumption in adult C57BL/6J mice and measured expression of TLRs and their signaling molecules immediately following consumption and 24 hours after removing alcohol. We focused on the prefrontal cortex where neuroimmune changes are the most robust and also investigated the nucleus accumbens and amygdala. *Tlr* mRNA and components of the TRIF-dependent pathway (mRNA and protein) were increased in the prefrontal cortex 24 hours after ethanol and *Cxcl10* expression increased 0 hour after ethanol. Expression of *Tlr3* and TRIF-related components increased in the nucleus accumbens, but slightly decreased in the amygdala. In addition, we demonstrate that the IKK ϵ /TBK1 inhibitor Amlexanox decreases immune activation of TRIF-dependent pathway in the brain and reduces ethanol consumption, suggesting the TRIF-dependent pathway regulates drinking. Our results support the importance of TLR3 and the TRIF-dependent pathway in ethanol-induced neuroimmune signaling and suggest that this pathway could be a target in the treatment of alcohol use disorders.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest regarding this research.

AUTHOR CONTRIBUTIONS

GMM, YAB and RAH were responsible for the study concept and design. YAB provided animals; YAB and GMM performed animal experiments. GMM and CRB collected tissue, and ASW performed tissue punches. GMM and CRB performed qPCR analysis, GMM performed western blots and ASW and CRB performed immunohistochemistry. GMM drafted the manuscript; ASW, CRB and RAH provided manuscript revisions. GMM and RAH performed data analysis and interpretation. All authors critically reviewed content and approved final version for publication.

Keywords

Amlexanox; chronic ethanol; neuroimmune; prefrontal cortex; Toll-like receptors; TRIF

INTRODUCTION

Chronic alcohol consumption leads to adaptive changes in the central nervous system (CNS) that contribute to dependence and the development of alcohol use disorders (AUDs) (Diamond & Gordon 1997; Harper 2009). The prefrontal cortex (PFC), a brain region central to cognitive behavior, is important in the development of AUDs (Mukherjee *et al.* 2008; Abernathy *et al.* 2010). Chronic alcohol use produces molecular adaptations in the innate immune system, and the PFC is particularly sensitive to these changes (Goral *et al.* 2008; Crews 2012; Stolyarova *et al.* 2015). Although the PFC shows the strongest immune response to ethanol, the nucleus accumbens (NAc) and amygdala (AMY) are also affected (He & Crews 2008; Bajo *et al.* 2014; Osterndorff-Kahanek *et al.* 2015). There is evidence that neuroimmune mechanisms cause CNS damage and also promote alcohol dependence, suggesting that these pathways are potential therapeutic targets for the deleterious effects of alcohol (Alfonso-Loeches *et al.* 2010; Zou & Crews 2010; Blednov *et al.* 2011; Blednov *et al.* 2012).

Toll-like receptors (TLRs) are innate immune proteins and key components of the neuroimmune response to ethanol, particularly TLR2, TLR3 and TLR4 (Alfonso-Loeches *et al.* 2010; Crews *et al.* 2013). TLRs are pattern recognition receptors, and their signaling is triggered through a variety of pathogen-derived ligands. TLR2 and TLR4 are found on the cell surface and respond to bacterial ligands while TLR3 and TLR7 are in endosomes and respond to viral ligands (Lehnardt 2010). In addition, TLRs can respond to endogenous ligands like high mobility group box 1 (HMGB1) (Crews *et al.* 2013; Anggayasti *et al.* 2017). TLRs signal through two pathways, the myeloid differentiation primary response (MyD88) and the TIR-domain-containing adapter protein (TRIF/TICAM-1) pathways. In the MyD88-dependent pathway, the adapter protein MYD88 binds to the Toll/IL-1 receptor (TIR) domain and recruits interleukin-1 (IL-1) receptor-associated kinase 4 (IRAK4), IRAK1 and TNF receptor-associated factor 6 (TRAF6). IRAK4 then phosphorylates IRAK1, which leads to the release of TRAF6 and the formation of the TRAF6 complex. Members of the TRAF6 complex phosphorylate the inhibitor of nuclear factor kappa-B kinase (IKK) complex, allowing release and activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) (Takeda & Akira 2005). NF- κ B activation leads to the transcription of proinflammatory cytokines, such as IL-1 β , tumor necrosis factor alpha (TNF- α) and interleukin-6 (IL-6). The TRIF-dependent pathway uses the adapter protein TRIF, which signals through IKK ϵ (also referred to as IKKi) and TANK-binding kinase 1 (TBK1), leading to the phosphorylation of interferon regulator factor 3 (IRF3) and the transcription of type I interferons. Activation of the TRIF-dependent pathway also leads to increased transcription of interferon inducible genes like C-X-C motif chemokine 10 (*Cxcl10/IP-10*) and chemokine (C-C motif) ligand 5 (*Ccl5/RANTES*) (Weighardt *et al.* 2004; Hirotani *et al.* 2005). All TLRs (except TLR3) signal through the MyD88-dependent

pathway while TLR3, TLR4 and possibly TLR2 signal through the TRIF-dependent pathway (Takeda & Akira 2005; Nilsen *et al.* 2015).

Although studies have shown increased TLR mRNA and protein levels with high dose ethanol exposure, few have examined the effects of voluntary ethanol consumption on the MyD88 versus TRIF pathway components in the brain (Crews *et al.* 2013; Lippai *et al.* 2013; Whitman *et al.* 2013). It is important to understand the downstream signaling events that mediate ethanol-induced changes in order to target the relevant pathways and simultaneously alter the activity of multiple TLRs. The goal of this study was to examine TLR signaling components after voluntary chronic ethanol consumption.

We investigated both mRNA and protein changes in TLRs and components of the MyD88-dependent and TRIF-dependent pathways using C57BL/6J mice undergoing an every-other-day two-bottle choice (EOD-2BC) paradigm, which is a voluntary drinking test that leads to escalation in drinking and changes in gene expression (Crabbe *et al.* 2012; Osterndorff-Kahanek *et al.* 2013). We examined three different brain regions and two different time points to compare changes that occur immediately after consumption, while alcohol is still present in low levels, with those that occur when the mice are anticipating reinstatement of alcohol. We present novel evidence for TRIF-dependent and brain region-dependent signaling during alcohol withdrawal that may influence craving. To determine the functional importance of TRIF signaling in regulating ethanol consumption, we show that the TRIF pathway inhibitor Amlexanox blunts the inflammatory response in the brain and reduces ethanol consumption. Our results suggest that TRIF-dependent signaling may have a role in the development of AUDs and is a potential therapeutic target.

MATERIAL AND METHODS

Ethics statement

All procedures were approved by the University of Texas at Austin Institutional Animal Care and Use Committee (animal protocol number AUP-2013-00061) and adhered to the NIH Guidelines. The University of Texas at Austin animal facility is accredited by the Association for Assessment and Accreditation of Laboratory animal Care.

Animals and voluntary ethanol consumption

Studies were conducted in adult (6–8 weeks old) drug-naïve C57BL/J (B6) male mice (Jackson Laboratories, Bar Harbor, ME, USA). Mice were individually housed and allowed to acclimate to upright bottles 1 week before the start of the experiment. The experimental rooms were maintained at an ambient temperature of $21 \pm 1^\circ\text{C}$, 40–60 percent humidity and a regular light/dark schedule (7 AM–7 PM). Food and water were available *ad libitum*.

An EOD-2BC paradigm was used (Crabbe *et al.* 2012; Osterndorff-Kahanek *et al.* 2013), and mice were randomly assigned to the control or treatment group. Treatment and control groups each contained 15 mice per time point (60 total). Control groups had access to water every day. Treatment groups had EOD access to water and a 15 percent (v/v) ethanol solution, and water only on off days. On days that ethanol was available, bottle positions were alternated to control for potential side preferences. Ethanol and water bottles were

weighed after drinking days, and animals were weighed once per week to calculate consumption (Supporting Information Fig. S1). The study concluded after 60 days (30 drinking days).

Blood alcohol measurements and tissue harvest

Mice were sacrificed either immediately after ethanol was removed (0-hour group) or 24 hours after ethanol was removed (24-hour group). For the 0-hour group, retro-orbital bleeds were performed before sacrifice to determine blood alcohol concentration. Blood alcohol concentration values, expressed as milligram ethanol per deciliter of blood (Supporting Information Table S1), were determined spectrophotometrically by an enzyme assay (Lundquist *et al.* 1959).

Five mice per group were used for immunohistochemistry. These mice were anesthetized using isoflurane and given intraperitoneal injections of Euthanasia III Solution (TW Medical, Lago Vista, TX, USA). Once death was confirmed, transcardial perfusion was begun with phosphate-buffered saline (PBS) and followed by 4 percent paraformaldehyde (PFA). The whole brain was removed and post-fixed in 4 percent PFA. The remaining 10 mice per group were used for RNA and protein isolation. Following rapid cervical dislocation, brains were removed and placed on ice. The PFC was dissected as previously described (Osterndorff-Kahanek *et al.* 2013), cut in half and flash frozen in liquid nitrogen. The remainder of the brain was flash frozen separately.

Tissue punches

Frozen brains were mounted in optimum cutting temperature compound (VWR International, Randor, PA, USA) and placed in isopentane on dry ice. Micropunches of the NAc and AMY were taken as previously described (Osterndorff-Kahanek *et al.* 2015). The following coordinates, anterior-posterior distance from bregma, were utilized: NAc (+1.8 mm to +0.6 mm) and AMY (−0.9 mm to −1.8 mm).

RNA isolation and quantitative reverse transcription polymerase chain reaction

Half of each frozen PFC and the tissue punches were used for RNA isolation using the MagMax-96 Total RNA Isolation Kit (Thermo Fisher Scientific Inc., Rockford, IL, USA). The RNA yield was quantified on a NanoDrop 1000 spectrophotometer and assessed for quality on an Agilent 2200 TapeStation (Agilent Technologies, Santa Clara, CA, USA). RNA was reverse transcribed into cDNA using the Applied Biosystems High-capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific Inc.). cDNA was tested for genomic DNA contamination and showed at least a 10 Cq difference between the reverse transcription (+RT and −RT) samples (Bustin *et al.* 2009). Applied Biosystems TaqMan® Gene Expression Assay (Thermo Fisher Scientific Inc.) primers were used, and specific assay IDs are shown in Supporting Information Table S2. Quantitative polymerase chain reactions (qPCRs) were performed using SsoAdvanced™ Universal Probes Supermix (Bio-Rad, Hercules, CA, USA) in 10-μl reactions containing 18 ng of cDNA. All reactions were performed in triplicate and included a negative control. qPCR reactions were carried out using the CFX384 Real-time System (Thermo Fisher Scientific Inc.). For the PFC, three reference genes (*Gusb*, *Gapdh* and *Hprt*) were tested for each time point, and the best two

genes were selected based on normalization factors in the qbase+ software (Biogazelle, Gent, Belgium). The 0-hour samples were normalized to *Gapdh* and *Hprt*; the 24-hour samples were normalized to *Gapdh* and *Gusb*. The NAc and AMY micropunches were normalized only to *Gapdh* due to smaller quantities of cDNA. Relative quantification of mRNA levels was determined using the qbase+ software.

Protein isolation and western blot analysis

Half of each PFC was used for protein isolation. Tissue was homogenized in 200 μ l of lysis buffer [150 mM NaCl, 50 mM Tris-HCl pH 7.4, 1 mM ethylenediaminetetraacetic acid, 1 percent Triton-X-100, 1 percent sodium deoxycholic acid, 0.1 percent sodium dodecyl sulfate and 1X Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific Inc.)], centrifuged for 10 minutes at $10\,000 \times g$, aliquotted and frozen at -80°C . Protein concentrations were determined using the DC Protein Assay (Bio-Rad). Cell lysates (40 μ g) were boiled for 5 minutes, run on 10 percent Mini-protean TGX Precast Gels (Bio-Rad) and transferred to polyvinylidene difluoride membranes using semi-dry transfer. Membranes were blocked with 5 percent dried milk in Tris-buffered saline with 0.5 percent Tween-20 (TBST) and incubated overnight at 4°C with primary antibody (Supporting Information Table S3a). Membranes were washed with TBST and incubated with horseradish peroxidase-conjugated secondary antibodies in 5 percent dried milk in TBST (Supporting Information Table S3b). Bands were visualized using enhanced chemiluminescence (Pierce) and imaged on film using G:BOX Chemi XX6 (Syngene, Cambridge, UK). Bands were quantified using ImageJ and normalized to GAPDH and β -actin. All blots were repeated at least once.

Immunohistochemistry

Brains were post-fixed for 24 hours in 4 percent PFA at 4°C , cryoprotected for 24 hours in 20 percent sucrose and mounted in molds with optimal temperature compound. Frozen brains were sectioned coronally (20 μ m thick) and placed free floating into PBS. Sections were permeabilized in optimized detergent (0.1 percent Triton-X-100 or 0.1 percent sodium dodecyl sulfate) and blocked in 10 percent goat or donkey serum for 1 hour at room temperature. Sections were incubated with primary antibody overnight at 4°C (Supporting Information Table S3c). The following day, sections were washed with PBS and incubated with secondary antibody (Supporting Information Table S3d) for 2 hours at room temperature. Appropriate quality control staining was performed for labeling and secondary antibodies (Supporting Information Fig. S2). Sections were mounted on slides using Vectashield containing 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) (Vector Labs, Burlingame, CA, USA).

Microscopy

Quantification of immunopositive cells was performed using a Zeiss Axiovert 200M fluorescent light microscope (Zeiss, Thornwood, NY, USA) equipped with an Axiocam b/w camera. Brain regions were identified using a mouse brain atlas as previously described (Zuloaga *et al.* 2014). Bilateral images of the PFC (distance from bregma: +2.8 to +2.24 mm), NAc (+1.10 to +0.8 mm) and AMY (-1.20 to -1.60 mm) were captured using a $20\times$ objective. All immunohistochemistry was quantified bilaterally within fixed area frames:

PFC (box, 645 μm $\times$ 645 μm), NAc (circle, 575 μm diameter) and AMY (circle, 675 μm diameter), using the ImageJ plug-in ITCN (<http://rsb.info.nih.gov/ij/plugins/itcn.html>). Areas used for quantification are shown in Supporting Information Fig. S2. Any auto-fluorescence around the perimeter of the tissue was not included. We quantified IRF3 and IKK ϵ immunoreactivity as the number of immunopositive cells per section divided by total cell count (i.e. DAPI). For all cell quantifications, cells were counted in both hemispheres for a given region and summed. Total cell counts for each animal were then averaged and are presented as percent immunopositive cells per section. For IBA1 staining, images were thresholded and then each microglial cell was counted (particle count), average particle size was determined and mean pixel intensity was quantified within the selected area (methods described in <https://www.unige.ch/medecine/bioimaging/files/1914/1208/6000/Quantification.pdf>). Selected area was calculated as sum area of particles/area fraction; density was calculated as particle count/selected area. Because of the diffuse staining pattern of GFAP, it was difficult to count individual cells, so the analysis was performed as previously described (Alfonso-Loeches et al. 2010). GFAP immunoreactivity was calculated as the percentage of thresholded area occupied by the specific staining in relation to the whole area. The results are expressed as a fold change over control values. Approximately three medial PFC slices from coronal brain sections of five animals per group were analyzed.

Amlexanox treatment

Mice consumed 15 percent ethanol for at least 8 weeks using the EOD-2BC paradigm described previously. After this period, ethanol consumption was measured for at least 4 days to ensure stable consumption. Ethanol intake was then measured after saline administration for 2 days, and mice were grouped to provide similar levels of ethanol intake and preference based on the consumption during these 2 days. On day 4, mice were administered saline or Amlexanox once daily, and results are presented as the average from 2-day periods of consecutive drinking using different bottle positions. Amlexanox (100 mg/kg) was administered orally by gavage (p.o.). Amlexanox was purchased from Abcam (ab142825). Drug was prepared as suspensions in saline with five to four drops of Tween-80 and administered once daily in a volume of 0.05 ml/10 g of body weight 30 minutes before drinking experiments. Saline containing four to five drops of Tween-80 was administered to control groups.

Drug effect on ethanol consumption and preference

Ethanol consumption, preference and total fluid consumption were measured as previously described (Blednov et al. 2003). Briefly, bottles containing 15 percent ethanol and water were weighed before they were placed in the cage and again after 5 hours. Ethanol consumption was measured based on g/kg body weight/5 hours. Preference for ethanol was calculated as the amount of ethanol consumed divided by the total amount of fluids consumed per day (a value >50 percent indicates preference for ethanol).

Amlexanox-Polyinosinic:polycytidylic acid treatment

Mice were divided into four groups ($n = 10$ per group) and were given an oral treatment (saline or Amlexanox 100 mg/kg) followed by an i.p. injection 30 minutes later [saline or

polyinosinic:polycytidylic acid (Poly I:C) 5 mg/kg]. Mice were sacrificed 3 hours after the i.p. injection, and the PFC was fresh harvested as described previously. RNA isolations and qPCR analysis were performed as described previously.

Statistical analysis

Results are reported as the mean $\pm$ standard error of the mean. Data were analyzed using GRAPHPAD software (GraphPad Software Inc., San Diego, CA, USA) and two-tailed Student's *t*-tests unless otherwise noted (unpaired parametric test assumes populations have the same standard deviation). Statistical outliers were identified using Grubb's test and removed from analysis. Consumption following Amlexanox or saline treatment was analyzed using a two-way analysis of variance and Tukey's multiple comparisons test. Gene expression changes following Poly I:C and Amlexanox treatments were analyzed using a one-way analysis of variance and Tukey's multiple comparisons test.

RESULTS

Chronic ethanol consumption increases expression of *Tlrs*

To determine whether chronic ethanol consumption changes mRNA levels in the TLR signaling pathways in the PFC, qPCR analysis was performed 0 and 24 hours after ethanol removal. Several TLRs are altered in other studies using different alcohol paradigms (Alfonso-Loeches *et al.* 2010; Crews *et al.* 2013; Lippai *et al.* 2013; Whitman *et al.* 2013). Consistent with previous studies, qPCR analysis confirmed that *Tlr2*, *Tlr3*, *Tlr4* and *Cd14* (TLR co-receptor) are increased following voluntary ethanol consumption at the 24-hour time point (Fig. 1a). Although *Tlr2* and *Tlr4* have been implicated in alcohol action in more studies than *Tlr3* (Robinson *et al.* 2014), the largest fold-change (2.12) was observed for *Tlr3*. Like *Tlr3*, *Tlr7* is another endocytic TLR that recognizes viral RNA and leads to the production of type I interferons, despite signaling through the MyD88-dependent pathway. *Tlr7* has been implicated in alcohol liver disease (Cha *et al.* 2012) and in the suppression of cytokines by acute ethanol exposure (Pruett *et al.* 2004). To determine whether *Tlr7* expression changed with chronic ethanol consumption, qPCR was performed at both time points. In contrast to the other TLRs, *Tlr7* expression increased 0 hour after ethanol removal.

Chronic ethanol consumption increases MyD88-related and TRIF-related mRNA levels in a time-dependent manner

The qPCR was then used to evaluate changes in components of the MyD88-dependent pathway, which is common to all TLRs except TLR3, and hypothesized to be affected by chronic drinking. Only modest increases were observed for *Myd88* and *Irak4* at 0 hour, and all MyD88 pathway components were unchanged at 24 hours (Fig. 1b). Investigation of the TRIF-dependent pathway revealed an increase in *Ikke* at 0 hour and an increase in *Trif* and *Irf3* 24 hours after ethanol removal (Fig. 1c). *Tbk1* mRNA expression was unchanged. These results suggest that chronic ethanol consumption mainly changes expression of TRIF-dependent pathway mRNA. There are also molecules that are common to both the MyD88-dependent and TRIF-dependent pathways. *Traf3* and *Irf7* are involved in both TRIF-dependent signaling and TLR7 signaling through the MyD88-dependent pathway while

Traf6 is involved in all MyD88-dependent and TRIF-dependent signaling. Measurement of these mRNAs using qPCR revealed that *Traf3* and *Traf6* were unchanged at both time points, but *Irf7* expression increased at 0 hour (Fig. 1d).

Chronic ethanol consumption increases *Il1b* and *Cxcl10* mRNA immediately after ethanol removal and *Il6* 24 hours after ethanol removal

The TLR pathway activation leads to transcription of cytokines, chemokines and interferons, and several cytokine mRNAs have been previously linked to alcohol exposure (*Il1b*, *Tnfa* and *Il6*) (Alfonso-Loeches *et al.* 2010; Zou & Crews 2010; Lippai *et al.* 2013; Whitman *et al.* 2013). Expression of these cytokines was measured to evaluate activation of the MyD88-dependent pathway. In addition, transcriptional outputs of the TRIF-dependent pathway (*Ifnb*, *Ccl15* and *Cxcl10*) were measured; however, only *Cxcl10* was expressed at high enough levels to be quantified. Expression of *Il1b* and *Cxcl10* mRNA increased at 0 hour while *Il6* expression increased 24 hours after ethanol removal (Fig. 1e). *Il1b* transcription is primarily induced by MyD88-dependent signaling, while *Cxcl10* is mostly induced by TRIF-dependent signaling and *Il6* requires both pathways (Hirotsu *et al.* 2005). These data suggest that both pathways may be activated in response to chronic ethanol exposure.

Chronic ethanol consumption increases a microglial, but not an astrocyte, marker at 24 hours

Several studies have shown that glial activation is an important component of ethanol-induced neuroimmune signaling (He & Crews 2008; Fernandez-Lizarbe *et al.* 2009; Lippai *et al.* 2013). Microglia and astrocytes respond to pathogens and danger signals (i.e. HMGB1) by releasing cytokines, which can then further activate glia (Crews & Vetreno 2015). To determine the impact of chronic voluntary ethanol consumption on glial activation, we measured the astrocyte marker *Gfap*, the microglial marker *Cd11b* and the activated microglial marker *Cd68*. *Cd68* expression decreased at 0 hour while *Cd11b* mRNA increased 24 hours after ethanol removal (Fig. 1f). Immunohistochemistry was also used to evaluate any morphological or protein level changes in microglia and astrocytes in the PFC. Immunohistochemistry revealed few changes, with only a decrease in microglial density at the 0-hour time point (Supporting Information Fig. S4).

Chronic ethanol consumption did not change protein levels in the prefrontal cortex

After gene expression changes were observed for TLRs and TRIF-dependent pathway transcripts, protein levels were measured from the same PFC tissue using western blots. No significant differences were seen in any of the proteins measured (Supporting Information Fig. S5). Due to the small fold-changes, it is possible that western blots are not sensitive enough to detect protein level changes or that protein expression is changing at different time points.

Chronic ethanol consumption leads to increased IKK ϵ and IRF3 immunopositive cells in the prefrontal cortex and nucleus accumbens

Immunohistochemistry, which may be a more sensitive measurement compared with western blots, was used to determine the number of IKK ϵ and IRF3 immunopositive cells. In the

PFC, there was a significant increase in the percentage of IKK ϵ immunopositive cells at 0 hour and a significant increase in the percentage of both IKK ϵ and IRF3 immunopositive cells 24 hours after ethanol removal (Fig. 2a). The increases were larger at the 24-hour time point, which is consistent with the qPCR data. It is interesting to note that changes in both mRNA and IKK ϵ protein are seen at 0 hour. In addition to the PFC, immunopositive cells were measured in the NAc and AMY. The NAc showed similar changes to the PFC, with a small increase in IKK ϵ and IRF3 at 0 hour and larger increases in both IKK ϵ and IRF3 at 24 hours (Fig. 2b). No changes were found in the AMY at 0 hour, but there was a decrease in IKK ϵ and an increase in IRF3 at 24 hours (Fig. 2c). These results suggest that components of the TRIF signaling pathway are increased on the protein level by chronic ethanol in a brain region-specific manner.

Chronic ethanol consumption leads to increased *Tlr3* mRNA in the nucleus accumbens and decreased *Tlr* mRNA in the amygdala

Because changes in the percentage of immunopositive cells in the NAc and AMY were observed, mRNA levels in these brain regions were measured from micropunch control, two-tailed *t*-test, ****P* < 0.001 compared with control, two-tailed *t*-test. AMY, amygdala; NAc, nucleus accumbens; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; SEM, standard error of the mean; TLRs, Toll-like receptors; TRIF, TIR-domain-containing adapter protein samples. Because of limited sample, only transcripts that changed in the PFC were measured. In the NAc, there was an increase in *Tlr3* mRNA at the 24-hour time point while there was a decrease in *Tlr3* and *Tlr4* at 24 hours in the AMY (Fig. 3 a). Despite changes in immunopositive cells, there were no changes in expression in the TRIF-dependent pathway components at either time point in either brain region (Fig. 3b). The AMY showed a decrease in *Cxcl10* at the 24-hour time point, consistent with the decreased expression of *Tlr3/4* and IKK ϵ immunopositive cells seen in that brain region (Fig. 3c). Like the PFC, both the NAc and AMY showed an increase in *Il1b* expression at the 0-hour time point (Fig. 3c). This early increase in *Il1b* has also been observed in the hippocampus and VTA (data not shown), suggesting a brain-wide increase in *Il1b* immediately after ethanol removal.

Inhibitor of TRIF-dependent pathway decreases ethanol consumption

To evaluate whether changes in TRIF-dependent signaling may regulate ethanol consumption, mice were treated with the drug Amlexanox. Amlexanox is considered a specific inhibitor of IKK ϵ and TBK1 (Reilly *et al.* 2013), although it does inhibit GRK5 (Homan *et al.* 2014) and the S100A13-FGF1 complex (Rani *et al.* 2010) at higher concentrations. Previous work showed that Amlexanox decreases kinase activity of IKK ϵ and TBK1 as well as phosphorylation of IRF3 in response to the TLR3 ligand Poly I:C (Reilly *et al.* 2013). At a dose of 100 mg/kg, Amlexanox reduced ethanol consumption and preference in mice undergoing a chronic EOD-2BC paradigm but did not significantly change total fluid consumption (Fig. 4). These data suggest a role for activation of the TRIF-dependent pathway in the regulation of ethanol consumption.

Amlexanox reduces polyinosinic:polycytidylic acid-induced increase in *Cxcl10* expression

Amlexanox decreases the Poly I:C response *in vitro* and decreases high-fat diet-induced expression of *Cxcl10* in liver (Reilly *et al.* 2013), but there is no evidence that it inhibits the TRIF-dependent pathway in the brain. To address this, we measured *Cxcl10* expression in the PFC of mice that were treated with saline only, Amlexanox only, the TLR3 ligand Poly I:C and both Poly I:C and Amlexanox. Administration of Poly I:C resulted in a ~2000-fold increase in *Cxcl10* expression; however, administration of Poly I:C with Amlexanox reduces the Poly I:C-induced increase by 40 percent (Supporting Information Fig. S7).

DISCUSSION

Emerging evidence supports the role of neuroinflammatory mechanisms in alcohol dependence and alcohol-induced brain damage. Our group and others have shown that ethanol upregulates inflammatory mediators in the brain, which activate neuroimmune pathways (Blednov *et al.* 2012; Crews *et al.* 2013; Lippai *et al.* 2013). Studies have focused on TLR4 because of its role in producing proinflammatory cytokines that can promote neuroinflammatory-dependent brain damage (Alfonso-Loeches *et al.* 2010; Robinson *et al.* 2014). Given that MyD88 is the adapter protein for TLR4 (and all other TLRs except TLR3), we hypothesized that chronic ethanol drinking would lead to increased expression of the MyD88-dependent pathway. However, our findings indicate that EOD ethanol consumption primarily increases expression of *Tlr3* and components of the TRIF-dependent pathway (Fig. 5). We observed upregulation of TRIF signaling components at both the mRNA and protein levels as well as increased expression of *Cxcl10*, a transcriptional output of TRIF pathway activation. Although it is not clear how endosomal TLRs (e.g. TLR3 and TLR7) or the TRIF-dependent pathway is targeted by alcohol, recent studies suggest that alcohol increases expression of endogenous ligands (HMGB1 and miRNAs) that may preferentially target specific TLRs (Crews *et al.* 2013; Coleman *et al.* 2017). The TRIF-dependent pathway changes appear to be both brain region-specific and time-specific, with the greatest increase in signaling components observed in the PFC 24 hours after the last ethanol exposure and an increase in transcriptional outputs at 0 hour (Table 1). Moreover, we provide evidence that the TRIF-dependent pathway may regulate drinking by showing that a IKK ϵ /TBK1 inhibitor decreases ethanol consumption. A potential role for the TRIF-dependent pathway in ethanol consumption points to the utility of drugs that target this system for treating AUDs.

Chronic alcohol abuse encompasses a relapsing cycle of intoxication, withdrawal and craving resulting in aberrant neuroplasticity in corticolimbic structures (e.g. PFC), mesolimbic (e.g. NAc) and the extended AMY circuit. Expression changes in immune-related genes in the PFC, NAc and AMY following chronic intermittent ethanol treatment showed little overlap between brain regions, suggesting that there are region-specific differences in immune signaling (Osterndorff-Kahanek *et al.* 2015). Our results also support brain region-specific differences in neuroinflammatory signaling. The PFC showed the largest increase in *Tlr* mRNA expression and in TRIF-related expression of mRNA and protein. Similar changes in protein were found in the NAc, but only *Tlr3* mRNA increased in this region. This could be due to differences in temporal signaling events in these brain

regions, suggesting a dominance of corticolimbic structures during craving. It is also possible that TLR3 is the predominant subtype altered in the NAc, while TLR2, TLR3 and TLR4 all increase in the PFC. Interestingly, the AMY showed decreased *Tlr3*, *Tlr4* and *Cxcl10* mRNA levels and decreased IKK ϵ protein at 24 hours. These results are consistent with data that showed psychological stress produced divergent cytokine gene expression in the medial PFC and the AMY (Vecchiarelli *et al.* 2016). However, increased IRF3 protein expression was observed in the AMY at 24 hours, potentially due to decreased TLR signaling and compensatory increased IRF3 signaling. Although *Tlr* and TRIF-dependent changes in the AMY were the opposite of those seen in the PFC and NAc, all three brain regions showed an increase in *Il1b* at the 0-hour time point suggesting that there may be distinct signaling events occurring. This increase in *Il1b* is noteworthy because of the involvement of the IL-1 system with GABAergic transmission and evidence that IL-1 is also involved in disorders, like depression, that have high comorbidity with AUDs (Bajo *et al.* 2015a; Bajo *et al.* 2015b; Barnes *et al.* 2016). Further studies are necessary to elucidate the differences in TLR expression and signaling in various brain regions.

The EOD drinking paradigm produces escalation of intake and maintains high alcohol consumption on drinking days (Crabbe *et al.* 2012). The 0- and 24-hour time points were selected to compare neuroimmune responses at different stages during chronic alcohol consumption (i.e. craving versus bingeing). The 0-hour time point assesses immune activation during the last ethanol exposure while ethanol is still present, albeit in low levels, whereas the 24-hour time point represents the postwithdrawal stage when the mice are anticipating the availability of alcohol. We observed effects of alcohol consumption that are dependent on the time after withdrawal with more MyD88-dependent pathway changes at the 0-hour time point while most of the TLR and TRIF-dependent pathway changes are at the 24-hour time point, consistent with the idea that the MyD88-dependent pathway is activated earlier than the TRIF-dependent pathway (Kawai & Akira 2007). In addition, we saw increased expression of *Cxcl10*, a transcriptional output of TRIF signaling, at the 0-hour time point in the PFC. These results suggest that the pathway components are increasing expression at 24 hours, leading to increased activation of the pathway that results in transcription induction later, at the 0-hour time point. However, few studies have looked at TRIF pathway components and outputs at multiple time points, and future work would be needed to verify this time course. Because we observed the largest increase in TRIF-dependent components after 24 hours, we suggest that this pathway may be involved in the post-withdrawal craving stage. Amlexanox reduced ethanol consumption when administered at this 24-hour time point (right before drinking was reinstated), further suggesting that TRIF signaling is involved in craving. Interestingly, *Cxcl10* expression decreased at the 0-hour time point in the AMY, possibly due to differences in temporal regulation between brain regions or differences in the timeline for transcription versus degradation of mRNA. There are some timing inconsistencies, such as *Ikke* increasing at the 0-hour time point and not the 24-hour time point or *Il6* and *Il1b* changing at different time points. TLR signaling is complex and involves many levels of regulation, including negative and positive feedback as well as components that are common to multiple pathways (Medzhitov & Horng 2009). It is likely that any perturbation of this pathway could impact expression of other signaling

molecules involved and that the timing of these changes would depend on the speed and level of regulation.

In addition to identifying time-specific and brain region-specific changes in the TRIF-dependent pathway, our results suggest a causal role for TRIF signaling in the regulation of consumption. We utilized Amlexanox, an immunomodulatory drug that was recently found to be a potent inhibitor of IKK ϵ /TBK1. Although it has not been demonstrated that Amlexanox can cross the blood brain barrier, Lipinski's rule of five (Lipinski *et al.* 2001; Lipinski 2004) suggests it does and we show that it inhibits PFC induction of *Cxcl10* in response to a TLR3 agonist. Our Amlexanox results show that global inhibition of the TRIF pathway reduces ethanol consumption. Previous work has suggested that TLR signaling regulates ethanol consumption, with TLR2 and CD14 null mice showing decreased ethanol consumption in several tests (Blednov *et al.* 2017). However, no previous studies have investigated components of the TRIF-dependent pathway. Interestingly, MyD88 null mice increase ethanol consumption, possibly because of upregulation of TRIF-dependent signaling (Blednov *et al.* 2017). It is important to note that inhibition of TLR4 signaling does not reduce alcohol consumption emphasizing the importance of the TLR3/TRIF pathway (Harris *et al.* 2016; Blednov *et al.* 2017).

Many of the TLR pathway components measured here have been studied following other methods of alcohol exposure. *Il1b* increased in mouse cortex after an ethanol-based diet, and *Il6* increased in mouse cerebellum after binge ethanol exposure (Whitman *et al.* 2013; Kane *et al.* 2014). *Tlr2* and *Tlr4* have been shown to increase after various drinking paradigms, and *Tlr3* was increased following chronic intragastric ethanol exposure (Crews *et al.* 2013; Lippai *et al.* 2013; Whitman *et al.* 2013). *In vitro* studies in microglia and astrocytes have shown that ethanol can activate both the MyD88-dependent and TRIF-dependent pathway, consistent with our detection of changes in components of these pathways *in vivo* (Fernandez-Lizarbe *et al.* 2009; Pascual-Lucas *et al.* 2013). In contrast to our results, some studies reported increases in MyD88 and TLR protein, *Gfap* mRNA, TNF- α (mRNA and protein) and IL-1p protein (Alfonso-Loeches *et al.* 2010; Crews *et al.* 2013; Lippai *et al.* 2013; Whitman *et al.* 2013). These differences are likely due to the differing alcohol consumption paradigms, which are known to produce distinct effects on gene expression (Osterndorff-Kahanek *et al.* 2013; Osterndorff-Kahanek *et al.* 2015). Interestingly, previous rodent ethanol studies have shown marked changes in glial morphology and marker expression (Alfonso-Loeches *et al.* 2010; Lippai *et al.* 2013), while this study showed few. Thus, it is worth noting that even though it did not induce dramatic glial changes, the voluntary ethanol paradigm still led to changes in TLR and cytokine expression. These results suggest that gene expression changes can occur in the absence of obvious glial morphological changes. Although controversial, it is also possible that CNS cell types other than microglia and astrocytes are contributing the gene expression changes.

A caveat of this study is that we were unable to show increased phosphorylation of IRF3 as a measure of pathway activation. We attempted to measure phosphorylated IRF3 and interferon expression, but were unable to detect them at high enough levels in brain (Supporting Information Fig. S6). However, we were able to measure the interferon inducible gene *Cxcl10*, which is transcribed in response to TRIF pathway activation

(Weighardt *et al.* 2004; Hirotani *et al.* 2005). Our data showing that the TLR3 ligand Poly I:C leads to increased *Cxcl10* expression in the PFC, further supports this chemokine as a measure of pathway activation. Because of the transient nature of phosphorylation states, changes in transcriptional outputs are a more reliable measure of pathway activation. The increased *Cxcl10* expression in the PFC at the 0-hour time point supports the idea that the TRIF-dependent pathway is being activated in response to chronic ethanol. CXCL10 is a chemokine that is responsible for recruiting cells that express CXCR3 (microglia, dendritic cells and T lymphocytes) and has roles in regulating apoptosis, cell growth and proliferation, and synaptic activity (Liu *et al.* 2011; Gruol 2016). Increased CXCL10 therefore could increase the immune response to ethanol as well as contribute to the alcohol-induced damage or changes in neuronal activity. In addition, the fact that an inhibitor of this pathway decreases drinking and partially rescues Poly I:C-induced *Cxcl10* increases, further supports the idea that TRIF pathway activation is involved in the ethanol response as well as regulation of consumption.

The fold changes reported here are relatively small, and it is important to consider that there may be a dilution effect when examining heterogeneous cell populations. If immune signaling molecules are preferentially expressed in glial cells, studying the entire brain region would minimize any cell type-specific changes that may be present (Mayfield *et al.* 2013). We have observed greater differences in gene expression in more homogenous cell fractions, which can reveal discrete, localized changes (Most *et al.* 2014). In addition, the drinking paradigm we used here is voluntary and does not produce as extreme changes as those seen with high dose paradigms. For example, 5 g/kg intragastric ethanol daily for 10 days (a binge model) produced increases in *Tlr* mRNA that ranged from 1.5 to 2.5 fold (Crews *et al.* 2013).

In summary, voluntary chronic ethanol consumption increases expression of brain *Tlrs*, particularly *Tlr3* and components of the TRIF-dependent pathway, 24 hours after ethanol exposure. These changes could promote ethanol consumption and dependence, supported by the fact that a TRIF pathway inhibitor decreased ethanol consumption.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Abernathy K, Chandler LJ, Woodward JJ. Alcohol and the prefrontal cortex. *Int Rev Neurobiol.* 2010; 91:289–320. [PubMed: 20813246]
- Alfonso-Loeches S, Pascual-Lucas M, Blanco AM, Sanchez-Vera I, Guerri C. Pivotal role of TLR4 receptors in alcohol-induced neuroinflammation and brain damage. *J Neurosci.* 2010; 30:8285–8295. [PubMed: 20554880]

- Anggayasti WL, Mancera RL, Bottomley S, Helmerhorst E. The self-association of HMGB1 and its possible role in the binding to DNA and cell-membrane receptors. *FEBS Lett.* 2017; 591:282–294. [PubMed: 28027393]
- Bajo M, Madamba SG, Roberto M, Blednov YA, Sagi VN, Roberts E, Rice KC, Harris RA, Siggins GR. Innate immune factors modulate ethanol interaction with GABAergic transmission in mouse central amygdala. *Brain Behav Immun.* 2014; 40:191–202. [PubMed: 24675033]
- Bajo M, Herman MA, Varodayan FP, Oleata CS, Madamba SG, Harris RA, Blednov YA, Roberto M. Role of the IL-1 receptor antagonist in ethanol-induced regulation of GABAergic transmission in the central amygdala. *Brain Behav Immun.* 2015a; 45:189–197. [PubMed: 25479427]
- Bajo M, Varodayan FP, Madamba SG, Robert AJ, Casal LM, Oleata CS, Siggins GR, Roberto M. IL-1 interacts with ethanol effects on GABAergic transmission in the mouse central amygdala. *Front Pharmacol.* 2015b; 6:49. [PubMed: 25852553]
- Barnes J, Mondelli V, Pariante CM. NPPR: genetic contributions of inflammation to depression. *Neuropsychopharmacology.* 2016
- Blednov YA, Benavidez JM, Geil C, Perra S, Morikawa H, Harris RA. Activation of inflammatory signaling by lipopolysaccharide produces a prolonged increase of voluntary alcohol intake in mice. *Brain Behav Immun.* 2011; 25:S92–S105. [PubMed: 21266194]
- Blednov YA, Black M, Chernis J, Da Costa A, Mayfield J, Harris RA. Ethanol consumption in mice lacking CD14, TLR2, TLR4, or MyD88. *Alcohol Clin Exp Res.* 2017; 41:516–530. [PubMed: 28146272]
- Blednov YA, Ponomarev I, Geil C, Bergeson S, Koob GF, Harris RA. Neuroimmune regulation of alcohol consumption: behavioral validation of genes obtained from genomic studies. *Addict Biol.* 2012; 17:108–120. [PubMed: 21309947]
- Blednov YA, Walker D, Alva H, Creech K, Findlay G, Harris RA. GABAA receptor alpha 1 and beta 2 subunit null mutant mice: behavioral responses to ethanol. *J Pharmacol Exp Ther.* 2003; 305:854–863. [PubMed: 12626647]
- Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem.* 2009; 55:611–622. [PubMed: 19246619]
- Cha A, Choi Y, Jin Y, Sung MK, Koo YC, Lee KW, Park T. Antilipogenic and anti-inflammatory activities of *Codonopsis lanceolata* in mice hepatic tissues after chronic ethanol feeding. *J Biomed Biotechnol.* 2012; 2012:141395. [PubMed: 22013387]
- Coleman LG Jr, Zou J, Crews FT. Microglial-derived miRNA let-7 and HMGB1 contribute to ethanol-induced neurotoxicity via TLR7. *J Neuroinflammation.* 2017; 14:22. [PubMed: 28118842]
- Crabbe JC, Harkness JH, Spence SE, Huang LC, Metten P. Intermittent availability of ethanol does not always lead to elevated drinking in mice. *Alcohol Alcohol.* 2012; 47:509–517. [PubMed: 22717273]
- Crews FT. Immune function genes, genetics, and the neurobiology of addiction. *Alcohol Res.* 2012; 34:355–361. [PubMed: 23134052]
- Crews FT, Qin L, Sheedy D, Vetreno RP, Zou J. High mobility group box 1/Toll-like receptor danger signaling increases brain neuroimmune activation in alcohol dependence. *Biol Psychiatry.* 2013; 73:602–612. [PubMed: 23206318]
- Crews FT, Vetreno RP. Mechanisms of neuroimmune gene induction in alcoholism. *Psychopharmacology (Berl).* 2015
- Diamond I, Gordon AS. Cellular and molecular neuroscience of alcoholism. *Physiol Rev.* 1997; 77:1–20. [PubMed: 9016298]
- Fernandez-Lizarbe S, Pascual M, Guerri C. Critical role of TLR4 response in the activation of microglia induced by ethanol. *J Immunol.* 2009; 183:4733–4744. [PubMed: 19752239]
- Goral J, Karavitis J, Kovacs EJ. Exposure-dependent effects of ethanol on the innate immune system. *Alcohol.* 2008; 42:237–247. [PubMed: 18411007]
- Gruol DL. Impact of increased astrocyte expression of IL-6, CCL2 or CXCL10 in transgenic mice on hippocampal synaptic function. *Brain Sci.* 2016; 6:19.

- Harper C. The neuropathology of alcohol-related brain damage. *Alcohol Alcohol*. 2009; 44:136–140. [PubMed: 19147798]
- Harris RA, Bajo M, Bell RL, Blednov YA, Varodayan FP, Truitt J, de Guglielmo G, Lasek AW, Logrip ML, Vendruscolo LF, Roberts AJ, Roberts E, George O, Mayfield J, Billiar TR, Hackam DJ, Mayfield RD, Koob GF, Roberto M, Homanics GE. Genetic and pharmacologic manipulation of TLR4 has minimal impact on ethanol consumption in rodents. *J Neurosci*. 2016
- He J, Crews FT. Increased MCP-1 and microglia in various regions of the human alcoholic brain. *Exp Neurol*. 2008; 210:349–358. [PubMed: 18190912]
- Hirotani T, Yamamoto M, Kumagai Y, Uematsu S, Kawase I, Takeuchi O, Akira S. Regulation of lipopolysaccharide-inducible genes by MyD88 and Toll/IL-1 domain containing adaptor inducing IFN-beta. *Biochem Biophys Res Commun*. 2005; 328:383–392. [PubMed: 15694359]
- Homan KT, Wu E, Cannavo A, Koch WJ, Tesmer JJ. Identification and characterization of Amlexanox as a G protein-coupled receptor kinase 5 inhibitor. *Molecules*. 2014; 19:16937–16949. [PubMed: 25340299]
- Kane CJ, Phelan KD, Douglas JC, Wagoner G, Johnson JW, Xu J, Phelan PS, Drew PD. Effects of ethanol on immune response in the brain: region-specific changes in adolescent versus adult mice. *Alcohol Clin Exp Res*. 2014; 38:384–391. [PubMed: 24033454]
- Kawai T, Akira S. Signaling to NF-kappaB by Toll-like receptors. *Trends Mol Med*. 2007; 13:460–469. [PubMed: 18029230]
- Lehnardt S. Innate immunity and neuroinflammation in the CNS: the role of microglia in Toll-like receptor-mediated neuronal injury. *Glia*. 2010; 58:253–263. [PubMed: 19705460]
- Lipinski CA. Lead-and drug-like compounds: the rule-of-five revolution. *Drug Discov Today Technol*. 2004; 1:337–341. [PubMed: 24981612]
- Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv Drug Deliv Rev*. 2001; 46:3–26. [PubMed: 11259830]
- Lippai D, Bala S, Petrasek J, Csak T, Levin I, Kurt-Jones EA, Szabo G. Alcohol-induced IL-1beta in the brain is mediated by NLRP3/ASC inflammasome activation that amplifies neuroinflammation. *J Leukoc Biol*. 2013; 94:171–182. [PubMed: 23625200]
- Liu M, Guo S, Hibbert JM, Jain V, Singh N, Wilson NO, Stiles JK. CXCL10/IP-10 in infectious diseases pathogenesis and potential therapeutic implications. *Cytokine Growth Factor Rev*. 2011; 22:121–130. [PubMed: 21802343]
- Lundquist F, Fugmann U, Klaning E, Rasmussen H. The metabolism of acetaldehyde in mammalian tissues: reactions in rat-liver suspensions under anaerobic conditions. *Biochem J*. 1959; 72:409–419. [PubMed: 14418937]
- Mayfield J, Ferguson L, Harris RA. Neuroimmune signaling: a key component of alcohol abuse. *Curr Opin Neurobiol*. 2013; 23:513–520. [PubMed: 23434064]
- Medzhitov R, Horng T. Transcriptional control of the inflammatory response. *Nat Rev Immunol*. 2009; 9:692–703. [PubMed: 19859064]
- Most D, Workman E, Harris RA. Synaptic adaptations by alcohol and drugs of abuse: changes in microRNA expression and mRNA regulation. *Front Mol Neurosci*. 2014; 7:85. [PubMed: 25565954]
- Mukherjee S, Das SK, Vaidyanathan K, Vasudevan DM. Consequences of alcohol consumption on neurotransmitters —an overview. *Curr Neurovasc Res*. 2008; 5:266–272. [PubMed: 19133404]
- Nilsen NJ, Vladimer GI, Stenvik J, Orning MP, Zeid-Kilani MV, Bugge M, Bergstroem B, Conlon J, Husebye H, Hise AG, Fitzgerald KA, Espevik T, Lien E. A role for the adaptor proteins TRAM and TRIF in Toll-like receptor 2 signaling. *J Biol Chem*. 2015; 290:3209–3222. [PubMed: 25505250]
- Osterndorff-Kahanek EA, Becker HC, Lopez MF, Farris SP, Tiwari GR, Nunez YO, Harris RA, Mayfield RD. Chronic ethanol exposure produces time- and brain region-dependent changes in gene coexpression networks. *PLoS One*. 2015; 10:e0121522. [PubMed: 25803291]
- Osterndorff-Kahanek E, Ponomarev I, Blednov YA, Harris RA. Gene expression in brain and liver produced by three different regimens of alcohol consumption in mice: comparison with immune activation. *PLoS One*. 2013; 8:e59870. [PubMed: 23555817]

- Pascual-Lucas M, Fernandez-Lizarbe S, Montesinos J, Guerri C. LPS or ethanol triggers clathrin- and rafts/caveolae-dependent endocytosis of TLR4 in cortical astrocytes. *J Neurochem*. 2013; 129:448–462.
- Pruett SB, Zheng Q, Fan R, Matthews K, Schwab C. Ethanol suppresses cytokine responses induced through Toll-like receptors as well as innate resistance to *Escherichia coli* in a mouse model for binge drinking. *Alcohol*. 2004; 33:147–155. [PubMed: 15528012]
- Rani SG, Mohan SK, Yu C. Molecular level interactions of S100A13 with Amlexanox: inhibitor for formation of the multiprotein complex in the nonclassical pathway of acidic fibroblast growth factor. *Biochemistry*. 2010; 49:2585–2592. [PubMed: 20178375]
- Reilly SM, Chiang SH, Decker SJ, Chang L, Uhm M, Larsen MJ, Rubin JR, Mowers J, White NM, Hochberg I, Downes M, Yu RT, Liddle C, Evans RM, Oh D, Li P, Olefsky JM, Saltiel AR. An inhibitor of the protein kinases TBK1 and IKK- ϵ improves obesity-related metabolic dysfunctions in mice. *Nat Med*. 2013; 19:313–321. [PubMed: 23396211]
- Robinson G, Most D, Ferguson LB, Mayfield J, Harris RA, Blednov YA. Neuroimmune pathways in alcohol consumption: evidence from behavioral and genetic studies in rodents and humans. *Int Rev Neurobiol*. 2014; 118:13–39. [PubMed: 25175860]
- Stolyarova A, Thompson AB, Barrientos RM, Izquierdo A. Reductions in frontocortical cytokine levels are associated with long-lasting alterations in reward valuation after methamphetamine. *Neuropsychopharmacology*. 2015; 40:1234–1242. [PubMed: 25409594]
- Takeda K, Akira S. Toll-like receptors in innate immunity. *Int Immunol*. 2005; 17:1–14. [PubMed: 15585605]
- Vecchiarelli HA, Gandhi CP, Gray JM, Morena M, Hassan KI, Hill MN. Divergent responses of inflammatory mediators within the amygdala and medial prefrontal cortex to acute psychological stress. *Brain Behav Immun*. 2016; 51:70–91. [PubMed: 26260453]
- Weighardt H, Jusek G, Mages J, Lang R, Hoebe K, Beutler B, Holzmann B. Identification of a TLR4- and TRIF-dependent activation program of dendritic cells. *Eur J Immunol*. 2004; 34:558–564. [PubMed: 14768061]
- Whitman BA, Knapp DJ, Werner DF, Crews FT, Breese GR. The cytokine mRNA increase induced by withdrawal from chronic ethanol in the sterile environment of brain is mediated by CRF and HMGB1 release. *Alcohol Clin Exp Res*. 2013; 37:2086–2097. [PubMed: 23895427]
- Zou J, Crews F. Induction of innate immune gene expression cascades in brain slice cultures by ethanol: key role of NF- κ B and proinflammatory cytokines. *Alcohol Clin Exp Res*. 2010; 34:777–789. [PubMed: 20201932]
- Zuloaga DG, Johnson LA, Agam M, Raber J. Sex differences in activation of the hypothalamic-pituitary-adrenal axis by methamphetamine. *J Neurochem*. 2014; 129:495–508. [PubMed: 24400874]

Figure 1.

RT-qPCR analysis of the PFC 0 and 24 hours after ethanol removal ($n = 10/\text{group}$). (a) The 0-hour group showed increased *Tlr7* expression while the 24-hour ethanol group showed increased *Tlr2*, *Tlr3*, *Tlr4* and *Cd14* mRNA expression. (b) The 0-hour ethanol group showed increased *Myd88* and *Irak4* expression. (c) Increased *Ikk1* expression was observed in the 0-hour group, while increased *Trif* and *Irf3* expression was found in the 24-hour ethanol group. (d) The 0-hour group showed increased *Irf7* expression with ethanol. (e) The 0-hour ethanol group showed increased *Irf7* and *Cxcl10* expression while the 24-hour ethanol group showed increased *Irf6* expression. (f) The 24-hour ethanol group showed increased expression of *Cd11b*, a microglial marker. All values are expressed as fold change over control $\pm$ SEM. $P < 0.05$ compared with control, one-tailed t -test; $*P < 0.05$ compared with control, two-tailed t -test; $**P < 0.01$ compared with control, two-tailed t -test; $***P < 0.001$ compared with control, two-tailed t -test. PFC, prefrontal cortex; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; SEM, standard error of the mean; TLRs, Toll-like receptors; TRIF, TIR-domain-containing adapter protein

(a) Prefrontal Cortex

(b) Nucleus Accumbens

(c) Amygdala

Figure 2.

Immunohistochemistry for IRF3 and IKKε 0 and 24 hours after ethanol removal.

Representative images are shown from one control and one ethanol sample. Graphs show quantification from all samples ($n = 5/\text{group}$), and values represent the average $\pm$ SEM percent of cells positive for either IKKε or IRF3 over the total number of DAPI positive cells. Scale bar for prefrontal cortex and nucleus accumbens = 50 μM and for amygdala = 100 μM . (a) In the prefrontal cortex, there is an increase in the percentage of IKKε positive cells at 0 hour and of both IKKε and IRF3 positive cells at 24 hours. (b) In the nucleus accumbens, there is an increase in the percentage both IKKε and IRF3 positive cells at 0 and 24 hours. (c) In the amygdala, there are no changes at 0 hour, and at 24 hours, there is a decrease in IKKε positive cells and an increase in IRF3 positive cells. # $P < 0.05$, one-tailed

t -test, $*P < 0.05$ compared with control, two-tailed t -test; $**P < 0.01$ compared with control, two-tailed t -test; $***P < 0.001$ compared with control, two-tailed t -test [Colour figure can be viewed at wileyonlinelibrary.com]

Figure 3.

RT-qPCR analysis from the NAc and AMY 0 and 24 hours after ethanol removal (for the 0-hour time point $n = 10$ /group and for the 24-hour time point $n = 10$ for the control group and $n = 9$ ethanol group). (a) The NAc shows increased *Tlr3* mRNA levels while the AMY shows decreased *Tlr3* and *Tlr4* at 24 hours. (b) No changes were seen in the TRIF pathway genes. (c) Both the NAc and AMY showed increased *Il1b* expression at 0 hour, and the AMY showed decreased *Cxcl10* expression at 24 hours. All values are expressed as fold change over control $\pm$ SEM. # $P < 0.05$ compared with control, one-tailed t -test; * $P < 0.05$ compared with control, two-tailed t -test; ** $P < 0.01$ compared with control, two-tailed t -test, *** $P < 0.001$ compared with control, two-tailed t -test. AMY, amygdala; NAc, nucleus accumbens; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; SEM, standard error of the mean; TLRs, Toll-like receptors; TRIF, TIR-domain-containing adapter protein

Figure 4.

Measures for saline and Amlexanox (100 mg/kg) treatment groups under baseline (both groups received saline injections) and drug conditions. All measurements are taken 5 hours following treatment. (a) The Amlexanox group shows decreased ethanol consumption. (b) The Amlexanox group shows decreased preference for ethanol. (c) The Amlexanox group shows no change in total fluid intake after treatment compared to baseline. Bars labeled with the same letter are not statistically different while bars with different letters are (two-way analysis of variance with Tukey's multiple comparisons test, $n=11$ per group)

Figure 5. Schematic of the Toll-like receptor (TLR) signaling pathways in the prefrontal cortex. Dark blue represents RNA changes at 24 hours, purple represents RNA changes at 0 hour, black dots indicate that polyinosinic:polycytidylic acid-induced mRNA was decreased with Amlexanox, checkered hatching represents protein increases at 0 and 24 hours while diagonal hatching represents protein increases at 24 hours. Gray represents no change, and white indicates that the pathway component was not measured in this study. Light blue means that expression was too low to measure in this study [Colour figure can be viewed at wileyonlinelibrary.com]

Table 1

Effects of chronic ethanol on mRNA and protein levels in (a) the PFC, (b) the NAc and (c) the AMY were examined 0 and 24 hours after ethanol removal.

(a) mRNA and protein changes in PFC				
	PFC			
	mRNA		protein-IHC	
	0 hour	24 hours	0 hour	24 hours
Toll-like receptors and co-receptors				
<i>Tlr2</i>	—	↑(1.26)		
<i>Tlr3</i>	—	↑(2.12)		
<i>Tlr4</i>	—	↑(1.49)		
<i>Tlr7</i>	↑(1.15)	—		
<i>Cd14</i>	—	↑(1.19)		
MyD88-dependent pathway signaling molecules				
<i>Myd88</i>	↑(1.07)	—		
<i>Irak1</i>	—	—		
<i>plrak1</i>				
<i>Irak4</i>	↑(1.19)	—		
<i>Ikkb</i>	—	—		
TRIF-dependent pathway signaling molecules				
<i>Trif</i>		↑(1.23)		
<i>Ikki</i>	↑(1.17)		↑(1.08)	↑(1.24)
<i>Irf3</i>	—	↑(1.56)	—	↑(1.68)
Molecules involved in both pathways				
<i>Irf7*</i>	↑(1.18)	—		
<i>Traf3*</i>	—	—		
<i>Traf6</i>	—	—		
Transcriptional outputs of MyD88-dependent pathway				
<i>Il1b</i>	↑(4.45)	—		
<i>Tnfa</i>	—	—		
<i>Il6</i>	—	↑(1.58)		
Transcriptional outputs of the TRIF-dependent pathway				
<i>Cxcl10</i>	↑(2.11)	—		
<i>Ccl5</i>	—	—		
<i>Ifnb</i>	—	—		
Glial markers				
<i>Cd11b/Iba1</i>		↑(1.26)		
<i>Gfap</i>	—	—		
<i>Cd68</i>	↓(0.89)	—		

(b) mRNA and protein changes in NAc				
	NAc			
	mRNA		protein-IHC	
	0 hour	24 hours	0 hour	24 hours
Toll-like receptors				
<i>Tlr2</i>	—	—		
<i>Tlr3</i>	—	↑(1.15)		
<i>Tlr4</i>	—	—		
MyD88-dependent pathway signaling molecules				
<i>Myd88</i>	—	—		
TRIF-dependent pathway signaling molecules				
<i>Trif</i>	—	—		
<i>Ikk1</i>	—	—	↑(1.05)	↑(1.36)
<i>Irf3</i>	—	—	↑(1.10)	↑(1.31)
Cytokines and chemokines				
<i>Il1b</i>	↑(4.24)	—		
<i>Cxcl10</i>	—	—		

(c) mRNA and protein changes in AMY				
	AMY			
	mRNA		protein-IHC	
	0 hour	24 hours	0 hour	24 hours
Toll-like receptors				
<i>Tlr2</i>	—	—		
<i>Tlr3</i>	—	↓(0.87)		
<i>Tlr4</i>	—	↓(0.87)		
MyD88-dependent pathway signaling molecules				
<i>Myd88</i>	—	—		
TRIF-dependent pathway signaling molecules				
<i>Trif</i>	—	—		
<i>Ikk1</i>	—	—	—	↓(0.88)
<i>Irf3</i>	—	—	—	↑(1.15)
Cytokines and chemokines				
<i>Il1b</i>	↑(3.25)	—		
<i>Cxcl10</i>		↓(0.37)		

Effects are shown as increases, decreases or no change (—); blank cells indicate that mRNA or protein was not measured. Gene names are indicated in the left column, and the numbers in parentheses indicate fold change compared with control group. AMY = amygdala; IHC = immunohistochemistry; NAc = nucleus accumbens; PFC = prefrontal cortex. Genes with * are only used in the non-canonical MyD88-dependent pathway that leads to the transcription of type I interferons.