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Time course of microglia activation and brain and blood cytokine/chemokine levels following chronic ethanol exposure and protracted withdrawal in rats

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Abstract

Alcohol produces complex effects on the immune system. Moderate alcohol use (1–2 drinks per day) has been shown to produce anti-inflammatory responses in human blood monocytes, whereas, the post-mortem brains of severe alcoholics show increased immune gene expression and activated microglial markers. The present study was conducted to evaluate the time course of alcohol effects during exposure and after withdrawal, and to determine the relationship between microglial and cytokine responses in brain and blood. Forty-eight adult, male Wistar rats were exposed to chronic ethanol vapors, or air control, for 5 weeks. Following ethanol/air exposure blood and brains were collected at 3 time points: 1) while intoxicated, following 35 days of air/vapor exposure; 2) following 24 hours of withdrawal from exposure, and 3) 28 days after withdrawal. One hemisphere of the brain was flash frozen for cytokine analysis, the other was fixed for immunohistochemical analysis. The ionized calcium-binding adapter molecule 1 (Iba-1) was used to evaluate microglia activation at the three time points, rat cytokine / chemokine Magnetic Bead Panels (Millipore) were used to analyze frontal cortex tissue lysate and serum. Ethanol induced a significant increase in Iba1 that peaked at day 35, remained significant after 1 day of withdrawal and was elevated at day 28 in frontal cortex, amygdala and substantia nigra.

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Conflicts of Interest
None

Ethanol exposure was associated with a transient reduction of the serum level of the major pro- and anti-inflammatory cytokines and chemokines and a transient increase of effectors of sterile inflammation. Little or no changes in these molecules were seen in the frontal cortex except for HMG1 and fractalkine that were reduced and elevated, respectively, at day 28 following withdrawal. These data show that ethanol exposure produces robust microglial activation, however, measures of inflammation in the blood differ from those in the brain over a protracted time course.

Keywords

Alcohol; cytokines; microglial activation; Wistar rats

Introduction

The immune system has a complex and diverse set of responses to alcohol consumption that appear to depend on the amount consumed, length of time a person has been drinking, that persons age, and the particular organ system that is under study (Crews et al., 2006; 2017; Gao, 2012; Szabo et al., 2007; Szabo & Saha, 2015). Moderate levels of alcohol drinking, that are within the NIAAA guidelines for consumption (e.g. 1 drink per day for women, 2 drinks a day for men), have been demonstrated to have health benefits such as reduced cardiovascular mortality and increased life expectancy (Gunzerath et al., 2004; Mukamal & Rimm, 2001; Thun et al., 1997). An acute dose of alcohol, designed to mimic this level of consumption, has also been demonstrated to produce dual anti-inflammatory effects that include attenuation of monocyte inflammatory responses involving inhibition of NF- κ B, as well as augmentation of IL-10 in peripheral blood (Mandrekar et al., 2006). Further work established that a rapid variation occurs in serum cytokine levels that follows the time course of alcohol intake and abstinence (Gonzalez-Quintela et al., 2000) indicating that an acute active process of cytokine modulation is involved. Studies in cultured human hepatocytes and monocytes further emphasized this time course by demonstrating that after acute EtOH exposure cytokine expression could be up-regulated (e.g. IL-1 alpha, IL-6 and TNF-alpha) as compared to cells in control conditions (Girouard et al., 1998; Neuman et al., 1998; Perez et al., 1984).

Alcohol use disorders and heavy drinking have been associated with mainly proinflammatory effects (Beech et al., 2012; Manzardo et al., 2016; Szabo & Iracheta-Vellve, 2015). Early work in this area was conducted in patients with alcoholic liver disease, who had no signs of acute infection, and never-the-less showed a marked infiltration of the liver by polymorphonuclear neutrophil leucocytes. Further studies demonstrated that a neutrophil activators, cytokines, as well as chemotactic signaling molecules were enhanced in the serum and liver of patients with alcoholic liver disease (Devalaraja et al., 1999; Deviere et al., 1989; Maltby et al., 1996; Miller et al., 2011; Nagy, 2015). Studies evaluating the plasma of patients in acute alcoholic hepatitis (AAH) showed that pro-inflammatory cytokines were markedly increased and that they closely correlated with the severity of the disease (Sheron et al., 1993; Taieb et al., 1998). Thus, chronic alcoholism, complicated by alcoholic liver disease, was shown to be characterized by alterations in the balance between pro-

inflammatory and antiinflammatory cytokine signaling (Daniluk et al., 2001; Laso et al., 1999; 2007; Neuman et al., 2001).

Chronic alcohol use and use disorders can also lead to physiological disruptions and damage to the brain during hepatic encephalopathy (Dennis et al., 2014). However, independent of any known hepatic involvement, neuroimmune signaling has been demonstrated to influence brain functioning (Crews et al., 2017). Studies in animal models have shown that some immune proteins can act as both immunesignaling molecules in the periphery and signaling molecules in the brain (Crews et al., 2006). Glial cells are one of the key immune effector cells in brain and as such are important regulators of neuroimmune responses (Ransohoff & Brown, 2012). One important indicator of enhanced neuroimmune function is the activation of glial cells which can then secrete a number of pro-inflammatory and neurotoxic factors which include cytokines and chemokines (Block et al., 2007). In the post-mortem brain of human alcoholics, microglia activation markers, most notably Iba-1, have been shown to be increased (He & Crews, 2008). There have also been a number of studies in animal models that have demonstrated that alcohol exposure alters the microglial activation spectrum, often producing a “partial” activation of microglia (Marshall et al., 2013; McClain et al., 2011). Microglia can present at least four distinct morphologies that represent different stages of activation. Specifically, resting ramified microglia move towards activation by becoming first hyper-ramified then bushy and eventually amoeboid. Post-mortem brains from alcoholics contain microglia at all four stages but are enriched in hyper-ramified microglia suggesting that ethanol sensitizes these cells (see (Crews & Vetreno, 2016). Further studies have demonstrated that microglial responses vary according to the duration, dose and pattern of alcohol exposure (Henriques et al., 2018; McCarthy et al., 2018; Yang et al., 2014). Very large doses of alcohol (Crews, 2008; Peng et al., 2017; Zahr et al., 2010), repeated cycles of ethanol exposure (Marshall et al., 2016; Zhao et al., 2013) or prior ethanol exposure are known to produce elevated levels of serum cytokines as well as microglia activation and long lasting increases in neuroimmune gene induction suggesting microglia priming (Crews & Vetreno, 2016; Perry & Holmes, 2014; Zou & Crews, 2010). Recently, stress has also been shown to affect the neuroimmune response to ethanol (Doremus-Fitzwater et al., 2018). How more modest doses of alcohol may impact peripheral markers and brain measures is less well understood as is the relationship between microglia ramification and other immune signaling measures during alcohol exposure and following protracted withdrawal.

The aims of the present study were to characterize the dynamic changes in microglial morphology in blood and brain, and cytokine and chemokine signaling in frontal cortex, in adult Wistar rats, following moderate chronic ethanol exposure and acute and prolonged alcohol withdrawal.

Experimental Procedures

Animal subjects

Forty-eight adult, male, Wistar rats were obtained from Charles River (USA) and arrived on postnatal day (PD) 90. Rats were pair-housed in standard plastic cages in a temperature-controlled room with a 12h light/dark cycle. Food and water were available *ad libitum*. The work described herein adheres to the guidelines stipulated in the NIH *Guide for the Care and*

Use of Laboratory Animals (NIH publication No. 80–23, revised 1996) and was reviewed and approved by The Scripps Research Institute's animal care and use committee.

Ethanol vapor exposure

Ethanol vapor exposure has been shown to reliably allow for the titration of blood ethanol concentrations (BECs) that are sufficient for inducing physical ethanol dependence. The ethanol vapor inhalation procedure and the chambers used in this study were previously described (Slawecki, 2002). Ethanol vapor chambers were calibrated to produce high to moderate BECs between 150–200 mg/dL. Rats were randomly divided into two groups; ethanol or control group. Ethanol rats were housed in sealed chambers, infused with vaporized 95% ethanol from 8 p.m. to 10 a.m. For the remaining 10 hrs of the day, ethanol vapor was not infused into the chambers. Rats (PD 91–127) were exposed to this vapor cycle for 5 weeks (35 days). Blood samples were collected from the tip of the tail every 3–4 days during the 5-week exposure period to assess BECs (5-week average, 162.6 ± 8.3 mg/dL). Control animals were housed in same cages in same room as vapor exposed animals. Blood was collected at the same time of day beginning at 0:800 from both ethanol and control rats. BECs were determined using the Analox micro-statAM1 (Analox Instr. Ltd., Lunenburg, MA). Following the 5-week exposure, ethanol animals were transferred to standard housing for the duration of the experiment.

Tissue collection and preparation

Brains and serum were obtained from rats at three time-points after 5 weeks of ethanol vapor exposure or air control. Time points used were: air/vapor 35 days (P127), 1 day after cessation of ethanol vapor (24 hours of withdrawal, P128) and 28 days after cessation of ethanol vapor (28 days withdrawal, P154). At each time-point animals were anesthetized with Fatal-Plus and blood was collected via cardiac puncture prior to perfusion with phosphate buffered saline (PBS). One hemisphere of the brain was used for dissecting specific brain regions that were flash frozen for subsequent cytokine analysis. The other hemisphere was placed in 4% para-formaldehyde for 24 hours then cryoprotected in 30% sucrose for 72 hours at 4°C for immunohistochemical analysis.

Tissue was prepared for both ELISA and Multiplex assay using flash frozen sections of the brain that were homogenized using RIPA buffer (Thermo Fisher Scientific, cat no. 89900) with 1X Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific, cat no. 87786). Homogenized tissue mixture was then placed on ice and shaken gently before being centrifuged at $14,000 \times g$ for 15 minutes at 4°C, and the supernatant was used for the assays.

Immunohistochemical analysis

Iba-1 DAB immunohistochemical analysis was performed on 35 μ m brain slices obtained using a Leica cryostat machine. Endogenous peroxidases were deactivated with 3 % hydrogen peroxide in 0.1 M PB for 15 min. Sections were washed twice in 0.1 M PB then blocked in normal serum goat 5%, BSA 1%, Tween 0,1% in 0.1 M PB. Sections were incubated with primary antibody rabbit anti-Iba1 (1:500, Wako, cat no. 019–19741) in blocking buffer o/n at 4°C. After overnight incubation, sections were washed three times

with 0.5 % BSA in 0.1 M PB and incubated with secondary antibody (biotinylated goat-anti rabbit, 1:400, Vector labs, cat no. BA-1000) in 0.5 % BSA in 0.1 M PB for 1 h at room temperature. Sections were washed twice in 0.5 % BSA in 0.1 M PB and incubated with ABC elite vectastatin (Vector labs, cat no. PK-6100) for 1 h and then washed twice with 0.1 M PB. Sections were stained with Impact DAB (Vector labs, cat no. SK-4105) for 5 min. Sections were transferred to excess 0.1 M PB after immunoreactivity and mounted in VectaMount.

Microscopic Quantification and Image Analysis

By using the Paxinos and Watson rat brain atlas (Paxinos & Watson, 1986). The following regions of interest were selected for image analysis: frontal cortex, hippocampus CA1, central nucleus of amygdala (CeA), substantia nigra pars reticulata (SNpr) and white matter fiber tracts of the cerebellum. Three slices, one every 6th section, were analyzed. Slices were scanned and acquired electronically by Leica Aperio AT2, and a total of 4 pictures under identical conditions were taken for each slice, at a 20X magnification. Activated microglia pixel density (cells with an area > 200 μm^2) were quantified using the ImageJ software (National Institute of Health, version 1.45) by invoking a stitching algorithm (Preibisch et al., 2009).

Multiplex Assays of cytokine/Chemokine concentrations

For multiplexing experiments, rat cytokine / chemokine magnetic bead panels (RECYTMAG-65K, EMD Millipore, Billerica, MA) were used to analyze frontal cortex brain tissue lysate and serum. Frontal cortex was selected because previous experiments in human alcoholics had suggested that neuroimmune signaling was impacted in this area (Crews et al., 2013; Vetreno et al., 2013). The assay was run in accordance with the provided instructions. In brief, samples and pre-mixed antibody beads were combined and incubated at room temperature (RT) for 2 hours on a plate shaker. Contents of the wells were washed, and detection antibodies were added and incubated for 1 hour at room temperature. Without aspirating the contents, streptavidinphycoerythrin was added and incubated for 30 mins. Contents were removed and washed and sheath fluid/drive fluid/assay buffer was used to re-suspend the bead. Plates were either kept at 4° C before being read using a MAGPIX plate reader. The targets measured were eotaxin/CCL11, fractalkine, high-mobility group protein 1 (HMGB1), interleukin 1 alpha (IL-1 α), interleukin 1 beta (IL-1 β), interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 10 (IL-10), interleukin 12 (IL-12(p70)), interleukin 13 (IL13), interleukin 17A (IL-17A), interleukin 18 (IL-18), interferon gamma-induced protein 10 (IP-10), leptin, lipopolysaccharide-inducible CXC chemokine (LIX), nuclear factor kappa beta (NF- κ B) regulated on activation, normal T cell expressed and secreted (RANTES), tumor necrosis factor alpha (TNF- α), and vascular endothelial growth factor (VEGF).

ELISA

For ELISA experiments, rat NF- κ B1 / NF- κ B elisa kit (LSBio, cat no. LS-F21577), rat TLR4 elisa kit (LSBio, cat no. LS-F22360) and rat HMGB1 elisa kit (LSBio, cat no. LS-F23582) were used to analyze the frontal cortex brain tissue lysate and serum. Blood and

brain tissue samples were diluted in their respected buffer and added onto the 96 well sandwich ELISA kit plate. The kit was incubated at 37° C while gently shaking for 90 mins. Detection antibodies were added on top of the mixture in each well and then incubated for another hour. Wells were washed and incubated with a HRP complex for 30 mins. A final wash procedure was followed by the addition of TMB substrate. Stop solution was added to each well after 15 mins of incubation. The plate was then read on a colorimetric spectrophotometer (Molecular Devices VERSAmax tunable microplate reader) calibrated at the 450 wave length.

Statistical analyses—Statistical analyses were performed using SPSS (IBM Corp, Armonk, NY). The effects of treatment (EtOH-exposed vs. control) on microglia activation over time was assessed using a two factor ANOVA (ethanol vs. control $\times$ 3 different time points) and when significant main effects were found, one-way analysis of variance (ANOVA) was used as a post-hoc analysis. Values are presented as mean $\pm$ standard error of the mean (SEM) and significances were considered at $p < 0.05$. The effects of treatment (EtOH-exposed vs. control) on cytokine expression was analyzed by a nonparametric method (Mann-Whitney), to account for a non-normal distribution of data, with significances considered at $p < 0.05$. Values for cytokine expression are presented as a percentage of control animals (set to 100%).

Results

Effects of Chronic EtOH Exposure on microglia at vapor/air 35 days, 1 day and 28 days of withdrawal.

Iba-1 immunoreactivity and cytokine profiles were performed on adult animals exposed to air or ethanol vapors for 5 weeks. Analysis was done at 3 time-points after cessation of the exposure: immediately after cessation (Air/Vapor 35d), 1 day (WD 1d) or 4 weeks (WD 28d) later. The following five brain regions were investigated: frontal cortex (FCTX), the hippocampus (HC), the amygdala (AMYG), the substantia nigra (SN) and the cerebellum (CB) (Figure 1). Two factor ANOVA showed significant main effects of treatment in all of the brain areas studied (FCTX: $df=1,40$, $F=32.0$, $p<0.01$; AMYG: $df=1,45$, $F=75.6$, $p<0.01$; HC: $df=1,37$, $F=58.8$, $p<0.01$; SN: $df=1,33$, $F=58.1$, $p<0.01$; CB: $df=1,44$, $F=20.8$, $p<0.01$). Significant main effects of time were also seen in all brain regions (FCTX: $df=2,39$, $F=26.8$, $p<0.01$; AMYG: $df=2,44$, $F=26.8$, $p<0.01$; HC: $df=2,36$, $F=18.7$, $p<0.01$; SN: $df=2,32$, $F=21.0$, $p<0.01$; CB: $df=2,43$, $F=3.6$, $p=0.04$). Treatment times time interactions were also found to be significant in all brain regions except cerebellum (FCTX: $df=2,39$, $F=5.4$, $p<0.01$; AMYG: $df=2,44$, $F=9.8$, $p<0.01$; HC: $df=2,36$, $F=12.9$, $p<0.01$; SN: $df=2,32$, $F=12.2$, $p<0.01$; CB: $df=2,43$, $F=2.5$, $p=0.1$).

Post hoc ANOVA on Iba-1 density revealed significant effects of alcohol with increases in the EtOH-exposed group as compared to the control (Table 1). Significant increases were seen at 35 days of vapor exposure in all five brain regions. In the animals that were sacrificed at 24 hours following withdrawal, Iba-density was reduced compared to levels seen in the animals that were sacrificed during exposure but remained significantly elevated in all brain areas compared to controls. At 28 days following the termination of vapor

exposure, Iba-1 levels were not significantly different from controls in hippocampus or cerebellum but remained significantly elevated in frontal cortex, amygdala and substantia nigra.

Effects of Chronic EtOH Exposure on frontal cortex cytokine profile at vapor/air 35 days, 1 day and 28 days of withdrawal.

The relative amount of 15 cytokines/chemokines were measured in the serum and the frontal cortex using a multiplex system (Millipore) and ELISA kit for TLR4, NF- κ B and HMGB1. The results of the ethanol group are expressed as relative percentage of the control group's mean (set to 100%) and are presented in table 2. Significant pvalues presented are from Mann-Whitney nonparametric analysis.

LIX showed a slight decrease at day 1, HMGB1 showed a reduction and fractalkine showed an increase at 28 days withdrawal. The pro-inflammatory cytokines typically produced (that include IL-1 α , IL-1 β , IL-12, IL-18, TNF- α , and IL-17) and the anti-inflammatory cytokines (that include IL-4, IL-5, IL-10, IL-13) did not show significant variation at any time point (Figure 2). NF- κ B and TLR4 were also measured because these molecules are involved in the modulation of inflammation that can occur either in response to a pathogen (infectious inflammation) or to stimuli that can cause tissue stress and injury even when no pathogen is present (sterile inflammation), such as, for instance, exposure to ethanol. Neither one changed significantly (Figure 2).

Effects of Chronic EtOH Exposure on serum cytokine profile at vapor/air 35 days, 1 day and 28 days of withdrawal.

The results of the ethanol group are expressed as relative percentage of the control group's mean (set to 100%) and are presented in table 3. The following cytokines/chemokines showed a statistically significant reduction following 35 days of vapor: pro-inflammatory cytokines: IL-1 α , IL-1 β , IL-12p70, IL-17A, IL-18, and TNF α ; anti-inflammatory cytokines: IL-4, IL-5, IL-10, IL-13; chemokines: eotaxin, IP-10, LIX. The chemokines fractalkine and RANTES, and the signaling proteins HMGB1, TLR4, NF- κ B did not show significant variations (See Figure 3).

At day 1 withdrawal there was a significant reduction of pro-inflammatory cytokines: IL-1 α , IL-1 β , IL-12p70 and TNF α ; anti-inflammatory cytokines: IL-4 and IL-13; chemokine eotaxin, and increases in the proteins NF- κ B and TLR4. No significant changes were observed for IL-17A, IL-18, IL-5, IL-10, fractalkine, IP-10, LIX, RANTES, and the signaling protein HMGB1.

No significant differences in the cytokine profile were observed 28 days after cessation of ethanol vapor exposure compared to controls.

Discussion

Microglial activation was investigated in the present study as it is recognized to be involved as a possible target of ethanol-mediated brain changes in neuroimmune signaling and possibly neurodegeneration (He & Crews, 2008; Obernier et al., 2002; Qin et al., 2008; Saito

et al., 2016). We found that exposure to chronic intermittent ethanol vapors for 5 weeks robustly increased Iba-1 immunoreactivity in the frontal cortex, amygdala, hippocampus, substantia nigra and cerebellum.

Iba1 is a cytoplasmic calcium-binding protein with actin-crosslinking activity involved in aspects of motility-associated rearrangement of the actin cytoskeleton. It is found in several cell types but in the brain is uniquely observed in microglia where its increased expression is considered an index of activation (Ito et al., 1998). Microglia normally exists in a “resting” state and can become activated in response to several insults or stimulation including exposure to ethanol (For review see (Crews et al., 2017)). Although the exact relationship between the morphological and functional activation states during alcoholism is currently unclear, the data collected here clearly indicate that microglia responded to ethanol exposure by upregulating Iba-1. The changes in Iba-1 expression were similar but not identical across the brain. Immunoreactivity appeared to be most striking in the amygdala and the hippocampus followed by the substantia nigra, the frontal cortex and the cerebellum. Since ethanol is known to penetrate all brain areas equally the observed region-specific differences in microglia responses to alcohol indicate that Iba-1 elevation may result not only from a direct action of ethanol on these cells but include more complex interactions with other cell types. This idea is supported by data showing differences in microglial activation between hippocampus and cerebellum following neonatal alcohol exposure (Topper et al., 2015).

The intensity of Iba-1 immunoreactivity decreased rapidly upon ethanol withdrawal dropping by nearly 40% in 24 hours in all brain regions investigated, although it remained significantly elevated in all brain areas investigated. This sharp decrease can be considered as an indication that Iba-1 production in microglia is mainly dependent on the presence of alcohol. However, in frontal cortex, amygdala and substantia nigra, Iba-1 remained significantly higher even after 28 days of withdrawal. Although, the data collected here do not shed light on the biological significance of this prolonged expression, it is evident that ethanol exposure can have effects on Iba-1 expression that are still present long after the alcohol has been withdrawn.

Among the regions showing prolonged Iba-1 immunoreactivity, the frontal cortex provided the largest amount of material and was thus chosen as used to measure the most representative pro-(IL-1 α , IL-1 β , IL-12, IL-18, TNF- α , IL-17), anti- (IL-4, IL-5, IL-10, IL-13), inflammatory cytokines, chemokines (eotaxin/CCL11, fractalkine, IP-10, LIX, RANTES), as well as three molecules that are at the intersection between sterile and infectious inflammation (HMGB1, NF- κ B, TLR4). Several studies prior to this one investigated the effects of ethanol on the level of circulating and central cytokines (reviewed in (Crews & Vetreno, 2014)). While the results provide clear evidence that ethanol can alter cytokine levels, they also show that such changes are not always consistent and depend on the experimental model, the route of administration and level of ethanol exposure as well as the time points investigated. It should also be noted that these measurements were only performed in the frontal cortex and thus it cannot be assumed that they are similar in other brain regions. In general, levels of serum cytokines are elevated only by large doses of alcohol, possibly as part of the toxic effects of ethanol on liver, but are otherwise often found to be reduced (Mandrekar et al., 2006; Szabo & Iracheta-Vellve, 2015). The model utilized

here also found similar results. Moderate alcohol exposure reduced the level of pro- and anti-inflammatory cytokines investigated during alcohol exposure. These differences were still partially observed 1 day after withdrawal for eotaxin, IL-1 α , IL-1 β , IL-12 but also IL4 and IL-13 that were still lower compared to the control group. However, none of the results remained significant 28 days after withdrawal of the ethanol.

Cytokines are highly inducible proteins and their levels can fluctuate by severalfold within hours. Thus, it is difficult to speculate on the biological significance of these changes, however, one exception may be represented by the dynamic profile of NF- κ B, TLR4 and HMGB1, three molecules that are pivotal in the regulation of sterile inflammation. HMGB1 is a ubiquitously expressed nuclear protein that is released during necrotic cell death and can activate TLR4 receptors leading to NF- κ B mediated transcription to potentiate cytokine responses (Crews & Vetreno, 2016; Vetreno & Crews, 2014; Wang et al., 2001). Notably, *in vitro* and *in vivo* studies showed that the ability of ethanol to regulate pro-inflammatory genes was severely reduced in TLR-4 knock-out mice (Montesinos et al., 2016). The levels of NF- κ B, TLR4 or HMGB1 were unchanged in the serum during ethanol exposure. However, NF- κ B, and TLR4 were elevated at 24 hours of withdrawal, but were not significantly elevated at 28 days following withdrawal. The profile of this time course suggests that these molecules may increase as a sterile inflammation response to ethanol withdrawal following moderate exposure levels.

Only a few of these molecules showed differences in the frontal cortex in response to moderate ethanol exposure. Statistically significant results were seen only for HMGB1, which was found to be reduced at day 28 of withdrawal, and for the chemokine LIX which was lower after 24 hours of withdrawal and fractalkine that was elevated at 28 days of withdrawal. The possible significance of the reduction in HMGB1 is difficult to interpret. In fact, although it would be reasonable to assume this as an indication of a reduction in pro-inflammatory signal, measurements carried out on postmortem human alcoholic brains indicate that ethanol can mediate neuronal damage in the hippocampus by regulating not necessarily HMGB1 level but its action by increasing its binding to the endogenous miRNA let-7b to form a complex capable of activating TLR7 (Coleman et al., 2017). The elevation of fractalkine is particularly interesting since this chemokine is believed to be released by dying neurons following ethanol-induced apoptosis serving as a signal promoting microglial migration, clearance and local inflammation (Sokolowski et al., 2014). In this context, the observation that fractalkine is elevated at 28 days of withdrawal suggests that exposure to ethanol vapor may have resulted in delayed neuronal toxicity and possible inflammation.

Several studies have found lasting changes induced by chronic ethanol exposure that involve dysregulation of immune signaling systems and associated microglial activation (Felver et al., 1990; He & Crews, 2008; Qin et al., 2008). Some limitations of the present study include the fact that only frontal cortex was investigated and only male rats were studied, and it is important to note that sex specific differences have been reported in neuro-immune responses to stress (Hudson et al., 2014). However, the data presented here confirm the notion that chronic moderate ethanol exposure activates microglia cells and further demonstrates that a dynamic and regional profile of activation occurs during acute and prolonged withdrawal. Our data additionally provide additional evidence that moderate

alcohol exposure can reduce cytokine levels in serum while producing cytokine elevations in brain.

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Highlights

- Chronic ethanol exposure induces microglial activation, which remained elevated at day 28 of withdrawal in some brain areas.
- Ethanol exposure is associated with a transient reduction of the serum level of the major pro- and anti-inflammatory cytokines/chemokines and a transient increase of effectors of sterile inflammation.
- Chronic ethanol exposure induces little or no changes of the major pro- and anti-inflammatory cytokines/chemokines in the frontal cortex.
- Ethanol exposure produces robust microglial activation, however, measures of inflammation in the blood differ from those in the brain over a protracted time course.

Figure 1:

Effects of ethanol and its withdrawal on microglia. (Left) Histograms showing the quantification of the relative intensity of Iba-1 immunoreactivity in the indicated brain regions of animals exposed for 5 weeks to intermittent ethanol vapors collected before withdrawal (Air/Vapor 35 days), 1 day (WD1d) or 28 (WD 28d) days after withdrawal. (Right) Representative immunoreactivity of Iba-1 for adjacent histograms, indicated scale is 50µm. Experiments were performed on n= 8 animals per group. Data are expressed as mean pixel intensity with significances indicated from post hoc ANOVA. Error bars = S.E.M. *p< 0.05.

Figure 2:

Histograms showing the cytokine profile in the frontal cortex collected from rats exposed to intermittent ethanol vapors for 5 weeks (2A: Air/Vapor 35 days), 1 day (2B: withdrawal 1 day) or 28 days after withdrawal (2C: withdrawal 28 days). Experiments were performed on $n = 8$ rats per group. Data and SEM presented as percentage of the controls (reference set at 100%) that were not exposed to ethanol vapors. * $p < 0.05$.

Figure 3:

Histograms showing the peripheral level of cytokines. Analysis was carried out on serum collected from rats exposed to intermittent ethanol vapors for 5 weeks (3A: Air/Vapor 35 days), 1 day (3B: withdrawal 1 day) or 28 days after withdrawal (3C: withdrawal 28 days). Experiments were performed on n= 8 rats per group. Data and SEM presented as percentage of the controls (reference set at 100%) that were not exposed to ethanol vapors. *p< 0.05.

Table 1

Iba-1 measures of microglia activation.

	Control	Ethanol	df	F Stat	P value
Vapor/Air 35 Days	Mean ± SEM	Mean ± SEM			
Frontal Cortex	178.8 ±13.0	561.0 ±49.7	1,14	62.5	<0.001
Amygdala	254.5 ±23.0	802.9 ±62.4	1,15	68.0	<0.001
Hippocampus	221.0 ±28.82	817.9 ±92.5	1,11	50.5	<0.001
Substantia Nigra	201.8 ±31.12	705.8 ±92.4	1,11	35.1	<0.001
Cerebellum	157.2 ±20.32	327.8 ±42.0	1,15	13.4	0.003
Withdrawal 1 Day					
Frontal Cortex	118.7 ±22.3	395.6 ±106.6	1,12	5.5	0.038
Amygdala	212.2 ±20.4	593.1 ±85.7	1,14	16.4	0.001
Hippocampus	223.0 ±22.6	466.3 ±65.3	1,10	18.7	0.002
Substantia Nigra	156.7 ±21.8	371.4 ±59.4	1,9	15.6	0.004
Cerebellum	129.1 ±4.4	224.7 ±13.9	1,14	38.1	0
Withdrawal 28 days					
Frontal Cortex	99.5 ±11.9	151.8 ±20.7	1,12	5.2	0.044
Amygdala	116.0 ±12.9	233.6 ±31.3	1,14	10.8	0.006
Hippocampus	167.2 ±26.3	279.5 ±64.5	1,14	2.9	0.114
Substantia Nigra	138.5 ±13.6	235.0 ±18.7	1,11	17.1	0.002
Cerebellum	156.8 ±30.5	203.6 ±37.0	1,13	1.0	0.345

Means and S.E.M. shown for post hoc ANOVA by treatment group.

Table 2

Frontal Cortex cytokine profile

	Vapor (35 days)				Withdrawal (1day)				Recovery (28 days)			
	% Ctrl	MWU	Z	P	% Ctrl	MWU	Z	P	% Ctrl	MWU	Z	P
IL-1 α	148.9	11	-0.3	0.74	84.7	6.5	-1.2	0.23	87.4	7.5	-0.6	0.52
IL-1 β	94.8	20	-0.6	0.57	95	17.5	-0.5	0.62	84	5	-1.8	0.07
IL-12p70	112.3	23.5	-0.1	0.9	82	8.5	-1.9	0.06	90.5	9	-1.1	0.26
IL-17A	99.1	24	-0.1	0.95	87.5	12	-1.3	0.2	87.3	9.5	-1	0.31
IL-18	92.1	19	-0.7	0.48	107.9	18	-0.4	0.67	93.2	12	-0.5	0.58
TNF α	118.7	17	-1	0.32	92.4	10	-1.7	0.09	91.6	8.5	-1.2	0.22
	% Ctrl	MWU	Z	P	% Ctrl	MWU	Z	P	% Ctrl	MWU	Z	P
IL-4	101.7	22.5	-0.3	0.8	92.3	16	-0.7	0.47	88.1	6.5	-1.6	0.12
IL-5	127.8	12	-0.9	0.36	72.5	8.5	-1.6	0.1	90.7	7	-0.5	0.59
IL-10	103.4	22	-0.3	0.75	87	12	-1.3	0.2	77.4	8	-1.3	0.2
IL-13	141.5	17.5	-0.1	0.94	69.5	6	-1.7	0.09	101	14	-0.2	0.85
	% Ctrl	MWU	Z	P	% Ctrl	MWU	Z	P	% Ctrl	MWU	Z	P
Eotaxin	104.2	23.5	-0.1	0.9	95.1	9.5	-1.7	0.09	96.9	7.5	-1.5	0.13
Fractalkine	92.5	14	-1.3	0.18	98.7	18	-0.4	0.67	114.3	3	-2.2	0.03
IP-10	70.8	11	-1.7	0.09	120.7	8	-1.9	0.06	96.9	13	-0.4	0.72
LIX	102.8	21.5	-0.4	0.7	97.2	6.5	-2.1	0.04	99.3	8.5	-1.2	0.23
RANTES	101.6	19	-0.7	0.48	105	13	-1.1	0.25	92.6	8	-1.3	0.2
	% Ctrl	MWU	Z	P	% Ctrl	MWU	Z	P	% Ctrl	MWU	Z	P
HMGB1	103.2	24	-0.1	0.95	74.1	10	-1.5	0.14	75.6	5	-2.2	0.03
NF- κ B	112.1	16	-1.1	0.28	88.6	16	-0.6	0.56	91.1	18	-0.3	0.77
TLR4	121.7	17	-1	0.34	87.1	14	-0.9	0.38	98.4	20	0	1

Percent control (% ctrl) equivalent of the means presented in vapor exposed animals for each cytokine at three time points. Non-parametric statistical results from Mann Whitney U (MWU), Zscore (Z) and P value (p) shown. P values less than 0.05 are shown in bold.

Table 3

Serum cytokine profile

	Vapor (35 days)				Withdrawal (1day)				Recovery (28 days)			
	% Ctrl	MWU	Z	P	% Ctrl	MWU	Z	P	% Ctrl	MWU	Z	P
IL-1 α	52.2	0	-3.1	<0.01	73	7	-2	0.05	93.5	11	-0.7	0.47
IL-1 β	77	9	-2	0.05	55.6	3	-2.6	0.01	80.7	7	-1.5	0.14
IL-12p70	66.2	1	-3	<0.01	81.2	3.5	-2.5	0.01	94.5	11	-0.7	0.46
IL-17A	52.1	0	-3.1	<0.01	74.1	9	-1.7	0.09	86.9	10	-0.9	0.36
IL-18	58.5	5.5	-2.4	0.02	53.6	14	-1	0.32	141.1	10	-0.9	0.36
TNF α	69.1	0	-3.1	<0.01	86.5	7.5	-1.9	0.05	85.6	9.5	-1	0.31
	Vapor (35 days)				Withdrawal (1day)				Recovery (28 days)			
	% Ctrl	MWU	Z	P	% Ctrl	MWU	Z	P	% Ctrl	MWU	Z	P
IL-4	61.9	0	-3.2	<0.01	78.7	6	-2.2	0.03	85.1	10.5	-0.8	0.41
IL-5	78.3	0	-3.1	<0.01	92	12	-1.3	0.2	95.4	11.5	-0.6	0.52
IL-10	52.7	1	-3	<0.01	63	9	-1.7	0.09	90.9	9	0	1
IL-13	51.7	0	-3.1	<0.01	75.9	6.5	-2.1	0.04	88.5	12	-0.6	0.57
	Vapor (35 days)				Withdrawal (1day)				Recovery (28 days)			
	% Ctrl	MWU	Z	P	% Ctrl	MWU	Z	P	% Ctrl	MWU	Z	P
Eotaxin	79	0	-3.1	<0.01	82.5	6	-2.2	0.03	96	12	-0.5	0.58
Fractalkine	87	10	-1.9	0.06	108.2	15	-0.9	0.39	93.9	15	0	1
IP-10	57.7	2	-2.9	<0.01	141.2	14	-1	0.32	116.8	8	-1.3	0.2
LIX	67.8	5	-2.5	0.01	91.1	15	-0.9	0.39	127	11	-0.7	0.47
RANTES	100.9	22	-0.3	0.75	119.7	16	-0.7	0.48	139.9	9	-1.1	0.27
	Vapor (35 days)				Withdrawal (1day)				Recovery (28 days)			
	% Ctrl	MWU	Z	P	% Ctrl	MWU	Z	P	% Ctrl	MWU	Z	P
HMGB1	89.4	24	-0.1	0.95	124.1	13	-1	0.31	100.3	19	-0.1	0.88
NF- κ B	113.2	19	-0.7	0.48	174.5	1	-2.8	0.01	101.7	17	-0.4	0.66
TLR4	102.9	20	-0.6	0.57	146.3	5	-2.2	0.03	128	7	-1.9	0.06

Percent control (% ctrl) equivalent of the means presented in vapor exposed animals for each cytokine at three time points. Non-parametric statistical results from Mann Whitney U (MWU), Zscore (Z) and P value (p) shown. P values less than 0.05 are shown in bold.