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# Increased expression of M1 and M2 phenotypic markers in isolated microglia after four-day binge alcohol exposure in male rats

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## Abstract

Microglia activation and neuroinflammation are common features of neurodegenerative conditions, including alcohol use disorders (AUDs). When activated, microglia span a continuum of diverse phenotypes ranging from classically activated, pro-inflammatory (M1) microglia/ macrophages to alternatively activated, growth-promoting (M2) microglia/macrophages. Identifying microglia phenotypes is critical for understanding the role of microglia in the pathogenesis of AUDs. Therefore, male rats were gavaged with 25% (w/v) ethanol or isocaloric control diet every 8 h for 4 days and sacrificed at 0, 2, 4, and 7 days after alcohol exposure (e.g., T0, T2, etc.). Microglia were isolated from hippocampus and entorhinal cortices by Percoll density gradient centrifugation. Cells were labeled with microglia surface antigens and analyzed by flow cytometry. Consistent with prior studies, isolated cells yielded a highly enriched population of brain macrophages/microglia (>95% pure), evidenced by staining for the macrophage/microglia antigen CD11b. Polarization states of $CD11b^+CD45^{low}$ microglia were evaluated by expression of M1 surface markers, major histocompatibility complex (MHC) II, CD32, CD86, and M2 surface marker, CD206 (mannose receptor). Ethanol-treated animals begin to show increased expression of M1 and M2 markers at T0 ($p$ = n.s.), with significant changes at the T2 time point. At T2, expression of M1 markers, MHC-II, CD86, and CD32 were increased ($p < 0.05$) in hippocampus and entorhinal cortices, while M2 marker, CD206, was increased significantly only in entorhinal cortices ($p < 0.05$). All effects resolved to control levels by T4. In summary, four-day binge alcohol exposure produces a transient increase in both M1 (MHC-II, CD32, and CD86) and M2 (CD206) populations of microglia isolated from the entorhinal cortex and hippocampus. Thus, these findings that both pro-inflammatory and potentially beneficial, recovery-promoting microglia phenotypes can be observed after a damaging exposure of alcohol are critically important to our understanding of the role of microglia in the pathogenesis of AUDs.

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## Introduction

According to new Diagnostic and Statistical Manual V criteria, an even larger portion of the U.S. population, nearly 14%, meets the diagnostic criteria for an alcohol use disorder (AUD) in any given year (Grant et al., 2015). Excessive alcohol (ethanol) use, characteristic of an AUD, causes significant neuropathology throughout the brain, including the limbic system, cerebellum, and cerebral cortex, according to both human studies and animal models (Crews & Nixon, 2009; de la Monte & Kril, 2014; Sullivan & Pfefferbaum, 2005). While human imaging studies have shown that both white and gray matter loss occurs in a variety of brain regions in alcoholics (Beresford et al., 2006; de la Monte & Kril, 2014; Mechtcheriakov et al., 2007; Pfefferbaum et al., 1992; Sullivan & Pfefferbaum, 2005), animal models of AUDs have been necessary to support the causal relationship between high blood alcohol concentrations characteristic of binge/bender pattern drinking and neurodegeneration (Collins, Corso, & Neafsey, 1996; Crews, Braun, Hoplight, Switzer, & Knapp, 2000; Crews & Nixon, 2009; Hunt, 1993; Kelso, Liput, Eaves, & Nixon, 2011). Binge drinking has been defined as imbibing 4 (women) or 5 (men) standard drinks within 2 h to produce blood ethanol concentrations (BECs) greater than 0.08 mg/dL (NIAAA; 2017), whereas a bender is repeated days of excessive intake including binge drinking. Over the last several years, studies have revealed that excessive alcohol exposure results in immune activation in the central nervous system (CNS), a phenomenon that several groups suggest is driving the pathogenesis of AUDs (Chastain & Sarkar, 2014; Crews & Vetreno, 2014; Cui, Shurtleff, & Harris, 2014; Davis & Syapin, 2004; Marshall et al., 2013; Vallés, Blanco, Pascual, & Guerri, 2004).

Microglia are the myeloid-lineage, resident immune cells of the CNS that become activated in response to insult (Ransohoff & Perry, 2009). Similar to what has been described in macrophages, microglia are often characterized as two distinct activated phenotypes: the M1 pro-inflammatory/classically activated phenotype, and the M2 anti-inflammatory/alternative activated phenotype (Benarroch, 2013; Beynon & Walker, 2012; Carson et al., 2007; C. Colton & Wilcock, 2010; Graeber, 2010; Raivich et al., 1999). While the M2 phenotypes promote tissue repair and phagocytosis of protein aggregates and cell debris, the M1 phenotypes are more likely to be detrimental to the brain by inducing neuronal toxicity through secretion of pro-inflammatory cytokine and chemokine and production of reactive oxygen species (ROS). Although we and others have documented the effect of ethanol on various neuroimmune activation markers and microglia in animal models of alcohol use and abuse (Alfonso-Loeches, Pascual-Lucas, Blanco, Sanchez-Vera, & Guerri, 2010; Blednov et al., 2005; Crews, Zou, & Qin, 2011; Fernandez-Lizarbe, Pascual, & Guerri, 2009; Marshall et al., 2013; McClain et al., 2011; Nixon, Kim, Potts, He, & Crews, 2008; Qin et al., 2008; Suk, 2007; Vallés et al., 2004), specific phenotypic M1 versus M2 markers have not been examined in the context of determining microglia phenotype. Therefore, the specific role of microglia in alcoholic neuropathology remains unclear. Identifying microglia activation

states (phenotypes) is critical for understanding the role of microglia in the pathogenesis of AUDs.

Microglia are routinely enumerated and classified by morphology and cell-surface markers using immunohistochemistry, and indirectly through assessment of cytokine expression (though multiple cell types could be the source, such as astroglia; Bedi, Smith, Hetz, Xue, & Cox, 2013; Beynon & Walker, 2012; Colton & Wilcock, 2010). For example, upregulation of ionized calcium binding adaptor molecule 1 (Iba-1) and monocyte chemotactic protein 1 (MCP1) immunoreactivity (He & Crews, 2008) and observation of proliferating microglia (Dennis et al., 2013; Sutherland et al., 2013) in human alcoholic brain provide the most compelling evidence for at least some level of activation, but neither of these phenomena are associated specifically with an M1 state (Raivich et al., 1999). Our recent work using these two methodologies has shown that four-day binge alcohol exposure, a model of an AUD, shows that microglia are activated but potentially not to a classically activated or M1 phenotype (Marshall et al., 2013; McClain et al., 2011). However, the specific and well-accepted phenotypic markers have not been examined. Therefore, the current study was designed to use Percoll separation/enrichment followed by three-color flow cytometric analysis of fluorescently labeled surface markers on microglia to enumerate microglia phenotypes from different brain regions, 48 h after four-day binge alcohol exposure. The fresh isolation of enriched cell suspensions enabled us to accurately quantify microglia activation states in entire populations of cells from regions of interest (hippocampus and entorhinal cortex) without reliance on manual morphometric counting of serial immunohistochemistry slides.

## Materials and Methods

### Rat Model of an AUD

All procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee and adhered to the Guide for the Care and Use of Laboratory Animals (NRC, 1996). Thirty-three adult, male Sprague Dawley rats (275–300 g, Charles River Laboratories, Raleigh, NC) were pair-housed in a University of Kentucky AALAC accredited vivarium with a 12-h light:dark cycle. Rats were allowed to acclimate to the vivarium for 2 days followed by 3 days of handling before any experimentation. Except during the binge periods when chow was removed, animals had *ad libitum* access to food and water. Following acclimation, rats were gavaged with ethanol (25% ethanol w/v in Vanilla Ensure Plus®, Abbott Laboratories, Abbott Park, IL; n = 17) or isocaloric control diet (added dextrose; n = 16) every 8 h for 4 days following a procedure modified from Majchrowicz (1975), as reported previously (Morris, Kelso, Liput, Marshall, & Nixon, 2010). Following an initial 5-g/kg ethanol dose, subsequent doses were titrated according to a 6-point intoxication behavior scale. BECs were determined in serum from tail blood collected 90 min following the seventh dose by an AM1 Alcohol Analyser (Analox, London, UK). Starting 10 h after the last dose of ethanol, withdrawal was observed for 30 min every 4 h for 16 h. Behaviors were scored based on a scale modified from Majchrowicz (1975) but identical to that reported previously (Morris et al., 2010).

### Isolation of microglia

Microglia were isolated from brain tissue by Percoll gradient centrifugation as described previously (Frank, Wieseler-Frank, Watkins, & Maier, 2006), with slight modification. Based on the time course of microglia activation in this model of an AUD, rats were humanely killed at 0, 2, 4, and 7 days following the last dose of ethanol (i.e., T0, T2, T4, and T7): rats were deeply anesthetized and transcardially perfused with 0.9% NaCl containing heparin. Brains were harvested and the hippocampus and entorhinal cortex were dissected on ice. Tissue was finely minced with a razor blade and gently homogenized in Dulbecco's phosphate-buffered saline (DPBS), pH 7.4, then passed through a 70-μm nylon cell strainer (VWR, Batavia, IL). Resulting homogenates were centrifuged at $400 \times g$ for 6 min and cell pellets were resuspended in 2 mL 50% isotonic Percoll (GE Healthcare, Piscataway, NJ). Two milliliters of 50% isotonic Percoll was gently layered on top of 1 mL 70% layer and then 1 mL 1× PBS was layered on top of the 50% Percoll layer. The density gradient was centrifuged at $1200 \times g$ for 45 min (minimum acceleration and brake) at 20 °C. Microglia were collected from the interphase between the 70% and 50% isotonic Percoll phases (Frank et al., 2006). Cells were washed in 1× PBS and then resuspended in sterile DPBS.

### Microglia staining and flow cytometry

Microglia were surface-stained with conjugated monoclonal antibodies to assess microglia purity (CD11b-FITC, BD Pharmingen, San Jose, CA; CD45-APC, eBioscience, San Diego, CA) and M1 activation markers (CD86-PE, FcγRIII[CD32]-PE, or MHC-II-PE, BD Pharmingen) as previous described (Bedi et al., 2013). For the M2 marker, microglia were stained with rabbit anti-rat CD206 (Abcam, Cambridge, MA), followed by a secondary incubation with donkey anti-rabbit-PE (BD Pharmingen). In brief, microglia were suspended in 50-μL incubation buffer (1× PBS + 0.1% bovine serum albumin) for 30 min on ice, and Fc receptors were blocked with anti-CD32 antibody (except for CD32 staining, eBioscience). Cells were incubated with antibodies for 30 min on ice in the dark. Cells were washed with 1× PBS and fixed with a formaldehyde-based fixation buffer (eBioscience #00-8222) on ice.

Data were acquired with an Attune Acoustic Focusing Cytometer (ABI, Carlsbad, CA) and analyzed with Attune Acoustic software (ABI). Before each run, the cytometer was calibrated with commercially available beads. Fluorescence spillover compensation values were generated using pooled non-stained cells and single-color staining as a control. Debris and aggregates were eliminated from the analysis by forward- and side-scatter characteristics. Myeloid cells identified as CD11b^{+} cells were divided into CD45^{low} microglia and a small CD45^{high} subpopulation (Fig. 1A). Polarization states of CD11b^{+}CD45^{low} microglia were evaluated for expression of M1 markers CD32, CD86, or MHC-II and M2 marker, CD206 (Fig. 3A, Figs. 3–6). For each sample, approximately 10,000 singlets were analyzed.

### Statistical analysis

All values are represented as mean ± SEM. To verify that alcohol exposure between time point groups was relatively similar, subject data of BEC and mean dose per day were analyzed by one-way ANOVA. As the primary question of interest in these studies is

whether the ethanol group differed from controls at each time point, planned comparisons were performed. Therefore, all comparisons were two-factors, control versus ethanol groups at each time point, and conducted via *t* tests. A *p* value <0.05 was accepted as statistically significant.

## Results

### Binge data

During four days of alcohol exposure, rats received an overall mean dose of 9.9 ± 0.7 g/kg/day of ethanol, which produced a peak blood ethanol concentration of 396.1 ± 82.5 mg/dL, measured at the third day of exposure. Subject data for each time point are shown in Table 1, and are generally similar between groups with the exception of the T0 BEC, which was significantly less than other time points ($F_{(3,12)} = 17.65$, $p < 0.05$). All other parameters are similar between time points. BEC is higher than that observed with the same model historically in the laboratory. However, the range of BEC values and the mean dose per day are remarkably similar to that reported previously (Marshall et al., 2013; Morris et al., 2010).

### Small CD45hi myeloid population increased after four-day binge

Myeloid cells were isolated from the hippocampus and entorhinal cortex at 0, 2, 4, and 7 days after alcohol exposure. These time points were selected based on our previous observations of microglial activation according to PK-11195 receptor autoradiography, microglia morphological change, and expression of cytokines in four-day binge ethanol-exposed rats versus controls at these times (Marshall et al., 2013). A simple mechanical dissociation was chosen to homogenize CNS tissue versus using an enzymatic digestion procedure in order to better preserve the immunophenotype of microglia and macrophages. Percoll-isolated cells were stained for CD11b (a component of complement receptor 3), which is constitutively expressed by microglia and macrophages and thus can be used to determine cell purity. Consistent with prior studies, isolated cells yielded highly enriched microglia and macrophages for immediate functional characterization *ex vivo* (Frank et al., 2006): over 95% pure as evidenced by staining for the microglia and macrophage antigen CD11b (Fig. 1A).

CD11b+ myeloid cells were further divided into microglia (CD11b$^+$CD45$^{low}$) and a small population of CD11b$^+$CD45$^{high}$ cells based on their CD45 expression (Bedi et al., 2013). CD11b$^+$CD45$^{high}$ cells have been described as endogenous CNS macrophages in a non-inflammatory context (Ford, Goodsall, Hickey, & Sedgwick, 1995) or as a combination of infiltrating monocytes/macrophages, neutrophils, and activated CNS microglia/macrophages in CNS injury scenarios (Jin, Ishii, Bai, Itokazu, & Yamashita, 2012; Stirling & Yong, 2008). We observed a small population of CD11b$^+$CD45$^{high}$ in hippocampus and entorhinal cortex of both control and alcohol-exposed rats. The frequency of these cells increased significantly in alcohol-exposed groups (3.42% ± 1.35 at T0, 4.64% ± 1.00 at T2) versus controls (1.25% ± 0.42 at T0, 1.35% ± 0.36 at T2) in hippocampus as well as in the entorhinal cortex in alcohol-exposed rats (2.28% ± 0.62 at T0, 6.85% ± 3.49 at T2) versus controls (1.51% ± 0.64 at T0, 1.57% ± 0.40 at T2), while complementary changes in

frequency of the majority $CD11b^+CD45^{low}$ microglial population were also noted in hippocampus at T2 (80.87% ± 7.88 in alcohol-exposed rats versus 93.02% ± 2.05 in control rats) and entorhinal cortex at T2 (84.24% ± 6.98 in alcohol-exposed rats versus 92.74% ± 0.89 in control rats).

CD11b is constitutively expressed by microglia, though expression increases with microglia activation (Hynes, 1992; Marshall et al., 2013; Morioka, Kalehua, & Streit, 1992). Four-day binge alcohol exposure significantly increased the expression of CD11b on $CD11b^+CD45^{low}$ microglia in both hippocampus (Fig. 2A) and entorhinal cortex (Fig. 2B) at T2. Alcohol exposure also increased CD45 expression on microglia in both hippocampus (Fig. 2C) and entorhinal cortex (Fig. 2D) at T0, with the most dramatic changes at the T2 time point, all of which resolved to control levels by T4.

### Increased expression of neuroimmune activation markers on the surface of microglia after four-day binge alcohol exposure

Activated microglia can be classified as either M1 or M2 based on morphology and/or cell surface antigens (Beynon & Walker, 2012; Colton & Wilcock, 2010; Ransohoff & Perry, 2009). Markers such as major histocompatibility complex (MHC) II, CD86, CD16/32, and iNOS have been used to identify M1-polarized cells (David & Kroner, 2011). M2 microglia, however, express the macrophage mannose receptor 1 (MMR, or CD206) on the cell membrane and Arginase-1, a prototypical M2 marker, intracellularly (Graeber, 2010; Nimmerjahn, Kirchhoff, & Helmchen, 2005). To assess global microglia/macrophage activation states after four-day binge alcohol exposure, we isolated myeloid cells from the hippocampus and the entorhinal cortices at T0, T2, T4, and T7 days after alcohol exposure. To characterize the activation profile of microglia ($CD45^{low}$) and CNS macrophages ($CD45^{high}$) after alcohol exposure, these cells were analyzed for several M1 (MHC-II and CD86, CD32) and M2 (CD206) surface markers (Fig. 3A).

Two markers involved in antigen presentation, MHC-II and CD86, were identified on both $CD11b^+CD45^{low}$ microglia and $CD11b^+CD45^{hi}$ cell populations (Fig. 3A). For MHC-II, as expected, the majority of microglia ($CD11b^+CD45^{low}$) were negative for MHC-II expression. After four-day binge alcohol exposure, the frequency of the $MHC-II^+$ cells increased slightly but not significantly in both hippocampus and entorhinal cortex at T0 ($p =$ n.s.), with significant changes at the T2 time point (Fig. 3; $p < 0.05$). At T2, we observed increased expression of $MHC-II^+$ cells in the hippocampus (2.73% in control rats to 11.28% in ethanol-exposed rats; $p = 0.009$, Fig. 3B) and in the entorhinal cortex (2.19% in control rats to 11.52% in ethanol-exposed rats; $p = 0.009$, Fig. 3C), all of which resolved to control levels by T4. The overall expression of MHC-II on $CD11b^+CD45^{high}$ cells (macrophages or neutrophils) is much higher than on $CD11b^+CD45^{low}$ microglia. Ethanol exposure does not affect the expression of MHC-II in the $CD11b^+CD45^{high}$ cell population (Fig. 3D & E).

For CD86 expression, the majority of microglia ($CD11b^+CD45^{low}$) were negative for CD86 expression (Fig. 4). After four-day binge alcohol exposure, the frequency of the $CD86^+$ cells in microglia increased slightly at T0 ($p =$ n.s.) only in entorhinal cortex (Fig. 4B), with the most dramatic changes at the T2 time point in both hippocampus and entorhinal cortex (Fig. 4). At T2, we observed increased expression of $CD86^+$ cells in the hippocampus (3.16% in

control rats to 9.14% in ethanol-exposed rats; $p = 0.011$, Fig. 4A), and in the entorhinal cortex (2.48% in control rats to 9.23% in ethanol-exposed rats; $p = 0.001$, Fig. 4B), all of which resolved to control levels by T4. The overall expression of CD86 in CD11b$^+$CD45$^{high}$ cells is much higher than CD11b$^+$CD45$^{low}$ microglia (Fig. 4C & D). Ethanol exposure increased the expression frequency of CD86 in the CD11b$^+$CD45$^{high}$ cell population in entorhinal cortex from 22.49% to 32.58% ($p = 0.04$).

While the functional role of the Fcγ receptors on microglia remains to be illuminated, CD16/32 has been widely used as an M1 marker in the CNS (Bedi et al., 2013; Kigerl et al., 2009). For CD32 (FcγRIII) expression, results showed that after four-day binge alcohol exposure, the frequency of the CD32$^+$ cells in CD11b$^+$CD45$^{low}$ microglia increased slightly at T0 ($p$ = n.s.), with the most dramatic changes at the T2 time point (Fig. 5). At T2, we observed increased expression of CD32$^+$ cells in the hippocampus (16.16% in control rats to 41.56% in ethanol-exposed rats; $p = 0.01$, Fig. 5A), and in the entorhinal cortex (21.07% in control rats to 47.74% in ethanol-exposed rats; $p = 0.004$, Fig. 5B). Although there was an increased frequency of CD32+ cells in CD11b$^+$CD45$^{low}$ microglia at T4 (from 21.55% in control rats to 33.66% in ethanol-exposed rats), it was not significant in hippocampus ($p = 0.20$; Fig. 5A), though there was a solid trend in entorhinal cortex (from 18.32% in control rats to 38.51% in ethanol-exposed rats; $p = 0.055$, Fig. 5B). The levels in ethanol-exposed rats were identical to the levels in control rats by T7. The overall expression of CD32 on CD11b$^+$CD45$^{high}$ cells is much higher than on CD11b$^+$CD45$^{low}$ microglia (Fig. 5C & D). Ethanol exposure increased the expression frequency of CD32 in CD11b$^+$CD45$^{high}$ cell population in entorhinal cortex (from 49.47% to 69.21%, $p = 0.035$).

CD206, macrophage mannose receptor 1, is the prototypical anti-inflammatory surface marker and has been used to identify M2 microglia in other CNS insults (Bedi et al., 2013; Beynon & Walker, 2012; Cherry, Olschowka, & O'Banion, 2014; Kigerl et al., 2009). In this study, it was used to evaluate the M2 marker expression on microglia/macrophages following alcohol exposure. The results showed that after four-day binge alcohol exposure, the frequency of CD206$^+$ cells in CD11b$^+$CD45$^{low}$ microglia in the hippocampus increased at the T2 time point, from 1.55% in control rats to 16.94% in ethanol-exposed rats, ($p = 0.105$, Fig. 6A), and in the entorhinal cortex from 1.97% to 15.81% ($p = 0.007$, Fig. 6B), all of which resolved to control levels by T4. The overall expression of CD206 on CD11b$^+$CD45$^{high}$ cells is much higher than on CD11b$^+$CD45$^{low}$ microglia (Fig. 6). Ethanol exposure did not affect the expression of CD206 on the CD11b$^+$CD45$^{high}$ cell population (Fig. 6C & D).

## Discussion

In this study, Percoll gradient centrifugation was used to isolate myeloid cells, followed by flow cytometric methods to evaluate the activation state (phenotype) of the macrophage/ microglia population in the hippocampus and entorhinal cortex in a four-day binge model of an AUD. To our knowledge, this is the first report using these combined methodologies to identify microglia phenotype within the alcohol research field. By using monoclonal antibodies against surface antigens to identify macrophages/microglia and characterize their polarization state, we found that alcohol exposure induces a significant increase of both

classically activated M1 and alternatively activated M2 microglia in hippocampus and entorhinal cortex after four-day binge alcohol exposure, with the most extensive changes at T2. This method has been shown to provide reproducible, sensitive, and quantitative measurement of microglia phenotypes in whole brain and/or brain regions of interest (Frank et al., 2006).

For cell isolation, we used mechanical dissociation of tissue followed by Percoll density gradient centrifugation to rapidly isolate microglia and macrophages from brain tissue. This non-enzymatic procedure allowed for the characterization of the cells without concern that their surface features had been enzymatically altered during tissue processing (Frank et al., 2006). This well-tested method has proven to be a powerful tool to study basic microglia biology and microglia immunophenotype and/or activation state under pathological conditions. The isolated cell population is not only sufficiently enriched with microglia and macrophages while excluding other cell types such as astrocytes, but these microglia/ macrophages also maintain their functional responsiveness after isolation (Barrientos et al., 2015; Frank, Baratta, Sprunger, Watkins, & Maier, 2007; Frank et al., 2006). Consistent with prior studies, our analysis revealed that purity and enrichment efficiency of myeloid cell suspensions yields over 95% CD11b+ cells (Fig. 1). Quiescent microglia typically display an MHC II-negative and CD86-negative immunophenotype (Colton & Wilcock, 2010; Frank et al., 2006; Guillemin & Brew, 2004). Flow cytometric analysis of isolated microglia from control rat brain also demonstrated that MHC II and CD86 expression was very low in control conditions (less than 3%), which provides additional evidence that the isolation procedure preserves the *in situ* activation state of microglia. One potential limitation of the study is that we used an anti-CD32 antibody to block Fc-mediated non-specific antibody binding instead of an isotype control (an antibody raised against an antigen not present on the cell type being analyzed). An isotype control will determine the non-specific binding of antibody to Fc receptors and ensure the observed staining is due to specific binding rather than an artifact. Our choice of the Fc receptor antibody to block nonspecific binding is common in studies with multiple antibodies used. To further reduce non-specific antibody binding, we also added bovine serum albumin to the staining buffer and titrated the antibody concentrations. Both the M1 and M2 marker expression is low in the control animals, which suggests that the background expression of these antibodies is very low. Without an isotype control, we cannot fully determine the background of our antibodies used, but the results generated from control and ethanol groups give us the relative quantification of the marker expression between control and ethanol-exposure groups.

Traditionally, the CNS has been described as an immune-privileged site, where the blood brain barrier limits movement of peripheral immune cells into the CNS. However, the blood brain barrier may be compromised by excessive alcohol consumption and become more permissive to immune cell extravagation (Alikunju, Abdul Muneer, Zhang, Szlachetka, & Haorah, 2011; Haorah, Knipe, Leibhart, Ghorpade, & Persidsky, 2005). Although there is no evidence of blood brain barrier compromise in the four-day binge model used (Marshall et al., 2013), a small population of CD11b$^+$CD45$^{high}$ cells was observed in both the hippocampus and entorhinal cortex, and the frequency of these cells increased in the ethanol-exposed group versus control animals (Fig. 1B & C). These cells displayed higher mean intensity values of CD11b and CD45 (Fig. 2) and a higher percentage of MHC-II$^+$/

$CD86^+/CD32^+/CD206$ staining than the $CD45^{low}$ microglial population (Figs. 3–6). $CD11b^+CD45^{high}$ cells have been described as endogenous CNS macrophages in a non-inflammatory context (Ford et al., 1995). In injured brains, $CD11b^+CD45^{high}$ cells are likely a mixed population of inflammatory myeloid cell types, such as infiltrating monocyte/macrophages, activated CNS microglia/macrophages, and possibly a few dendritic cells and neutrophils (Bedi et al., 2013). To further discriminate neutrophils from microglia/macrophages, a neutrophil marker such as Gr-1 could be used to eliminate neutrophils from this population before analysis.

Phenotypic markers such as MHC-II, CD86, iNOS, and CD16/32 have been used widely to identify M1-polarized cells (David & Kroner, 2011; Kigerl et al., 2009; Ponomarev, Veremeyko, Barteneva, Krichevsky, & Weiner, 2011), while CD206 (MMR) and arginase-1 have been used to differentiate M2 microglia (Graeber, 2010; Kigerl et al., 2009; Nimmerjahn et al., 2005). In this study, we chose to examine surface markers (MHC-II, CD86, CD32, and CD206) versus the intracellular markers (such as iNOS and arginase-1) to avoid permeabilization for intracellular staining to retain ability to sort live cells for future functional experiments. Although the heterogeneity of microglia has been well described in other neuroinflammatory diseases (Carson et al., 2007; Colton et al., 2006; Kigerl et al., 2009; Mikita et al., 2011; Zhang & Gensel, 2014), we are the first to identify various phenotypes of microglia under alcohol exposure using flow cytometric analysis of M1 and M2 surface markers. Our results show that four-day binge alcohol exposure induced a transient increase of classically activated M1 microglia (as indicated by the increased expression of MHC-II, CD86, and CD32), as well as alternatively activated M2 microglia as indicated by the increased expression of CD206.

Evidence that ethanol activates microglia to a pro-inflammatory stage has emerged from several studies showing increased expression of pro-inflammatory cytokines, chemokines, and danger-associated molecular patterns (DAMPs) in human alcoholic brain as well as in rodent models of AUDs (Antón et al., 2016; Blednov et al., 2012; Crews, Qin, Sheedy, Vetreno, & Zou, 2013; Flatscher-Bader et al., 2005; Liu et al., 2006; Vetreno & Crews, 2012; Vetreno, Qin, & Crews, 2013; Wang et al., 2015; see also Crews et al., 2006; Crews & Vetreno, 2014; Robinson et al., 2014 for review). For example, chronic alcohol exposure induces microglia expression of pro-inflammatory factors, including TNF-α, IL-1β, and IL-6 (Boyadjieva & Sarkar, 2010; Fernandez-Lizarbe, Montesinos, & Guerri, 2013), factors that are associated with alcohol drinking and preference (Bajo et al., 2014; Blednov et al., 2012; Robinson et al., 2014). Intermittent exposure to alcohol in rodent models activates microglia and stimulates production of pro-inflammatory and neurotoxic molecules including NO and COX2, cytokines such as TNF-α and IL-1β, and chemokines including MCP1 and MIP-1α and β (Alfonso-Loeches et al., 2010; Blanco & Guerri, 2007; He & Crews, 2008; Pascual, Blanco, Cauli, Miñarro, & Guerri, 2007; Qin et al., 2008; Vallés et al., 2004). In adult mice, chronic ethanol administration increases TNF-α levels in the brain and potentiates the LPS-induced increase in IL-1β levels in the brain (Qin et al., 2008). Some of the most compelling data for a role of innate immune activation in AUDs come from evidence of increased DAMP signaling, specifically high mobility group box 1 (HMGB1), which is evident in human brain tissue as well as animal models (Antón et al., 2016; Crews et al., 2013; Vetreno & Crews, 2012; Vetreno et al., 2013; Wang et al., 2015). The increased

expression of pro-inflammatory markers in these published studies supports the increase in M1 phenotypic markers observed here (Figs. 3–5). The microglia morphological changes were most robust on T2, which is also consistent with the increase in M1 marker expression as well as the timeline of the acute phase of neuroinflammation following brain injury (Ansari, 2015; Bedi et al., 2013; Ransohoff & Perry, 2009). Indeed, the relatively few effects at T0 may be due, in part, to blunted immune and/or neuroimmune function during ethanol intoxication (Aroor & Baker, 1998; Gano, Doremus-Fitzwater, & Deak, 2016; Goral, Karavitis, & Kovacs, 2008). Intriguingly, however, our previous studies showed that the same AUD model as used in the current experiments results in morphological changes in microglia consistent with activation (cellular processes become shorter, broader, and less branched while the somas exhibited hypertrophy), but without the induction of pro-inflammatory cytokines in entorhinal cortex or hippocampus (Marshall et al., 2013; McClain et al., 2011), an observation confirmed by others (Zahr, Luong, Sullivan, & Pfefferbaum, 2010). Thus, based on the morphological, immunohistochemical, and ELISA data in these reports, we concluded that microglia activation in this model was not pro-inflammatory (Marshall et al., 2013). While the immunohistochemical approaches are necessary for the assessment of cell morphology, flow cytometric analysis of the Percoll-enriched microglia reveals the global activation state of the *entire* microglial population of the region examined. For example, the increased expression of MHC-II in the current experiment was not expected based on negative MHC-II immunohistochemistry in our prior report (Marshall et al., 2013). However, immunohistochemistry is performed on only one out of every 12 brain sections, which makes it theoretically possible that MHC-II+ cells could be missed, especially if they were localized to a particular, small region (e.g., Ward et al., 2009). Therefore, the flow cytometric technique used in these studies is proving to be a more sensitive and quantitative measure, which may provide a more accurate characterization of an entire cell population (Bedi et al., 2013; Ransohoff & Perry, 2009). Although the observation of increased M1-like marker expression may appear contradictory, the ratio of M1 to M2 microglia would better define the contribution of these different phenotypes to the pro- or anti-inflammatory environment.

The increase in M2 marker, CD206, was predicted by our previous work and hypotheses that beneficial microglia may underlie plasticity and reparative responses following alcohol insult (Marshall et al., 2013; McClain et al., 2011). Multiple approaches – immunohistochemistry, morphology, and ELISAs of anti-inflammatory cytokines and growth factors – supported the conclusion that microglia after four-day binge alcohol exposure may be of the beneficial phenotype (Marshall et al., 2013; McClain et al., 2011). Indeed, the fold change in the percentage of CD11b+/CD45$^{lo}$ cells expressing CD206 is quite striking. When the current data are considered with our past work, namely ELISAs that consistently show increases in anti-inflammatory cytokine and growth factor expression, the data herein continue to support our hypothesis that microglia are of a beneficial phenotype. The finding of M2-type of microglia, however, is not inconsistent with innate immune induction via DAMPs eliciting primed microglia (Crews et al., 2013; Frank et al., 2016; Weber, Frank, Tracey, Watkins, & Maier, 2015). There is significant overlap between M2-like marker expression and primed microglia (Perry & Holmes, 2014; Ransohoff & Perry, 2009), and past work supports the idea that alcohol primes microglia (e.g., Qin et al., 2008),

including in the 4-day binge model (Marshall, Geil, & Nixon, 2016). Therefore, development of potential therapeutics that target microglia will need to be more specific than merely "inhibiting microglial activation". Ablating all microglia is often detrimental to brain recovery processes, which supports the conclusion that maintaining some microglia homeostatic functions or perhaps promoting the M2, reparative population, is necessary for plasticity and regenerative mechanisms (Cherry et al., 2014; Colton, 2009; Lalancette-Hebert, Gowing, Simard, Weng, & Kriz, 2007; Szalay et al., 2016; Yenari, Kauppinen, & Swanson, 2010). Therapeutic approaches that induce M2 polarization or specifically inhibit the pathological or chronic M1-like responses may be indicated. It is important to note, though, that some neuroimmune activation is necessary and perhaps beneficial; some TNF-α induction is required to elicit protective M2 responses (e.g., Lambertsen et al., 2009; Turrin & Rivest, 2006). It is possible that the increase in M1 markers reflects that point.

Microglia activation is a dynamic process that generates complex, overlapping patterns of surface marker expression in various neurodegenerative disease models (e.g., Hu et al., 2012; Kigerl et al., 2009) and, as we now describe, for a model of an AUD as well. Reproducible, quantitative measurement of microglia phenotypes in whole brains and/or regions of interest have made important contributions to our understanding of microglial biology in models of neurodegenerative and psychological diseases such as AUDs. This novel result of increased M2-like cells is critical to our understanding of the role of microglia in the development of and recovery from AUDs. Most especially, these data are important because microglial activation is an emerging therapeutic target for anti-inflammatory strategies in neurodegenerative disorders and CNS insults, including AUDs (Crews & Vetreno, 2014; Crews et al., 2011; Cui et al., 2014). It is critical to understand the phenotype of microglia induced by an insult in order to develop therapies specifically targeting pathological aspects of the neuroimmune response, likely the chronic pro-inflammatory M1 response, while maintaining or promoting beneficial M2 responses. As microglia phenotype has not been considered in the context of the pathogenesis of AUDs, these findings have important implications for drug discovery efforts on the pharmacotherapeutic treatment of AUDs.

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## Highlights

- Identifying microglia phenotypes is critical to understanding the role of microglia in AUDs.

- Flow cytometry of isolated microglia was used for the first time in alcohol research.

- Four-day binge alcohol exposure transiently increases M1 and M2 populations of microglia.
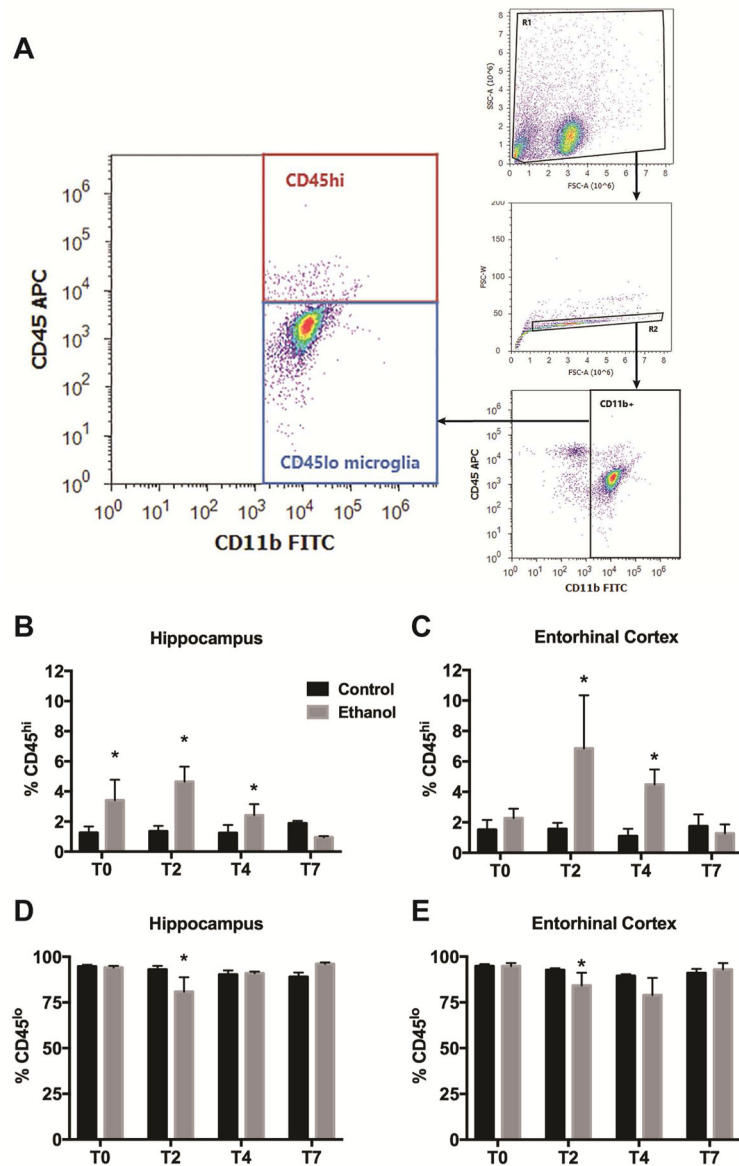
**Fig. 1. Two populations of CD11b$^+$ myeloid cells were identified from adult rat brains after a four-day binge alcohol exposure by flow cytometry**

At 0, 2, 4, and 7 days after the last dose of diet (i.e., T0, T2, T4, and T7), microglia were isolated from hippocampal and entorhinal cortex homogenates through Percoll density gradient centrifugation. Cells were labeled with microglia surface antigens and analyzed by flow cytometry. **A.** Debris and aggregates were eliminated from the analysis by forward- and side-scatter characteristics (small plots). Myeloid cells identified as CD11b$^+$ singlets were further divided into CD11b$^+$CD45$^{lo}$ microglia (blue box) and a small CD11b$^+$CD45$^{hi}$ cell subpopulation (red box). **B–E.** The relative frequencies of CD11b$^+$CD45$^{hi}$ cells **(B & C)** and CD11b$^+$CD45$^{lo}$ microglia **(D & E)** varied significantly between control and alcohol-exposed rat hippocampus **(B & D)** and entorhinal cortex **(C & E)**. *$p < 0.05$ versus control.
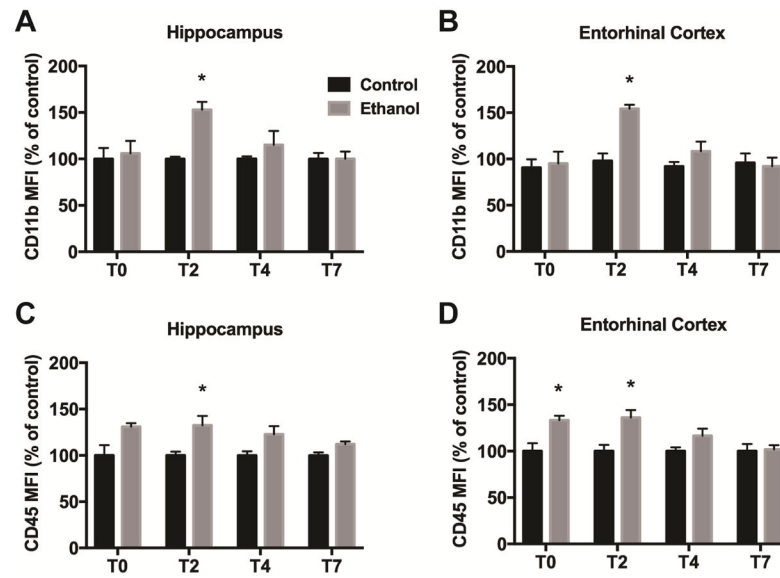
**Fig. 2. Mean fluorescent intensity (MFI) of CD11b and CD45 expression on CD11b$^+$CD45$^{lo}$ microglia**

**A–D.** Data presented show MFI of CD11b **(A & B)** and CD45 **(C & D)** on CD11b$^+$CD45$^{lo}$ microglia isolated from control and alcohol-exposed rat hippocampus **(A & C)** and entorhinal cortex **(B & D)** as a percent of control. *$p < 0.05$ versus respective control.
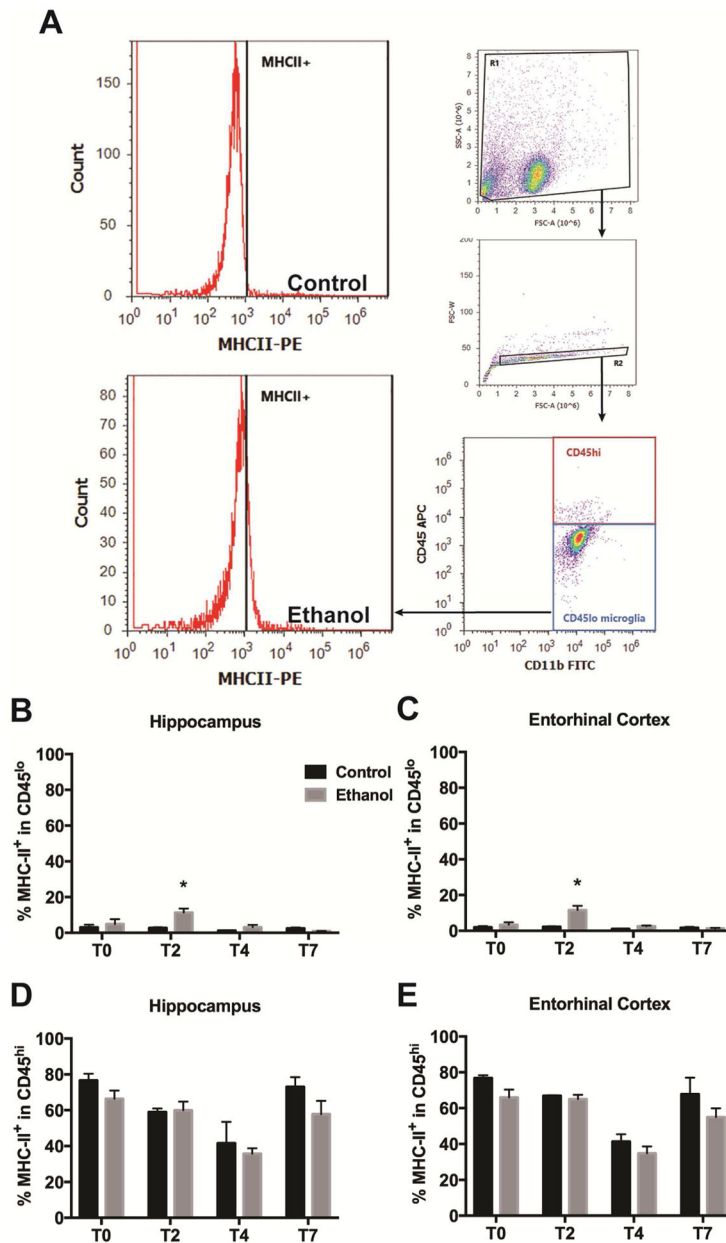
**Fig. 3. Increased expression of MHC-II on CD11b$^+$CD45$^{lo}$ cells after a four-day binge alcohol exposure**

**A.** MHC II$^+$ cells were enumerated in CD11b$^+$CD45$^{lo}$ microglia (blue box) and CD11b$^+$CD45$^{hi}$ cells (red box) after any remaining myelin debris and aggregates were eliminated by exclusion gates based on scatter characteristics (small plots). **B–E.** The percentage of MHC II$^+$ cells within CD11b$^+$CD45$^{lo}$ microglia (**B & C**) and CD11b$^+$CD45$^{hi}$ cells (**D & E**) varied significantly between control and alcohol-exposed rat hippocampus (**B & D**) and entorhinal cortex (**C & E**). *$p < 0.05$ versus respective control.
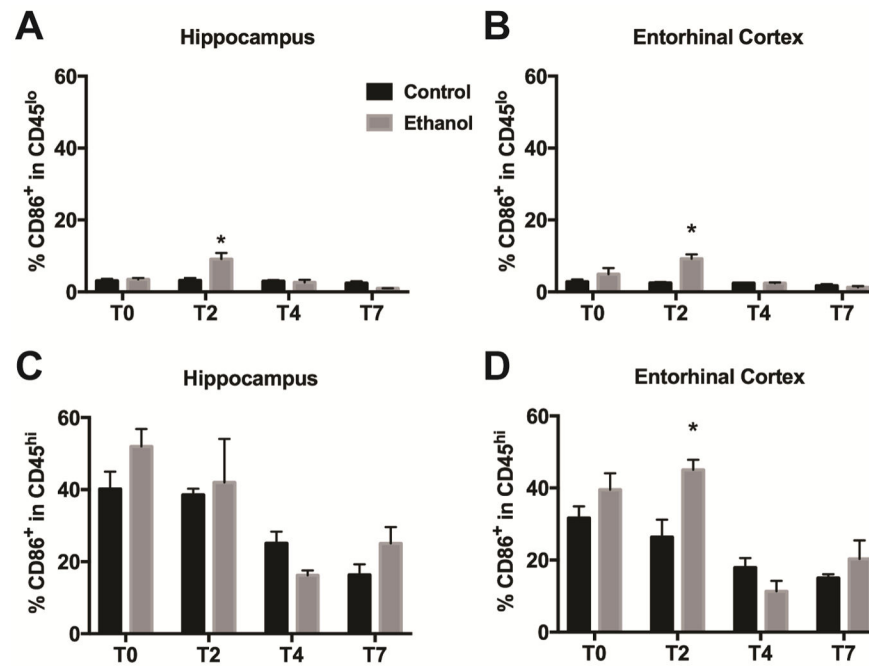
**Fig. 4. Increased expression of CD86 on CD11b$^+$CD45$^{lo}$ cells after a four-day binge alcohol exposure**

**A–D.** The percentage of CD86$^+$ cells within CD11b$^+$CD45$^{lo}$ microglia **(A & B)** and CD11b$^+$CD45$^{hi}$ cells **(C & D)** varied significantly between control and alcohol-exposed rat hippocampus **(A & C)** and entorhinal cortex **(B & D)**. $*p < 0.05$ versus respective control.
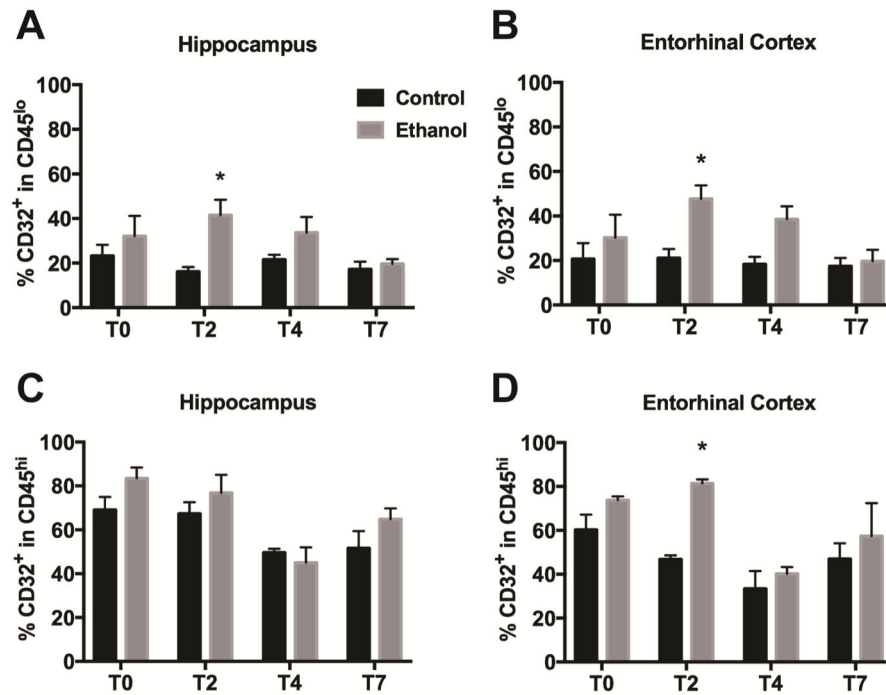
**Fig. 5. Increased expression of CD32 on CD11b$^+$CD45$^{lo}$ cells after a four-day binge alcohol exposure**

**A–D.** The percentage of CD32$^+$ cells within CD11b$^+$CD45$^{lo}$ microglia **(A & B)** and CD11b$^+$CD45$^{hi}$ cells **(C & D)** varied significantly between control and alcohol-exposed rat hippocampus **(A & C)** and entorhinal cortex **(B & D)**. *$p < 0.05$ versus respective control.

**Fig. 6. Increased expression of CD206 on CD11b$^+$CD45$^{lo}$ cells after four-day binge alcohol exposure**

**A–D.** The percentage of CD206$^+$ cells within CD11b$^+$CD45$^{lo}$ microglia **(A & B)** and CD11b$^+$CD45$^{hi}$ cells **(C & D)** varied significantly between control and alcohol-exposed rat hippocampus **(A & C)** and entorhinal cortex **(B & D)**. *$p < 0.05$ versus respective control.
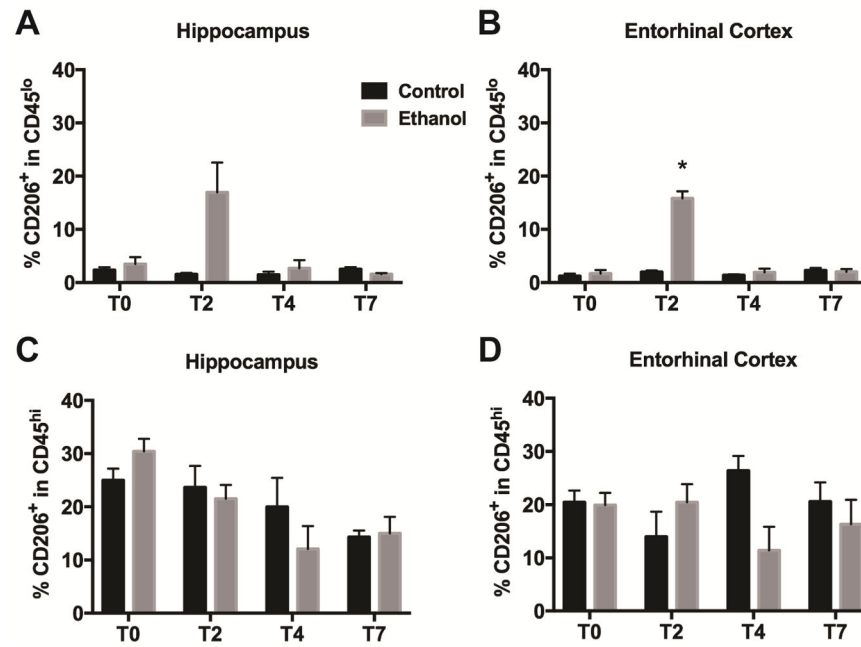
**Table 1**

Subject Data

| Time Point (Days) | Controls N | ethanol-exposed N | BEC (mg/dL) | Peak Withdrawal | Mean Withdrawal | Dose (g/kg/day) |
|---|---|---|---|---|---|---|
| T0 | 3 | 4 | $269.6 \pm 3.4^{*}$ | n/a | n/a | $10.2 \pm 1.0$ |
| T2 | 7 | 7 | $442.6 \pm 43.9$ | $3.1 \pm 0.3$ | $2.1 \pm 0.9$ | $9.7 \pm 0.4$ |
| T4 | 3 | 3 | $393.2 \pm 26.0$ | $3.1 \pm 0.6$ | $1.2 \pm 0.5$ | $10.6 \pm 0.6$ |
| T7 | 3 | 3 | $459.7 \pm 44.3$ | $3.3 \pm 0.5$ | $1.8 \pm 0.7$ | $9.6 \pm 0.8$ |

BEC = Blood ethanol concentration

*
$p < 0.05$