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A cross-sectional comparison of ethanol-related cytokine expression in the hippocampus of young and aged Fischer 344 rats

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Abstract

Our work in Sprague Dawley rats has shown rapid alterations in neuroimmune gene expression (RANGE) in the hippocampus and paraventricular nucleus of the hypothalamus (PVN). These manifest as increased Interleukin (IL)-6 and $\text{I}\kappa\text{B}\alpha$, and suppressed IL-1 β and Tumor necrosis factor alpha (TNF α) during acute ethanol intoxication. The present studies tested these effects across the lifespan (young adulthood at 2–3 months; senescence at 18 and 24 months), as well as across strain (Fischer 344) and sex. The hippocampus revealed age-dependent shifts in cytokine expression [IL-6, IL-1 β , Monocyte chemoattractant protein 1], but no changes observed in the PVN at baseline or following ethanol. RANGE in adults was similar across sex and comparable to effects seen in Sprague Dawley rats. Plasma corticosterone levels increased with age, while the blood ethanol concentrations and loss of righting reflex were similar in all groups older than 2 months. These findings indicate that the RANGE effect is largely conserved across strain and is durable across age, even in the face of a shifting neuroimmune profile that emerges during immunosenescence.

Keywords

cytokine; interleukin; immunosenescence; aging; ethanol; gene expression; sex differences

1. Introduction

A sizeable proportion of alcohol research is devoted to understanding drinking patterns and consequences in younger, at-risk populations (such as binge-drinking teenagers), as well as devising possible intervention strategies to curtail problematic drinking. However, substantially less attention is devoted to alcohol consumption on the other end of the

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developmental timeline, though the 65+ year old demographic makes up 13.4% of the American population (United States Census Bureau, 2012) and is considered by NIAAA to be a group particularly at risk due to harmful effects of alcohol consumption (NIAAA, assessed 2016). This segment of the population is steadily expanding, and though data regarding alcohol consumption in the elderly is limited, National Health and Nutrition Examination Surveys performed over the last two decades estimate older adult drinkers to consume alcohol on either a daily (17.9%) or at least weekly (57.0%) basis, with 42.1% of this age group participating in “harmful and/or hazardous drinking,” defined as consumption that may exacerbate or complicate existing alcohol-related problems or poses risks of future harm to individuals with pre-existing medical conditions (Wilson, Knowles, Huang, & Fink, 2014). Overall, these patterns of problematic alcohol consumption increase the likelihood of detrimental outcomes in this population not only because of the larger risk of falls and injuries (Immonen, Valvanne, & Pitkala, 2011), but also due to adverse interactions with chronic illnesses and/or the medications associated with them (for review, see Heuberger, 2009).

Aging is associated with profound changes in immune function, including increased signs of inflammation. For example, impaired antibody response is the leading cause of antibiotic-resistant diarrhea due to *Clostridium difficile* infection in the elderly (for review, see Shin, High, & Warren, 2016). Altered T cell ratios in old age have been linked with poor response to the influenza vaccine (for review, see Targonski, Jacobson, & Poland, 2007). Aging-related alterations in immune function frequently manifest as increased levels of pro-inflammatory cytokines in blood or specific organs. The cytokines most commonly associated with aging-related inflammatory alterations are Tumor necrosis factor alpha (TNF α) (Puchta et al., 2016), Interleukin (IL)-1 β (Malyshev, 2015), and IL-6 (Ferrucci, 1999). Of particular interest is the fact that some of these cytokines and chemokines, such as IL-6 and Monocyte chemoattractant protein (MCP)-1, are found to have increased basal presence in not only pathological cases, but in healthy aged individuals that have gone through what has been termed “successful aging” (Baggio et al., 1998; Valiathan, Ashman, & Deshratn, 2015). Together, these changes in the cytokine milieu that may be indicative of chronic low-grade inflammation have been referred to as “inflamm-aging,” with IL-6 playing a particularly important role in the study of gerontology (Ershler, 1993).

While these peripheral indices of aging and, more specifically, inflamm-aging, have been well characterized and related to certain disease states and risks, studies on brain-derived cytokines in aging have been comparatively limited. Animal studies have confirmed that peripheral inflamm-aging is accompanied by changes in the brain; for instance, it has been shown that increased levels of IL-6 are present in the brains of aged 24 month old BALB/c mice (Ye & Johnson, 1999; Ye & Johnson, 2001), likely as a result of spontaneous over-production by microglia in the CNS. It has also been found that IL-1 β , TNF α , CCL2/MCP-1, and IL-6 in the C57BL/6 mouse hippocampus are expressed at higher levels in senescence (24 months) as compared to lower levels during the earlier part of the lifespan (3–18 months) (Terao et al., 2002). However, not all pre-clinical studies support clear differences in peak inflammatory cytokine responses among aged rodents, but instead suggest that aged mice and rats may exhibit deficits in mechanisms that counter-regulate inflammation. For instance, blunted peripheral expression of anti-inflammatory cytokines

such as IL-10 has been shown in the aged animal following immune challenge and spinal cord injury models as compared to young controls (Williams, José, Brown, & Chambers, 2015; Zhang, Bailey, Braun, & Gensel, 2015). In the brain, exaggerated levels of cytokines are associated with aging, yet insensitivity to the effects of anti-inflammatory cytokines such as IL-10 has been reported, indicating shifts in the regulation of neuroinflammation (Norden, Trojanowski, Walker, & Godbout, 2016). Consistent with this, aged mice have been shown to exhibit sensitized cytokine responses and a protracted recovery from immune challenge (Huang, Henry, Dantzer, Johnson, & Godbout, 2008).

While alcohol abuse in the elderly is a significant health hazard, little has been done to examine the impact of ethanol on the neuroimmune system in senescence. In human postmortem studies, MCP-1 levels were found to be elevated in the amygdala, hippocampus, substantia nigra, and ventral tegmental area of alcoholics as compared to age-matched controls (He & Crews, 2008), though as the average age of the deceased was in the mid-60s, this examination did not cover the age group specified by NIAAA as being at a particularly high risk (NIAAA, assessed 2016). A more recent study examined MCP-1, IL-6, and TNF α expression in the aged rat hippocampus, cerebellum, and cerebral cortex following a 10-day binge ethanol exposure procedure and found no effects of ethanol on IL-6 and TNF α expression, though brain collection took place a day after the final exposure and was therefore not focused on changes induced by active intoxication or immediate withdrawal (Kane et al., 2013). However, MCP-1 was found to be elevated in the hippocampus and cerebellum at this time point. The hippocampus is a structure examined frequently in investigations of ethanol effects on the aged immune system, however, a large proportion of these studies focus on microglial morphology rather than cytokine expression (Dlugos & Pentney, 2001; Rintala et al., 2001).

Previous work from our laboratory has shown that acute ethanol exposure results in rapid alterations in neuroimmune gene expression (RANGE) across a variety of ethanol-responsive brain areas (paraventricular nucleus of the hypothalamus [PVN], bed nucleus of the stria terminalis [BNST], amygdala, and hippocampus). Prior studies have shown that across these brain regions, adult rats receiving acute doses of ethanol ranging from 3–5 g/kg i.p. or i.g. showed elevation of IL-6 and I κ B α (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, α ; a reporter of Nf- κ B activity) and suppression of TNF α and IL-1 β during intoxication (Doremus-Fitzwater et al., 2014; 2015; Gano et al., 2016). More recent work has shown that repeated ethanol administration (4 g/kg i.g.) resulted in sustained IL-6 and I κ B α expression in the hippocampus after as many as 6 once-daily exposures, whereas the amygdala and BNST showed signs of habituation. These alterations were independent of blood ethanol concentrations observed, and were seen after repeated daily administration as opposed to every-other-day exposure to the same dose, which did not produce changes in these brain regions, showing the schedule and site specificity of the RANGE effects (Gano et al., 2016). Interestingly, recent work indicated that adolescents display similar patterns of cytokine expression after acute ethanol challenge, though the effects were severely blunted relative to young adults in most structures (Doremus-Fitzwater et al., 2015). These intriguing findings suggest that developmental age might be a critical predictor of neuroimmune consequences of alcohol, and that the shifting of the neuroimmune profile towards an inflammatory state in old age may exacerbate the

immune response to ethanol. Thus, the primary goal of the present work was to perform a systematic examination of potential sex and aging-related differences in the neuroimmune response evoked by acute ethanol challenge. Experiment 1 extended RANGE characterization onto adult (~P100) Fischer 344 males and females during intoxication and withdrawal, as well as examining plasma corticosterone (CORT), progesterone (PROG), and blood ethanol concentrations (BECs). In Experiment 2, the adult Fischer 344 response to ethanol was compared to that of young adults (2 months) as well as aged rats (19 months) in the hippocampus and PVN. Loss of righting reflex (LORR) was assessed as a behavioral correlate in addition to examining blood ethanol concentrations and plasma corticosterone. Finally, in Experiment 3, adult animals were compared with middle-aged (9 months) and aged (18 and 24 months old) rats using hippocampal gene expression, plasma hormone response, loss of righting reflex, and blood ethanol concentrations.

2. Methods and materials

2.1 Subjects

Male and female Fischer (F) 344 rats were obtained from the National Institute of Aging (NIA) colonies at Taconic and Charles River. F344 rats are a strain commonly used in aging research due to their longevity and a general tendency to display symptoms of senescence (for instance, cognitive decline) that resemble certain aspects of human aging (Gallagher, Stocker, & Toh 2011; Sprott, 1991). Rats were housed in a colony maintained at $22\pm1^{\circ}\text{C}$ with 12:12 light–dark cycle, lights on at 0700. Rats were pair-housed (Exp. 1, 2) or single-housed (Exp. 3) in standard Plexiglas cages with *ad libitum* access to food and water and were handled briefly for two days prior to experimentation. Rats used in these studies were previously tested in a brief series of (non-invasive) social behavior tasks as described in Perkins et al. (2016). At all times, animals were maintained and treated in accordance with the guidelines set forth by the Institute of Laboratory Animal Resources (1996), and with protocols approved by the IACUC at Binghamton University.

2.2 Ethanol administration

The alcohol challenge consisted of 3 (Exp. 1) or 3.5 (Exp. 2, 3) g/kg ethanol (20% v/v). Sterile physiological saline (0.9%, TEKnova, Hollister, CA) was used to make the ethanol solution and vehicle-exposed rats were given equivolume saline only. Both solutions were given using intraperitoneal (i.p.) administration, and cage mates were assigned to the same experimental condition.

2.3 Blood and Tissue collection

Animals were rapidly decapitated (unanesthetized) 3 or 18 h after ethanol administration and trunk blood was collected into EDTA-coated Vacutainers. For all experiments, plasma was separated through refrigerated centrifugation and stored at -20°C . Brains were extracted and immediately flash-frozen via 15 sec submersion in methylbutane (VWR, cat no. JTQ223-8) on dry ice and stored at -80°C until use. Structures of interest were identified using a brain atlas (Watson & Paxinos, 2005) and dissected on a cold cryostat (Leica) with areas of interest collected using biopsy punches (VWR, Catalog # 100492-814,-816) and stored at -80°C . Regions of interest were as follows: the PVN and hippocampus for Exp. 1 and 2. In

Exp. 3, the hippocampus was collected into separate tubes bilaterally, with one side used for gene expression analysis with real-time RT-PCR.

2.4 Blood ethanol concentrations (BECs)

BECs were determined in 5- μ L plasma aliquots using an Analox AM-1 alcohol analyzer (Analox Instruments, Lunenburg, MA). The machine was calibrated every 15 samples using a 100 mg% industry standard, with BECs recorded in milligram per deciliter (mg%). The floor of assay sensitivity using the Analox is ~12–15 mg/dL, as evidenced by background BEC measurements obtained from rats never exposed to ethanol. As such, measurements at or below those observed in vehicle-injected rats should be interpreted as zero values (Gano et al., 2016).

2.5 Loss of righting reflex (LORR)

In Experiments 2 & 3, LORR data for the ethanol-injected animals were collected immediately following injection. A trough was formed by shifting bedding in the home cage, and animals were placed on their backs. Latency to lose the righting reflex was measured, defined as the inability to right one's position (turn over onto stomach) twice within 60 sec of being placed on its back, with the average of the two observations used as the time point. Once LORR was achieved, rats were checked every 10 min for return of righting reflex. Sleep time was calculated as wake time minus latency to sleep.

2.6 Plasma hormones

Plasma concentrations of corticosterone (CORT) and progesterone (PROG) were determined using commercially available EIA kits (Cat No: ADI-901-097 and ADI-901-011, respectively; Enzo Life Sciences, Farmingdale, NY) according to manufacturer's instructions, with the exception that samples were heat-inactivated to denature endogenous corticosteroid binding globulin (CBG) by immersion in 75°C water for 60 min (Buck et al., 2011). The CORT assay had a sensitivity of 27.0 pg/mL and inter-assay variability of 11.0%, the PROG assay had a sensitivity of 8.57 pg/mL and an inter-assay variability of 13.4%.

2.7 Real Time RT-PCR

Tissue was placed into a 2.0 mL Eppendorf tube containing 500 μ L Trizol® RNA reagent and a 5 mm stainless steel bead, and then homogenized using a Qiagen Tissue Lyser II. Following homogenization, RNA was extracted using Qiagen's RNeasy mini kit (catalog #74106), according to manufacturer's instructions. Synthesis and storage of cDNA was performed as described previously including a DNase treatment step, and probed cDNA amplification was performed and captured in real-time using the CFX384 Real-Time PCR Detection System (Bio-Rad, #185-5485) using our standard laboratory procedures (Doremus-Fitzwater et al., 2015). Primer sequences and accession numbers are listed in Table 1.

2.8 PCR normalization & statistical analysis

Across all experiments, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used a reference gene, with expression of GAPDH initially analyzed as a separate target to examine

its stability across experimental conditions. All data were adjusted relative to GAPDH using the 2⁻CT method (Schmittgen & Livak, 2008). Data were analyzed with Statistica software using analyses of variance (ANOVA) design, as described below. *Post-hoc* testing was done using Fisher's Least Significant Difference (LSD) for all observed main effects and interactions. An α -level of 0.05 was used as the criterion for significance for all effects. For brevity, only significant main effects and interactions are reported, and any main effect or interaction that is not explicitly described can be presumed to be non-significant.

2.9 Experiment 1 methods

The goal of Exp. 1 was to examine potential sex differences in RANGE effects during both intoxication and withdrawal in adult (~P100) Fischer 344 rats. Fischer 344 rats were utilized because eventual studies to be conducted in aged rats would use this strain. Two time points (3 h and 18 h) were assessed to ascertain potential differences in either intoxication or withdrawal-related changes in cytokines (Doremus-Fitzwater et al., 2014). The design of this experiment was a 2 (Sex: Male vs. Female) $\times$ 3 (Drug: Vehicle vs. 3 h EtOH vs. 18 h EtOH), N = 48; n = 8 per group. Rats were given 3 g/kg i.p. (20% v/v) ethanol injection or equivolume saline for the Vehicle control group either 3 or 18 hours before tissue collection. Vehicle injections were split evenly between the two time points and later collapsed into a single control group after being analyzed separately for baseline differences. Plasma concentrations of CORT and PROG were measured as general neuroendocrine indices of ethanol sensitivity, and ultimately correlated with BECs obtained in the same samples. In addition to examining genes that exemplified RANGE in prior work (i.e. IL-6, IL-1, TNF α , and I κ B α), c-Fos was examined as a marker of cellular activation. Cluster of differentiation (CD) 14 and MCP-1 were examined as markers of neuroimmune activation. Although CD14 is often used as a marker for myeloid-derived cells, its expression has been used as an index of immunological priming and has recently been linked to ethanol consumption behavior. The chemokine MCP-1, on the other hand, has been shown to be overexpressed in the brains of deceased alcoholics (Blednov et al., 2012; He and Crews, 2008) and elicits recruitment of immune cells.

2.10 Experiment 2 methods

The goal of Exp. 2 was to examine ethanol sensitivity via LORR and ethanol-induced cytokine expression changes in young and aged male Fischer 344 rats. The design of this experiment was a 2 (Drug: Control vs. Ethanol) $\times$ 3 (Age: 2 vs. 3.5 vs. 19 months), N = 48; n = 6–9 per group. In order to ensure that all rats would achieve LORR, the dose of ethanol administered was increased to 3.5 g/kg as compared to 3 g/kg in the previous experiment. Rats were given 3.5 g/kg i.p. (20% v/v) ethanol injection in the Ethanol group. The Control group consisted of Vehicle rats that were injected with equivolume saline and unmanipulated homecage control rats. No differences were observed between the control and vehicle-injected animals, so rats were combined into a single Control group. LORR data was collected for 3 h following injection, at which point tissue collection was performed. This time point was selected based on gene expression findings in Experiment 1, and as none of the rats had regained righting reflex at 3 h when tissue and blood were collected, it was not possible to collect data on BECs at awakening as an index of behavioral sensitivity to ethanol as awakening would have happened after this selected time point.

2.11 Experiment 3 methods

The results of Exp. 2 suggested modest differences in ethanol-induced cytokine expression in 19 month old rats relative to younger adults (3.5 mo). Because Exp. 2 was slightly under-powered, we performed a third experiment to further explore the apparent late-aging-related difference in ethanol-induced cytokine expression, including a broader age curve. Because previous studies revealed no significant sex differences in these effects, both males and females were included in this experiment in approximately equal proportions (3–5 males and 3–5 females in each group; Becker & Koob, 2016). The design of this experiment was a 2 (Drug: Ethanol vs. Vehicle) $\times$ 4 (Age: 3 vs. 9 vs. 18 vs. 24 months old), $n = 8$ –10 per group/ $N = 74$. Rats were given 3.5 g/kg i.p. (20% v/v) ethanol injection or equivolume saline for the vehicle control group. Three hours later, tissue was collected for analysis, with LORR data collected up until the point of tissue harvest.

3. Results

3.1 Experiment 1

3.1.1 Plasma—A 2×3 ANOVA revealed a main effect of Drug [$F(2, 42) = 1015.5$, $p < 0.0001$] on BECs. Rats at 18 h showed BECs comparable to those seen in the Vehicle group, indicating that clearance was achieved at this time for both sexes. A targeted t-test (planned *a priori*) was performed between Male and Female 3 h EtOH groups to probe potential sex differences at this time point, and showed comparable elevation in both groups [$t(14) = -1.53$, $p = 0.14$] (see Table 2).

A 2×3 ANOVA test of CORT (see Table 2) revealed main effects of Sex [$F(1, 39) = 18.62$, $p < 0.001$], Drug [$F(2, 39) = 262.26$, $p < 0.0001$], and a Sex $\times$ Drug interaction [$F(2, 39) = 3.56$, $p < 0.05$]. The Female Vehicle group exhibited moderately higher baseline CORT than the Male counterparts, higher peak CORT levels (3 h EtOH), but a return to their own relative baseline levels was seen in both Males and Females in the 18 h EtOH groups ($p < 0.001$ for all comparisons). There was a strong and significant correlation between BECs and CORT [$r(31) = 0.90$, $p < 0.0001$, $r^2 = 0.82$].

A 2×3 ANOVA test of PROG (see Table 2) revealed a similar pattern of effects compared to CORT; there were significant main effects of Sex [$F(1, 39) = 225.09$, $p < 0.0001$], Drug [$F(2, 39) = 447.14$, $p < 0.0001$], and a Sex $\times$ Drug interaction [$F(2, 39) = 3.99$, $p < 0.05$]. Higher baseline PROG levels were seen in Females as compared to Males in the Vehicle group, with peak PROG levels observed in the 3 h EtOH groups (higher in Females than Males), and return to their own relative baselines observed in the 18 h EtOH groups (all $p < 0.001$). PROG was correlated strongly with both BECs [$r(30) = 0.81$, $p < 0.0001$, $r^2 = 0.65$] as well as with CORT (Including Vehicle animals: [$r(43) = 0.93$, $p < 0.0001$, $r^2 = 0.86$]; when only Ethanol animals were analyzed: [$r(30) = 0.94$, $p < 0.0001$, $r^2 = 0.88$].

3.1.2 Hippocampus gene expression—A 2×3 ANOVA revealed similar patterns of expression of IL-6 (Figure 1A) and I κ B α (Figure 1B), with main effects of Drug [$F(2, 42) = 31.63$, $p < 0.0001$ and $F(2, 42) = 63.47$, $p < 0.0001$, respectively] in which the 3 h EtOH group exhibited elevation in comparison to Vehicle and 18 h EtOH (all $p < 0.0001$). IL-1 β

(Figure 1C) showed a significant effect of Drug [$F(2, 42) = 23.88, p < 0.0001$], and a Sex $\times$ Drug interaction [$F(2, 42) = 5.16, p < 0.01$], as well as a trend for a main effect of Sex that was not significant ($p = 0.057$). Both sexes showed a significant suppression in the 3 h EtOH group as compared to Vehicles ($p < 0.0001$) and 18 h EtOH ($p < 0.05$). Both Male and Female 18 h EtOH groups showed IL-1 β levels comparable to their own baseline Vehicle, however, the males had higher levels of IL-1 β in this group than did Females ($p < 0.01$). TNF α (Figure 1D) expression showed a main effect of Drug [$F(2, 42) = 58.46, p < 0.0001$], with 3 h EtOH groups showing suppression as compared to Vehicle and 18 h EtOH ($p < 0.0001$), and 18 h EtOH groups showing elevation compared to Vehicle ($p < 0.001$). There was a main effect of Drug on MCP-1 (Figure 1G) expression [$F(2, 41) = 20.09, p < 0.0001$], with suppression observed in the 3 h EtOH group as compared to both Vehicle and 18 h EtOH (both $p < 0.0001$). There were no effects observed in expression of c-Fos or CD14 (Figures 1E and F).

3.1.3 PVN gene expression— 2×3 ANOVA analysis revealed IL-6 (Figure 2A) to display a main effect of Drug [$F(2, 42) = 3.94, p < 0.05$], with 3 h EtOH group exhibiting an elevation as compared to Vehicles and 18 h EtOH ($p < 0.05$). I κ B α (Figure 2B) was similarly affected by Drug [$F(2, 42) = 25.89, p < 0.0001$], with the 3 h EtOH group exhibiting elevation compared to both others ($p < 0.0001$). A trend was observed in the expression of IL-1 β (Figure 2C) for an effect of Drug [$F(2, 42) = 2.67, p = 0.08$]. TNF α (Figure 2D) exhibited a main effect of Drug [$F(2, 42) = 35.78, p < 0.0001$] as well as a Sex $\times$ Drug interaction [$F(2, 42) = 3.44, p < 0.05$]; both sexes exhibited TNF α suppression in the 3 h EtOH group as compared to Vehicles (both $p < 0.0001$). Whereas the Females showed a return to baseline in the 18 h EtOH group, the Male 18 h EtOH group showed levels that were elevated above Vehicle levels for either Males ($p < 0.0001$) or Females ($p < 0.01$). There was a main effect of Drug on c-Fos (Figure 2E) expression [$F(2, 42) = 53.33, p < 0.0001$], with significant elevation seen in the 3 h EtOH group as compared to Vehicles and 18 h EtOH ($p < 0.001$). CD14 (Figure 2F) expression exhibited a main effect of Drug [$F(2, 40) = 46.86, p < 0.0001$], and an interaction [$F(2, 40) = 3.78, p < 0.05$], which, when further probed, revealed both sexes to show an elevation in the 3 h EtOH group as compared to Vehicles (both $p < 0.0001$), with the peak expression in the 3 h EtOH group in Females being higher than that of Males ($p < 0.05$). MCP-1 (Figure 2G) showed a main effect of Drug [$F(2, 39) = 15.68, p < 0.0001$] with the 3 h EtOH group showing suppressed levels as compared to the Vehicle and 18 h EtOH groups.

3.2 Experiment 2

3.2.1 Loss of Righting Reflex—A one-way ANOVA was used to analyze differences between 2, 3.5, and 19 month old animals' LORR data (Figure 3A). There was a main effect of Age on duration of LORR [$F(2, 14) = 8.96, p < 0.01$], with 2 month animals recovering LORR faster than either 3.5 or 19 month old groups ($p < 0.05$ for both). Two out of six of the 3.5 month animals and all six of the 19 months animals never recovered their LORR and were unresponsive at the 3 h timepoint set for tissue collection. Latency to lose the righting reflex was not affected by Age.

3.2.1 Plasma—Analysis of blood ethanol concentrations revealed a main effect of Drug [$F(1,42) = 144.00, p < 0.001$], with ethanol-injected animals displaying higher BECs than Controls ($p < 0.0001$), (Figure 3B). Analysis of plasma CORT levels revealed both a main effect of Drug [$F(1,42) = 250.13, p < 0.001$] and Age [$F(2,42) = 7.71, p < 0.01$]. *Post-hoc* analyses revealed higher corticosterone levels in 19 month old animals as compared to 2 and 3.5 month old animals ($p < 0.0001$ and 0.01 , respectively), an effect that reflects escalating baseline CORT as a function of age. Animals exposed to ethanol showed higher levels of corticosterone as compared to the Control group ($p < 0.01$) (see Table 2)

3.2.2 Hippocampus gene expression— 2×3 ANOVA analysis revealed main effects of Age [$F(2,36) = 4.23, p < 0.05$] and Drug [$F(1,36) = 117.09, p < 0.001$] on IL-6 expression (Figure 4A). The EtOH group displayed elevated expression of IL-6 as compared to Control ($p < 0.0001$). Nineteen month old animals displayed an overall higher level of IL-6 expression as compared to 3.5 month olds ($p < 0.05$), but narrowly missed significance as compared to slightly lower expression levels in the 2 month old rats ($p = 0.051$). There were significant effects of Age [$F(2, 36) = 7.82, p < 0.01$] and Drug [$F(1, 36) = 100.16, p < 0.0001$] on I κ B α expression (Figure 4B), as well as a significant Age $\times$ Drug interaction [$F(2, 36) = 5.24, p < 0.05$]. While all animals displayed ethanol-induced I κ B α elevation as compared to their age-matched Control group (all $p < 0.001$), the 19 month olds displayed an exaggerated response that was higher than those of the 2 ($p < 0.0001$) and 3.5 month old ($p < 0.01$) animals. There was a main effect of Age [$F(2,37) = 7.36, p < 0.01$] and Drug [$F(1,37) = 4.65, p < 0.05$] on IL-1 β expression (Figure 4C). The EtOH group displayed suppressed levels of IL-1 β as compared to Control ($p < 0.01$); 19 month old animals displayed higher levels of IL-1 β than 2 ($p < 0.01$), but not 3.5 ($p = 0.11$) month old animals. There was a main effect of Drug on the expression of TNF α [$F(1,37) = 61.17, p < 0.001$], with EtOH animals showing suppression as compared to Control ($p < 0.0001$) (Figure 4D). There were no effects of Age, Drug or interactions on c-Fos expression (Figure 4E). There was a main effect of Age on CD14 (Figure 4F) expression [$F(2,37) = 3.54, p < 0.05$], with higher expression seen in 19 month old animals as compared to both 2 and 3.5 month old groups ($p < 0.05$ for both). There was a main effect of Drug on MCP-1 (Figure 4G) expression [$F(1, 35) = 22.95, p < 0.0001$], with suppression observed in the EtOH group as compared to Control ($p < 0.0001$).

3.2.3 PVN gene expression—In 2×3 ANOVA analysis, IL-6 (Figure 5A) displayed a main effect of Drug [$F(1,42) = 21.62, p < 0.001$]. As expected, the EtOH group exhibited IL-6 elevation as compared to Controls ($p < 0.0001$). Similar patterns were observed in I κ B α (Figure 5B) with a main effect of Drug [$F(1, 39) = 71.10, p < 0.0001$]; elevation was observed in EtOH group as compared to Controls ($p < 0.0001$). There was an elevation of IL-1 β (Figure 5C) in the EtOH group as compared to Control ($p < 0.05$) due to a main effect of Drug [$F(1, 40) = 9.05, p < 0.01$]. TNF α (Figure 5D) likewise showed a significant main effect of Drug [$F(1,42) = 40.42, p < 0.001$], revealing a suppression in the EtOH group as compared to Control ($p < 0.0001$). There was a main effect of Drug on c-Fos expression, [$F(1,42) = 14.53, p < 0.001$], with elevated levels in EtOH as compared to Control ($p < 0.001$), (Figure 5E). There was a main effect of Drug on CD14 (Figure 5F) expression [$F(1,42) = 28.48, p < 0.001$], with the EtOH group showing elevation as compared to

Control ($p < 0.0001$). There was a main effect of Drug [$F(1, 35) = 8.56, p < 0.01$] on MCP-1 (Figure 5G) expression, with suppression observed in the EtOH group as compared to Control ($p < 0.01$).

3.3 Experiment 3

3.3.1 Loss of Righting Reflex—There was no effect of Sex or Age on the latency to lose righting reflex. All animals remained asleep until the tissue harvest 3 hours after injection, so sleep time was not analyzed (Table 3).

3.3.2 Plasma—Because sex differences in BECs and CORT were both expected and observed in previous experiments, sex was coded as a variable in initial analyses of blood measures. All EtOH animals displayed elevated BECs regardless of Age or Sex, however, there was a significant Age $\times$ Sex interaction when only ethanol-injected animals were analyzed [$F(3,30) = 3.14, p < 0.05$]; 3 month females displayed moderately lower BECs than any other group except the 24 month Males ($p < 0.05$ for all). When plasma CORT was analyzed in a full $2 \times 2 \times 4$ design, there was a main effect of Sex [$F(1, 58) = 10.16, p < 0.01$], Age [$F(3, 58) = 3.47, p < 0.05$, and Drug [$F(1, 58) = 354.73, p < 0.0001$], as well as a Sex $\times$ Drug interaction [$F(1, 58) = 9.66, p < 0.01$]. Eighteen month old animals displayed CORT levels significantly above 3 month old rats ($p < 0.01$). While saline-injected Males and Females showed equivalent CORT levels, the Female response to EtOH was higher than that of the Males ($p < 0.0001$). Analysis of plasma PROG likewise revealed main effects of Sex [$F(1, 58) = 58.18, p < 0.0001$], Age [$F(3, 58) = 7.96, p < 0.001$], and Drug [$F(1, 58) = 85.47, p < 0.0001$], as well as a Sex $\times$ Drug interaction [$F(1, 58) = 8.46, p < 0.01$]. Eighteen and 24 month animals showed PROG levels significantly above those seen in the 3 ($p < 0.01$) and 9 month old animals ($p < 0.05$). Saline-injected Females showed significantly higher PROG levels than their Male counterparts ($p < 0.0001$), and higher Ethanol-induced PROG than Males ($p < 0.0001$). For all these data, see Table 3.

3.3.3 Hippocampus gene expression—As the patterns of gene expression across sex were found to be similar in Experiment 1, both sexes were included in this experiment in equal proportions for a representative sample, and data were analyzed collapsed across sex using a 2 (Drug: EtOH vs. Vehicle) $\times$ 4 (Age: 3 vs. 9 vs. 18 vs. 24 months old) design. All gene expression data was adjusted to Vehicle 3 month old group as the ultimate control. IL-6 gene expression analysis revealed an Age $\times$ Drug interaction [$F(1,66) = 3.03, p < 0.05$], with baseline levels of IL-6 significantly higher at 24 months than any other age ($p < 0.05$). The overall response to ethanol was similar in magnitude across groups, but while 3, 9, and 18 month animals showed significant increases from their own within-age baselines ($p < 0.0001$), the 24 month rats had basal levels so high that they were not different from the 24 month old response to ethanol (Figure 6A). $\text{I}\kappa\text{B}\alpha$ (Figure 6B) exhibited a main effect of Drug [$F(1, 66) = 90.91, p < 0.0001$], with EtOH animals showing elevation as compared to Vehicle ($p < 0.0001$). IL-1 β (Figure 6C) revealed a main effect of Drug [$F(1,66) = 17.90, p < 0.0001$], with no effect of Age (trend: $p = 0.09$) observed. Ethanol administration resulted in a suppression of IL-1 levels across all age groups ($p < 0.001$). TNF α (Figure 6D) showed a main effect of Drug [$F(1,64) = 96.51, p < 0.0001$]. Ethanol administration resulted in a suppression of TNF α levels across all age groups ($p < 0.0001$). There was a main effect of

Drug [$F(1,66) = 7.91, p < 0.01$] on c-Fos expression, with Ethanol groups showing higher levels than Vehicles ($p < 0.01$), (Figure 6E). There was a main effect of Drug on CD14 (Figure 6F) expression [$F(1, 66) = 10.13, p = 0.01$], with higher levels observed in the EtOH group as compared to Vehicle ($p < 0.01$). There was a main effect of Age, with 18 and 24 month old animals showing elevation as compared to Vehicle ($p < 0.01$ and 0.001 , respectively), with 24 month olds' levels being higher as compared to 9 month olds as well ($p < 0.05$). There was a main effect of Drug [$F(1,64) = 5.45, p < 0.01$] on MCP-1 (Figure 6G) expression, with Ethanol groups showing suppression as compared to Vehicles ($p < 0.001$). There was also a main effect of Age [$F(3,64) = 5.45, p < 0.01$], with 9, 18, and 24 month animals showing higher levels of MCP-1 than 3 month animals ($p < 0.05, 0.01, 0.001$, respectively), but not showing differences between one another.

4. Discussion

Previous work from our laboratory has established a highly replicable pattern of Rapid Alterations in Neuroimmune Gene Expression (RANGE) produced by acute ethanol exposure (Doremus-Fitzwater et al., 2014; Doremus-Fitzwater et al., 2015; Gano, Doremus-Fitzwater, & Deak, 2016). Whereas previous studies have confirmed these effects under a variety of conditions, these utilized predominantly adult male Sprague Dawley rats, without consideration of subject characteristics such as potential sex- or late aging-related differences that might occur and how they might relate to the functional impact of ethanol exposure. Thus, the goal of the present work was to assess the generality of RANGE effects across strain, sex, and lifespan. Although some subtle differences were observed as a function of subject characteristics, the overall pattern of cytokine changes were reasonably consistent, thereby underscoring the generality – and perhaps importance—of future studies that will delineate the mechanisms by which alcohol exposure produces dynamic changes in neuroimmune gene expression across cycles of intoxication and withdrawal.

Strain differences were not directly compared in this work, yet several points can be made regarding ethanol effects observed here in F344 rats. First, the demonstration of a nearly identical pattern of cytokine gene expression changes (increased IL-6 and $\text{I}\kappa\text{B}\alpha$ and decreased IL-1 β and TNF α during intoxication) in F344 rats relative to previously published effects in Sprague Dawley rats underscores the highly reproducible nature of these effects and their generality across strain. Incidentally, we have observed this same pattern in Long Evans rats as well (data not shown). Second, the overall magnitude of cytokine changes in F344 rats was substantially larger relative to previously published effects in Sprague Dawley rats. This is consistent with the generally heightened sensitivity of F344 rats to stress challenges, an effect that is thought to be attributable to the “hyper-adrenergic” state of F344 rats (Sternberg et al., 1992), and prior studies showing enhanced cytokine responses in F344 rats relative to Sprague Dawley rats (Porterfield et al., 2011). In this way, the present findings fit well within both historical and contemporary aspects of the literature on strain differences.

In addition to a generalized “hyper-adrenergic” state, F344 rats also display hypothalamic-pituitary-adrenal (HPA) axis responses that are larger than other strains across the entire axis, including sensitized PVN expression of stress-related and immediate early genes, an

effect that has been shown to be influenced by cytokines (Rivest & Rivier, 1994; Sternberg et al., 1992). For these reasons, and because prior studies indicate robust RANGE effects in the PVN, the present studies targeted PVN cytokine expression and plasma CORT after acute ethanol challenge. The PVN is not known to be impervious to aging effects in general. Prior studies having shown that on various metrics, such as CRF mRNA expression and axonal/dendritic morphology (Cizza, Gold, & Chrousos, 1995; Itzev, Lolov, & Usunoff, 2003), it is a site sensitive to the effects of aging. However, the present studies show that in the F344 PVN, basal changes in cytokine expression were minimal, and RANGE effects were largely conserved across age. This suggests a certain resistance of the PVN to some of the signs of inflamm-aging, further supported by a lack of changes seen in the expression of immune activity markers such as CD-14 and MCP-1 across ages examined in these studies.

In contrast to the stability of the PVN, the functional hormone output of the HPA axis showed signs of aging-associated change, indicating that the aging-induced changes to HPA function are likely not governed by aspects of immunosenescence within the PVN. Corticosterone was assessed across sex and age as a marker of HPA sensitivity to ethanol, as well as a hormone previously shown to be affected by aging. PROG was measured in the same samples due to recent studies indicating a strong association between these two adrenal-derived hormones, even in male rats (Hueston & Deak, 2014). While the overall pattern of corticosterone and progesterone response to ethanol largely recapitulated findings from previous Sprague Dawley experiments, overall levels of corticosterone were high in the hyperadrenergic F344 strain relative to those typically reported in Sprague Dawley rats under comparable conditions. While the magnitude of the peak corticosterone response to ethanol was similar across age groups, 18 and 19 month old animals exhibited overall higher CORT levels, supporting accumulating evidence of an age-related hypersecretion of glucocorticoids that has been linked to impaired function of the limbic-hypothalamic brain circuits (Herman & Larson, 2001).

Examination of these hormones yielded the most profound sex differences observed across this series of studies. Females, as expected, exhibited higher basal levels of corticosterone and progesterone, as well as higher levels observed at the selected intoxication time point following ethanol. This differed strikingly from the lack of sex effect observed in central measures both at baseline as well as following ethanol administration. Overall, the lack of sex differences in RANGE effects produced by acute ethanol exposure were in stark contrast to differences observed in blood measures (CORT, PROG and to a lesser extent BECs). Indeed, sex differences in HPA axis responses are well precedented (for reviews, see Kudielka & Kirschbaum, 2005; Weinberg, Sliwowska, Lan, & Helleman, 2008) and, based on the present findings, do not appear to be predictive of neuroimmune gene alterations produced by acute ethanol exposure. With that said, CORT and PROG responses to acute ethanol challenge provide an important physiological context to the cytokine alterations, as previous studies have demonstrated a key role for neuroendocrine hormones in the regulation of cytokines (see Deak et al., 2015 for review).

There is not a wealth of information detailing the behavioral response to hypnotic doses of ethanol during senescence specifically, but reports indicate a likelihood of increased sensitivity to sedative drugs and altered pharmacokinetics in the elderly (Cherry & Morton,

1989). Blood ethanol concentrations following a 3 g/kg i.p. dose did not differ between sexes (Experiment 1), so in subsequent experiments, the higher 3.5 g/kg i.p. dose was used in order to reach a hypnotic state allowing for the measurement of loss of righting reflex as a behavioral correlate to intoxication. Young adult (2 months) animals used in Experiment 2 regained the righting reflex sooner than aged or adult counterparts, in concordance with research indicating no difference in latency to LORR in younger animals, but a shorter time spent asleep than adults (Broadwater, Varlinskaya, & Spear, 2011). Aged (18, 19, 24 months old) animals did not show differences in the propensity to lose the righting reflex nor in time spent asleep within this 3 hour window as compared to younger adults. Though the literature concerning the hypnotic effects of ethanol in aged rats is limited, there is evidence for acute ethanol increasing sleep time in aged versus young animals. Based on trajectories of BECs and accounting for the higher dose in our study, extending testing past our tissue collection point would have likely revealed differences in time of awakening as ethanol was further metabolized (Ornelas, Novier, Van Skike, Diaz-Granados, & Matthews, 2015).

A major emphasis of the present work was neuroimmune gene expression changes in the hippocampus. Overall, the pattern of ethanol induced changes was similar in aged rats (relative to young), though interpretation of these findings is obfuscated somewhat due to baseline, aging-related differences in the expression of cytokines, the chemokine MCP-1, and CD14 as a marker of innate immunity activity. Indeed, these changes were observed in both Exps 2 & 3, which supports the overall hypothesis of “inflamm-aging” that is now well-precedented in the literature (Stilling et al., 2014). Although the functional significance of ethanol-induced RANGE effects has not yet been established, aging-related changes in hippocampal gene expression have been implicated in connection with cognitive deficits and pre-disease states (Pawlowski et al., 2009; Verbitsky et al., 2004). An unbiased microarray probing of 24 month old F344 rats has revealed that upregulation of neuroinflammatory genes in the hippocampus was highly correlated with cognitive impairment (Blalock et al., 2003). By modifying gene expression in this area, ethanol exposure during late aging may be hypothesized to exacerbate certain cognitive deficits that accompany late aging.

As with any set of studies, certain limitations should be considered. The cross-sectional design of these studies has allowed us to get quick and full descriptions of RANGE effects across age and sex. However, having established this background, future studies should be longitudinal and include rats with a history of intermittent alcohol exposure across the lifespan in order to be more naturalistic. Prior work from our lab has shown adaptations in the PVN following prolonged exposure to ethanol in young adult rats, in contrast to the resiliency of cytokine expression in this brain region across aging (Doremus-Fitzwater et al., 2014); it is likely that while areas such as the PVN are protected from certain aging effects, the adaptations associated with prolonged ethanol use would compound dysregulated responses to immune challenge in an aged organism. Because the present studies utilized gene expression measures performed on brain punches, we cannot at present draw conclusions regarding whether the observed changes in gene expression reflect increased transcription, decreased degradation, or influx of peripheral immune cells into the CNS. Such studies are planned for the near future. Additionally, having examined RANGE at a previously well-characterized intoxication time point and knowing that pharmacokinetics shift across late development, collecting BECs and central inflammatory measures at the

time of awakening could be a priority for future studies. It is likely that while peak cytokine responses during intoxication could remain stable across age, the resolution of the inflammatory response could differ across ontogeny. Prior work has shown that the aged neuroimmune system can exhibit protracted neuroimmune activation following a peripheral immune challenge (Norden et al., 2016; Huang, Henry, Dantzer, Johnson, & Godbout, 2008). While a rapid resolution of RANGE is seen at 18 h in adult rats, examination of this time point in aged animals may yield alternative results that would help inform conclusions regarding alcohol effects on neuroimmune function in late aging. The extent to which these neuroimmune changes might reflect a response to tissue damage and/or repair-related responses produced by acute alcohol exposure is not yet clear. However, it can be noted that most studies examining the involvement of neuroimmune signaling pathways in Alcohol-Related Brain Damage utilize alcohol administration procedures that involve substantially greater alcohol load, often delivered over the course of days to weeks in order to elicit overt damage (Vetreno et al., 2016). Nevertheless, examination of damage- and repair-related processes should be a more directed consideration for future studies.

Although strain differences are likely to be observed in future studies where they are systematically manipulated, the overall pattern of the cytokine response to acute ethanol exposure appears to generalize across strain, sex, late aging, and to a large degree, brain structure. This underscores the importance of our recent findings showing blunted responses during adolescence (and the uniqueness of that developmental epoch), as well as the influence of a positive history of prior ethanol exposure at various windows in development (Doremus-Fitzwater et al., 2015). The complicated interaction between the shifting inflammatory milieu throughout developmental epochs and influence of ethanol consumption on top of that is important to untangle, as the affected key players (i.e. cytokines such as IL-6) are implicated in maintaining a healthy CNS. Recent years have shown immune-CNS interactions to be of vital interest not only as consequences but, potentially, as causes and therapeutic targets for addiction. Furthermore, the activity of cytokines shown to be affected by ethanol exposure may have relevant ties to other functions, for instance, in relation to motivated behavior (Yohn et al., 2016). Further investigation is necessary to identify potential mechanisms via which ethanol engenders cytokine adaptations, their precise relationship to the cognitive decline associated with senescence, and potential intervention strategies to alleviate these effects.

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Highlights

- Alcohol-induced neuroimmune changes generalize to Aged Fischer 344 rats.
- No sex differences in the neuroimmune response to alcohol were observed.
- Plasma CORT and PROG responses were higher in females than in males.
- The hippocampus may be more vulnerable to inflamm-aging than the PVN.
- Findings highlight the durability of alcohol effects across age, sex, and strain.

Hippocampus

Figure 1. Hippocampal gene expression Experiment 1

Experiment 1 hippocampal gene expression for (A) Interleukin-6, (B) nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (I κ B α), (C) Interleukin-1 β , (D) Tumor Necrosis Factor α , (E) c-Fos, (F) Cluster of differentiation 14 (CD14), and (G) Monocyte chemoattractant protein-1 (MCP-1). Tissue was collected either 3 or 18 h after 3.0 g/kg intraperitoneal ethanol injection (Vehicle animals injected with saline at both time points and combined). All data are expressed relative to the ultimate control group (Male Vehicle) and normalized to CyclophilinA. Main effect of Drug is indicated with an asterisk (*) to indicate significant differences in the 3 h EtOH group as compared to both Vehicle and

18 h EtOH, and various symbols to indicate groups that are different from one another (different symbols between bars indicate statistical difference). Significant interaction of Drug $\times$ Age is indicated by a lettering system in which bars that share a common letter are considered statistically comparable, whereas bars that do not share a common letter are identified as significantly different. All *post-hoc* comparisons were made using Fisher's Least Significant Difference post hoc test ($p < 0.05$).

Paraventricular nucleus of the hypothalamus

Figure 2. Paraventricular nucleus of the hypothalamus gene expression Experiment 1
 Experiment 1 paraventricular nucleus of the hypothalamus gene expression for (A) Interleukin-6, (B) nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα), (C) Interleukin-1β, (D) Tumor Necrosis Factor α, (E) c-Fos, (F) Cluster of differentiation 14 (CD14), and (G) Monocyte chemoattractant protein-1 (MCP-1). Tissue was collected either 3 or 18 h after 3.0 g/kg intraperitoneal ethanol injection (Vehicle animals injected with saline at both time points and combined). All data are expressed relative to the ultimate control group (Male Vehicle) and normalized to CyclophilinA. Main effect of Drug (all $p < 0.05$) is indicated with an asterisk (*) to indicate significant

differences in the 3 h EtOH group as compared to Vehicle and 18 h EtOH. Significant interaction of Drug $\times$ Sex is indicated by a lettering system in which bars that share a common letter are considered statistically comparable, whereas bars that do not share a common letter are identified as significantly different. All *post-hoc* comparisons were made using Fisher's Least Significant Difference post hoc test ($p < 0.05$).

Figure 3. Plasma and behavioral data from Experiment 2

In Experiment 2, rats of three Ages (2, 3.5, 19 months old) received 3.5 g/kg intraperitoneal ethanol injections or served as Controls (combined equivolume saline group and handled control group). Loss of righting reflex (LORR) data including (A) latency to lose LORR and time spent asleep was collected for 3 h following injection in the EtOH group. At the 3 h time point, trunk blood was collected and analyzed for (B) blood ethanol concentrations and (C) plasma corticosterone. Main effect of Drug ($p < 0.05$) is indicated with an asterisk (*) denoting a difference from the Control group. Main effect of Age ($p < 0.05$) is represented

with a pound sign (#) indicating a difference in that group from both other ages examined. All *post-hoc* comparisons were made using Fisher's Least Significant Difference post hoc test ($p < 0.05$).

Figure 4. Hippocampal gene expression Experiment 2

Experiment 2 hippocampal gene expression for (A) Interleukin-6, (B) nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα), (C) Interleukin-1β, (D) Tumor Necrosis Factor α, (E) c-Fos, (F) Cluster of differentiation 14 (CD14), and (G) Monocyte chemoattractant protein-1 (MCP-1). Rats of three Ages (2, 3.5, and 19 months old) were injected with 3.5 g/kg intraperitoneal Ethanol or served as Controls (combined equivolume saline group and handled control group), and tissue was collected 3 h after. All data are expressed relative to the ultimate control group (3.5 months Control) and

normalized to CyclophilinA. Main effect of Drug (all $p < 0.05$) is indicated with an asterisk (*) to indicate significant difference from the Control group. Age groups that are different from others are indicated by a pound sign (#; $p < 0.05$). Significant interaction of Drug $\times$ Age is indicated by a lettering system in which bars that share a common letter are considered statistically comparable, whereas bars that do not share a common letter are identified as significantly different. All *post-hoc* comparisons were made using Fisher's Least Significant Difference post hoc test ($p < 0.05$).

Paraventricular nucleus of the hypothalamus

Figure 5. Paraventricular nucleus of the hypothalamus gene expression Experiment 2
 Experiment 2 paraventricular nucleus of the hypothalamus gene expression for (A) Interleukin-6, (B) nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα), (C) Interleukin-1β, (D) Tumor Necrosis Factor α, (E) c-Fos, (F) Cluster of differentiation 14 (CD14), and (G) Monocyte chemoattractant protein-1 (MCP-1). Rats of three Ages (2, 3.5, and 19 months old) were injected with 3.5 g/kg intraperitoneal or served as Controls (combined equivolume saline group and handled control group) and tissue was collected 3 h after. All data are expressed relative to the ultimate control group

(3.5 months Control) and normalized to CyclophilinA. Main effect of Drug (all $p < 0.05$) is indicated with an asterisk (*) to indicate significant difference from the Control group. All *post-hoc* comparisons were made using Fisher's Least Significant Difference post hoc test ($p < 0.05$).

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Hippocampus

Figure 6. Hippocampal gene expression Experiment 3

Experiment 3 hippocampal gene expression for (A) Interleukin-6, (B) nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha ($I\kappa B\alpha$), (C) Interleukin-1 β , (D) Tumor Necrosis Factor α , (E) c-Fos, (F) Cluster of differentiation 14 (CD14), and (G) Monocyte chemoattractant protein-1 (MCP-1). Rats of four Ages (3, 9, 18, and 24 months) were given 3.5 g/kg intraperitoneal ethanol or equivolume saline injections and tissue was collected 3 h later. All data are expressed relative to the ultimate control group (3 months Vehicle) and normalized to CyclophilinA. Main effect of Drug (all $p < 0.05$) is indicated with an asterisk (*) to indicate significant difference from the Vehicle group. Main effect of Age (all $p < 0.05$) is indicated with a symbol system in which bar sets (Ages) that share a

common symbol are considered statistically comparable, whereas bar sets that do not share a common symbol are identified as significantly different. Significant interaction of Drug $\times$ Age is indicated by a lettering system in which bars that share a common letter are considered statistically comparable, whereas bars that do not share a common letter are identified as significantly different. All *post-hoc* comparisons were made using Fisher's Least Significant Difference post hoc test ($p < 0.05$).

Table 1
Primer sequences and accession numbers

Primers, accession numbers, and sequences used in real-time RT-PCR for all gene expression studies.

Primer	Accession Numbers	Oligo	Sequence
CD14 ¹	NM_021744	Forward	5'-AACAACGGATACCTGGCTCG-3'
		Reverse	5'-GTCCTTTCTCGCCCAACTCA-3'
c-Fos	NM_022197.2	Forward	5'-CCAAGCGGAGACAGATCAAC-3'
		Reverse	5'-AAGTCCAGGGAGGTCACAGA-3'
Gapdh ²	NM_017008	Forward	5'-ATGACTCTACCCACGGCAAG-3'
		Reverse	5'-AGCATCACCCATTGATGT-3'
IκBα ³	NM_080899	Forward	5'-CTGTTGAAGTGTGGGGCTGA-3'
		Reverse	5'-AGGGCAACTCATCTTCCGTG-3'
IL-1 ⁴	NM_031512	Forward	5'-AGGACCAAGCACCTTCTTT-3'
		Reverse	5'-AGACAGCACGAGGCATTTT-3'
IL-6 ⁵	NM_012589	Forward	5'-TAGTCCTTCTACCCAACTTCC-3'
		Reverse	5'-TTGGTCCTTAGCCACTCCTTC-3'
MCP-1 ⁶	NM_031530.1	Forward	5'-TCTCTGTACGCTTCTGGG-3'
		Reverse	5'-TGCTGCTGGTGATTCTTTG-3'
TNFα ⁷	NM_012675	Forward	5'-GGGGCCACCACGCTTCTTG-3'
		Reverse	5'-CGACGTGGGCTACGGGTTG-3'

¹Cluster of differentiation 14;

²Glyceraldehyde 3-phosphate dehydrogenase;

³Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha.

⁴Interleukin-1;

⁵Interleukin-6;

⁶Monocyte chemotactic protein 1;

⁷Tumor necrosis factor alpha.

Table 2

Adult (P100) male (σ) and female (φ) rats were injected with 3.0 g/kg i.p. ethanol or equivolume saline. Blood was collected 3 or 18 h after injection (Vehicle group collapsed across time points). Displayed are measures for blood ethanol concentrations (BECs; mg/dL), plasma corticosterone (CORT; $\mu\text{g/mL}$) and progesterone (PROG; ng/mL).

Measure		Vehicle	3 hours post-ethanol	18 hours post-ethanol
BECs	σ	1.83 $\pm$ 0.52	256.48 $\pm$ 8.23	3.03 $\pm$ 0.60
	φ	3.40 $\pm$ 0.58	233.263 $\pm$ 12.72	3.05 $\pm$ 0.49
Plasma CORT	σ	6.59 $\pm$ 1.20	59.37 $\pm$ 1.37	5.46 $\pm$ 1.06
	φ	14.81 $\pm$ 5.56	79.34 $\pm$ 4.17	9.93 $\pm$ 2.63
Plasma PROG	σ	0.29 $\pm$ 0.35	10.48 $\pm$ 0.30	0.64 $\pm$ 0.24
	φ	4.66 $\pm$ 0.39	16.90 $\pm$ 0.56	5.77 $\pm$ 0.68

Blood ethanol concentrations (BECs; mg/dL) taken 3 h after a 3.5 g/kg i.p. ethanol injection in 3, 9, 18, and 24 month old males (σ^E) and females (φ^E). Vehicle samples were also assessed (16.27 ± 1.27 collapsed across age and sex) but are not displayed in the table. Latency to lose the right reflex (LORR; min) is also shown for the ethanol-injected animals; time asleep is not shown as none of the animals awoke prior to tissue collection. Plasma corticosterone ($\mu\text{g/mL}$) and progesterone (ng/mL) are displayed for 3, 9, 18, and 24 month old males that received either vehicle or ethanol (σ^V , σ^E), and females that received either vehicle or ethanol (σ^V , σ^E).

Table 3

Measure		3 months old	9 months old	18 months old	24 months old
BECs	σ^E	385.20 $\pm$ 13.22	376.98 $\pm$ 12.79	382.20 $\pm$ 6.07	364.33 $\pm$ 10.66
	φ^E	335.14 $\pm$ 12.16	376.30 $\pm$ 14.12	370.74 $\pm$ 13.56	392.90 $\pm$ 17
Latency to LORR	σ^E	2.27 $\pm$ 0.10	8.50 $\pm$ 6.06	2.51 $\pm$ 0.15	2.63 $\pm$ 0.09
	φ^E	5.60 $\pm$ 3.08	11.68 $\pm$ 3.08	2.15 $\pm$ 0.14	2.29 $\pm$ 0.22
CORT Plasma	σ^V	9.58 $\pm$ 2.83	15.24 $\pm$ 4.72	24.35 $\pm$ 6.57	13.67 $\pm$ 4.20
	σ^E	51.24 $\pm$ 3.41	54.13 $\pm$ 3.57	59.66 $\pm$ 3.71	57.60 $\pm$ 5.90
	φ^V	10.38 $\pm$ 7.39	15.49 $\pm$ 3.78	19.59 $\pm$ 6.05	18.18 $\pm$ 5.00
	φ^E	70.08 $\pm$ 5.35	68.66 $\pm$ 3.16	80.84 $\pm$ 4.70	67.00 $\pm$ 7.08
PROG Plasma	σ^V	0.22 $\pm$ 0.14	0.02 $\pm$ 0.02	3.94 $\pm$ 1.15	3.49 $\pm$ 1.43
	σ^E	5.94 $\pm$ 0.43	5.48 $\pm$ 0.93	8.19 $\pm$ 0.62	8.74 $\pm$ 0.49
	φ^V	5.75 $\pm$ 1.26	5.26 $\pm$ 0.87	7.94 $\pm$ 1.03	6.64 $\pm$ 1.22
	φ^E	9.12 $\pm$ 0.23	9.06 $\pm$ 0.54	9.42 $\pm$ 0.40	8.78 $\pm$ 0.84