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## Astrocyte reactivity and neurodegeneration in the female rat brain following alcohol dependence

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### Abstract

Recent evidence suggests that alcohol use disorder (AUD) may manifest itself differently in women compared to men. Women experience AUDs on an accelerated timeline and may have certain regional vulnerabilities. In male rats, neuronal cell death and astrocyte reactivity are noted following induction of alcohol dependence in an animal model of AUD. However, the regional and temporal patterns of neurodegeneration and astrocyte reactivity have yet to be fully examined in females using this model. Therefore, adult female rats were exposed to a 4-day binge model of alcohol dependence followed by several different periods of abstinence. Immunohistochemical markers for FluoroJade B, a label of degenerating neurons, and vimentin, a marker for reactive astrocytes, were utilized. The expression of these markers in cortical and limbic regions was quantified immediately after their last dose (e.g., T0), or 2, 7, and 14 days later. Significant neuronal cell death was noted in the entorhinal cortex and the hippocampus, similar to previous reports in males, and also several cortical regions not previously observed. Vimentin immunoreactivity was noted in the same regions as previously reported, in addition to three novel regions. Vimentin immunoreactivity also occurred both at earlier and later time points in some cortical and hippocampal regions. These data suggest that both neuronal cell death and vimentin immunoreactivity, sensitive markers of astrocyte reactivity, could be more widespread in females compared to males. Therefore, this study provides a framework for specific regions and

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#### Author Contributions

SPG and KN designed the experiments. SPG, JM, HQD, and CAS performed the experiments. SPG performed the statistical analyses. SPG and KN interpreted data. SPG wrote the initial draft, edited the manuscript, and created the figures. KN wrote portions of and edited the manuscript. All authors reviewed and provided edits and comments on the manuscript.

#### Competing Interests

Declarations of interest: none

#### Ethics Approval and Consent to Participate

The animal study was reviewed and approved by the University of Kentucky Institutional Animal Care and Use Committee and The University of Texas at Austin Institutional Animal Care and Use Committee.

time points which should be examined in future studies of alcohol-induced damage that include female rats.

## Keywords

ethanol; vimentin; sex differences; alcohol use disorder; gliosis; GFAP

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## Introduction

The pervasiveness of alcohol misuse is startling; in the United States, nearly one-third of individuals will develop an Alcohol Use Disorder (AUD) at some point in their life, with about 14% of individuals meeting the criteria for an AUD in any given year (Grant et al., 2015). Although rates of AUD have historically been higher among men than women, this gap is shrinking, with an 84% increase in AUD in women compared to 35% in men in recent years (Grant et al., 2017). In lockstep with shifting societal norms, increasing numbers of women have adopted harmful patterns of alcohol use, such as binge drinking (Gruza et al., 2018; Keyes et al., 2008, 2011; Slade et al., 2016). Heavy alcohol use can result in brain volume reduction (Agartz et al., 1999; Pfefferbaum et al., 1992), neuronal cell death (Kril et al., 1997), and astrocyte reactivity (Franke, 1995; Miguel-Hidalgo, 2021), which may contribute to long-term cognitive deficits (Eckardt & Martin, 1986; Sullivan & Pfefferbaum, 2005). Despite initial evidence that females may be more sensitive to alcohol-induced brain damage than males (Hommer et al., 2001), few studies have sought to characterize how neuronal cell death and astrocyte reactivity may occur differently in these two populations.

Research on how AUD related cognitive deficits and neurotoxicity may differ between men and women have produced contradictory results. Initial studies indicated that compared to men, women with AUDs may experience alcohol-related medical problems on an accelerated timeline (Ashley et al., 1977; Mumenthaler et al., 1999; Piazza et al., 1989), develop cognitive and motor deficits with shorter drinking histories (Acker, 1985), and perform worse on some cognitive tests (Acker, 1986; Sullivan et al., 2002). Neuroimaging studies have sought to tie these behaviors to structural deficits, with mixed results (Cortez et al., 2020; Fama et al., 2020; Verplaetse et al., 2021). Some magnetic resonance imaging (MRI) studies have revealed that women are more susceptible to alcohol-related structural abnormalities than men, especially in corticolimbic regions (Agartz et al., 2003; Hommer et al., 2001; Mann et al., 2005; Pfefferbaum et al., 2001). Others indicate greater brain volume loss in men compared to women, not just in corticolimbic regions (Momenan et al., 2012; Sawyer et al., 2017) but in the cerebellum as well (Sawyer et al., 2016; Sullivan et al., 2010). Recent MRI studies with modern voxel based imaging techniques do not find sex differences in brain volumes of those with AUD (Demirakca et al., 2011; Mechtcheriakov et al., 2007). Although some evidence exists for sex differences, more than 80% of recent alcohol related neuroimaging studies either do not analyze by sex or are underpowered to do so, making interpretations of the human imaging literature challenging (Verplaetse et al., 2021).

Animal studies, which provide a means to directly investigate the causal effects of alcohol on brain structure and cognition, have yet to fully characterize neurodegeneration in females.

Most studies of alcohol-induced brain damage utilize the Majchrowicz model of alcohol dependence (Majchrowicz, 1975), which emulates the binge-like intoxication, dependence, withdrawal, and damage observed in persons with an AUD. In male rats, this model results in neuronal cell death in multiple regions of the brain, most extensively in corticolimbic regions such as the hippocampus and rhinal and piriform cortices (Collins et al., 1996; Crews et al., 2000). Damage begins while the animals are intoxicated, peaking just after the last dose of alcohol and continuing at low but detectable levels for up to a week of abstinence from alcohol (Collins et al., 1996; Crews et al., 2000; Hayes et al., 2013; Kelso et al., 2011; Obernier, Bouldin, et al., 2002). However, few such studies have been performed in females. In those that have, one study found neuronal cell death in similar brain regions compared to previous studies with males, but they only focused on these common sites of damage (Leasure & Nixon, 2010). Another found a greater number of degenerating neurons in the hippocampus of females compared to males (Maynard et al., 2018), again focusing on specific regions where damage had been observed previously. In both of these studies, limited time points were examined and/or only regions where damage was previously observed in male rats were examined.

Another facet of alcohol-induced neuropathology, astrocyte reactivity, may also occur differently in women compared to men. Astrocytes are key regulators of brain homeostasis and perform important functions such as removing neurotransmitters from the synaptic space, ion buffering, vascular coupling, and modulating immune signaling (Giovannoni & Quintana, 2020; Sofroniew & Vinters, 2010; Verkhratsky & Nedergaard, 2018). With insult or disease, astrocytes become reactive, meaning they change phenotypically and reflect a host of morphological, molecular, and functional differences (Escartin et al., 2019, 2021). Reactive astrocytosis has been observed in numerous neuropathologies, including stroke (Li et al., 2008), Alzheimer's Disease (Chun & Lee, 2018), and alcohol use (Franke, 1995; Sarkisyan et al., 2017). Though classically defined by the upregulation of intermediate filaments like GFAP and vimentin (Pekny & Nilsson, 2005), astrocyte reactivity also results in a variety of functional changes which may reflect the heterogenous phenotypes that result from each type of insult. For instance, neuroinflammation can produce reactive astrocytes capable of inducing neuronal cell death, while ischemia can produce a neuroprotective reactive phenotype (Liddelov et al., 2017). Therefore, in AUD, reactive astrocytes could play a functional role in both alcohol related neurodegeneration as well as recovery. The release of inflammatory mediators by reactive astrocytes (Blanco et al., 2004) may influence alcohol-induced neuronal cell death (Crews & Nixon, 2009). Vimentin, an intermediate filament protein expressed in low levels in astrocytes at baseline (Schnitzer et al., 1981), is a good tool to characterize reactive astrocytes (Kelso et al., 2011). For example, in animal models of traumatic brain injury (TBI), vimentin appears to be more specific than GFAP in identifying regions of necrosis or permanent ischemic injury (Petito et al., 1990; Schmidt-Kastner et al., 1990). It has similarly been used to suggest additional brain regions of vulnerability to alcohol in male rats (Kelso et al., 2011; Hayes et al., 2013) but has only been examined in female rats in one study using a once per week binge-like exposure model ((Hayes et al., 2013; Kelso et al., 2011; West et al., 2021). Vimentin expression has yet to be examined in female rats in a model of alcohol dependence.

Therefore, to understand the timeline of neurodegeneration and to perform an unbiased survey of areas of ethanol-induced neurotoxicity in females, we examined two measures in a time course following alcohol dependence. First, we utilized FluoroJade B (FJB) staining, which binds to dying neurons, to document the pattern of cell death in females during alcohol dependence, and across the first week of abstinence following ethanol. Then, immunohistochemistry for vimentin was employed to identify regions of astrocyte reactivity during these same time points.

## Experimental Procedures

### Animals

Adult female Sprague-Dawley rats ( $234 \pm 17$ g and ~70 days old; Charles River Laboratories, Raleigh, NC, United States) were used in this study. A total of 86 rats were used across two institutions, 32 rats at the University of Kentucky (UK; rats in the T0 and T7 time points) and 54 rats at The University of Texas at Austin (UT; rats in T2, T7-colabel, and T14 groups), all while following identical experimental procedures including some overlap in personnel (Morris, Kelso, et al., 2010). Further, only the animal work and brain tissue extraction were performed at UK; all other work, notably all histology and immunohistochemistry, was conducted at UT. Rats were allowed to acclimate for 3 days to the environment of the respective vivariums and double-housed in standard polycarbonate cages except for a 24-hour period when animals were single housed to observe withdrawal behaviors. Rats were given free access to rat chow (UK = Teklad 2018 diet, Envigo, Madison, WI, United States; UT = ProLab<sup>®</sup> RMH 1800 5LL2\*, LabDiet, St Louis, MO, United States) except for during the ethanol administration procedure, and always had access to water. The different standard diets between the two institutions do not appear to have impacted any outcome measured to date. The vivariums were kept on a 12-hour light-dark cycle, with lights on from 0700 h. For three days before the start of the ethanol administration, rats were handled for 3 minutes each. All experimental procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee and The University of Texas at Austin Institutional Animal Care and Use Committee and followed the National Institutes of Health guidelines for the Care and Use of Laboratory Animals.

### Ethanol Administration Model

Rats were administered ethanol via intragastric gavage in a binge model of an AUD (Majchrowicz, 1975; Morris, Eaves, et al., 2010). An ethanol-containing diet (25% ethanol w/v in Vanilla Ensure Plus<sup>®</sup>, Abbott Labs, Columbus, OH, United States) was administered 3 times per day (every 8 hours) for four days (Figure 1A). An initial dose of 5.0 g/kg was administered with subsequent doses administered based on the intoxication behavior of each rat (Figure 1B). Control rats received an isocaloric diet (Vanilla Ensure Plus<sup>®</sup> containing dextrose). The amount of diet received by control rats was calculated as the average volume of the diet received by ethanol-treated rats at each administration time point. Blood ethanol concentrations (BEC) were determined via tail blood taken 90 minutes following the 7<sup>th</sup> dose of the ethanol administration procedure. Tail blood was taken via heparinized capillary tubes and stored in microcentrifuge tubes with 3 $\mu$ l of heparin (Meitheal Pharmaceuticals,

Chicago, IL, United States), centrifuged for 5 minutes at 6500 rpm, and the plasma was stored at  $-20^{\circ}\text{C}$ . BECs were analyzed with an AM1 Alcohol Analyzer (Analox Instruments, Lunenburg, MA, United States). Withdrawal behaviors were measured by observing rats every 30 minutes, beginning 10 hours after the final dose of alcohol for 17 hours. Behaviors were scored based on a withdrawal severity scale (Figure 1C; see also (Morris, Kelso, et al., 2010)).

### Tissue Preparation

As prior work in male rats indicates that FJB staining occurs maximally directly following the last dose ( $T_0$ ) while vimentin immunoreactivity peaks following 2 to 7 days of abstinence (Kelso et al., 2011), rats were sacrificed at 0, 2, 7, and 14 days after ethanol exposure. At each time point, rats were given a lethal dose of sodium pentobarbital (i.p.; Fatal-Plus<sup>®</sup>, Vortech Pharmaceuticals, Dearborn, MI, United States) and perfused transcardially with 0.1M phosphate buffered saline (PBS, pH=7.4) followed by 4% paraformaldehyde (PFA). Brains were extracted and post-fixed overnight in 4% PFA, then rinsed and stored in PBS at  $4^{\circ}\text{C}$ . Brain tissue was sectioned in twelve series of  $40\mu\text{m}$  sections using a vibrating microtome (Leica VT100S, Wetzlar, Germany) starting randomly at approximately 2.4mm through 7.0mm Bregma. Tissue was stored in 24-well plates with cryoprotectant at  $-20^{\circ}\text{C}$ .

**FluoroJade B Staining**—FluoroJade B (FJB) is a fluorescent dye that has been shown previously to identify damaged neurons (Schmued & Hopkins, 2000) and with better signal to noise resolution in our hands than the newer FluoroJade C (Schmued et al., 2005). Although the stain does not provide information on the mode of cell death, past studies indicate that cell death is more necrotic in nature in this model of alcohol dependence (Morris, Eaves, et al., 2010; Obernier, Bouldin, et al., 2002). Every 12<sup>th</sup> section of each brain (adjacent sections to vimentin) as well as a positive control (rat stroke brain) were mounted on Superfrost Plus<sup>®</sup> slides (Fisher Scientific) and allowed to dry overnight. The next morning, slides were placed on a slide warmer at  $24^{\circ}\text{C}$  for an additional 3 hours before staining. The sections were processed through 1% NaOH in 80% EtOH for 5 minutes, 70% EtOH for 2 minutes, ddH<sub>2</sub>O for 2 minutes, and then 0.06% KMnO<sub>4</sub> for 10 minutes. Following a wash in ddH<sub>2</sub>O, the slides were incubated in the dark with FJB (MilliporeSigma) for 20 minutes before the final three ddH<sub>2</sub>O washes for 1 minute each. Finally, the tissue was dried on a slide warmer in the dark at  $24^{\circ}\text{C}$  for 30 minutes and then coverslipped in Cytoseal<sup>®</sup>.

**Vimentin Immunohistochemistry**—Immunohistochemistry was utilized to visualize vimentin, an intermediate filament protein expressed in some populations of astrocytes (Schnitzer et al., 1981). Every 12<sup>th</sup> section (a single well) was processed for free-floating immunohistochemistry as previously described (Kelso et al., 2011). Importantly, dilution curves were conducted to determine antibody dilutions and included negative controls (no primary; no secondary) to revalidate all methodology in the new lab location. Sections were first rinsed ( $3 \times 5$  min) in tris-buffered saline (TBS), then incubated in a 0.6% peroxidase solution for 30 minutes. Following more rinses of TBS, tissue was blocked (3% horse serum/ 0.1% Triton-X/ TBS), then incubated in anti-vimentin primary antibody (Chemicon,

1:750; determined by dilution curve) in blocking solution overnight at 4°C. Sections were washed 3 times for 10 minutes in blocking solution, then incubated in horse anti-mouse, rat adsorbed biotinylated secondary antibody (Vector Laboratories, 1:200). Slices were washed in TBS, followed by avidin-biotin-peroxidase complex (ABC Elite kit; Vector Laboratories), rinsed in TBS again, and peroxidases were detected and visualized by diaminobenzidine (DAB; Vector Laboratories, Burlingame, CA, United States). Sections were rinsed in TBS, mounted on slides, and coverslipped with Cytoseal<sup>®</sup> (Thermo Fisher Scientific, Waltham, MA, United States)

For the Vimentin and GFAP/Iba-1 co-immunofluorescent immunohistochemistry, sections were rinsed in TBS, blocked (10% goat serum/ 0.1% Triton-X/ TBS), and incubated overnight at 4°C in blocking solution with rabbit anti-GFAP (DAKO, 1:2500) or rabbit anti-Iba-1 (Wako, 1:400) and mouse anti-vimentin (Chemicon, 1:400). Sections were rinsed in blocking solution, and then incubated in secondary antibodies: Alexa Fluor 555 anti-mouse (Invitrogen, 1:200) and Alexa-Fluor 488 anti-rabbit (Invitrogen, 1:200). Slices were rinsed in TBS, mounted on slides, and coverslipped with ProLong Gold Anti-fade mounting medium (Invitrogen).

### Quantification

**FluoroJade B**—FJB-stained slides were coded, and each section from 1.92mm Bregma to -7.44mm Bregma was closely examined for FJB-positive (FJB+) cells. The brain regions which contained FJB+ cells were identified by visual comparison of each brain slice with a rat brain atlas (George Paxinos & Charles Watson, 2009). FJB+ cells were manually counted across each section with a BX-51 Olympus microscope (Olympus, Center Valley, PA, United States) under blue light excitation (includes 488nm) at 10X and up to 80X magnifications as needed for each region. Manual profile counts were utilized as with previous (Kelso et al., 2011; Leasure & Nixon, 2010) due to the lack of background staining necessary for thickness estimation or regional demarcation, low numbers of profiles per region, and the heterogeneous labeling pattern (Noori & Fornal, 2011). FJB+ cells were counted by brain region in which significant staining was observed.

**Vimentin immunohistochemistry**—Vimentin-stained slides were coded, and each section from 1.92mm Bregma to -7.44mm Bregma was closely examined for vimentin-positive cells. Areas of significant vimentin+ cells with a glial morphology were analyzed. A BX-51 microscope under brightfield illumination was used to take 4x images in cortical and limbic regions (Stereo Investigator<sup>®</sup>; MBF Biosciences, Williston, VT, United States). Each image was quantified for percent area staining using Fiji (Schindelin et al., 2012) in a method similar to that used to analyze GFAP as reported by others (Miguel-Hidalgo et al., 2000, 2010). As Vimentin may faintly label endothelial cells (Baldwin & Scheff, 1996) in addition to astrocytes and radial glia, an area of each section containing only endothelial cells was quantified and subtracted from the analyzed region of interest. For co-labeling assessments of vimentin and GFAP or Iba-1, 60x z-stack images were taken with an Olympus FV300 laser scanning confocal microscope. Imaris software (Oxford Instruments, Tubney Woods, United Kingdom) was utilized to measure colocalization via the Coloc tool.

## Statistical Analysis

All data were compiled in Microsoft Excel for Mac (Version 16.62) and analyzed with GraphPad Prism 9 (GraphPad Software, La Jolla, CA, United States). As all the regions in which FJB was found, and all regions except one where vimentin was quantified contained no cells of interest in control subjects, control subjects across the different time points were collapsed and data were analyzed using one-way ANOVA followed by Dunnett's posthoc test. As F-tests revealed non-homogenous variance between groups, data were log-transformed before analysis (zeros were replaced with 0.00000001). The granule cell layer of the dentate gyrus contained vimentin immunoreactive radial glial cells in control subjects, as expected, therefore these data were analyzed by two-way ANOVA followed by Bonferroni posthoc test. Co-localization data were analyzed via one-way ANOVA followed by Tukey's posthoc test. Any  $p < 0.05$  was considered a significant difference. All data are plotted using bar graphs of mean  $\pm$  SEM including individual data points.

## Results

### Rat Model of an AUD - Intoxication and Dependence

Ethanol intoxication parameters resulting from the binge model of an AUD are outlined in Table 1. Overall, ethanol-treated rats achieved an intoxication score of  $1.6 \pm 0.4$ , which resulted in an ethanol dose of  $9.6 \pm 1.3$  g/kg. BECs taken after the 7<sup>th</sup> ethanol dose measured  $379.9 \pm 82.0$  mg/dL. The mean withdrawal score (mean of the highest scoring behavior each hour over the 17 hours) was  $1.1 \pm 1.0$ , while the peak withdrawal score (highest/most severe withdrawal behavior observed) was  $2.7 \pm 1.0$ . While dose and mean withdrawal parameters differed slightly but significantly among a couple of time points, the critical indicator, BEC, did not differ between groups. Furthermore, all parameters were similar to past studies with male and female rats utilizing this model (Barton et al., 2017; Kelso et al., 2011; Leasure & Nixon, 2010; Nawarawong et al., 2021).

### Alcohol Increased FJB+ Cells in the Hippocampus and Cortical Regions

All sections of each brain were carefully surveyed for FJB+ cells. In controls, FJB was almost nonexistent, in line with previous studies (Hayes et al., 2013; Kelso et al., 2011; Leasure & Nixon, 2010). In ethanol-treated animals, 11 brain regions were found to contain at least some FJB+ cells: the insular cortex, piriform/amygdalopiriform transition area (apiriform) cortex, granule cell layer of the dentate gyrus, posterolateral cortical amygdaloid nucleus, cingulate/retrosplenial cortex, motor cortex, somatosensory cortex, parietal cortex, auditory cortex, visual cortex, and the peri/entorhinal cortex. In the granule cell layer, one-way ANOVA revealed an effect of treatment (Figure 2;  $F_{4,65}=36.08$ ,  $p < 0.0001$ ), and Dunnett's post hoc test showed that there was an increase at the T0 ( $p < 0.0001$ ), T2 ( $p < 0.0001$ ), and T7 ( $p = 0.0002$ ) time points. All remaining regions, except for the auditory cortex, had a statistically significant elevation of FJB+ cells in the ethanol group compared to controls (Figure 3): the cingulate/retrosplenial cortex (Figure 3;  $F_{4,65}=4.282$ ,  $p = 0.0039$ ) at the T2 ( $p = 0.0037$ ) time point; the insular cortex (Figure 3;  $F_{4,65}=46.87$ ,  $p < 0.0001$ ) at the T0 ( $p < 0.0001$ ), T2 ( $p < 0.0001$ ), and T7 ( $p < 0.0001$ ) time points; the motor cortex (Figure 3;  $F_{4,65}=3.528$ ,  $p = 0.0115$ ) at the T2 ( $p = 0.0041$ ) time point; the parietal cortex (Figure 3;  $F_{4,65}=5.593$ ,  $p = 0.0006$ ) at the T2 ( $p = 0.0006$ ) and T7 ( $p = 0.0329$ ) time points;

the peri/entorhinal cortex ( $F_{4,65}=58.12$ ,  $p<0.0001$ ) at the T0 ( $p<0.0001$ ), T2 ( $p<0.0001$ ), and T7 ( $p<0.0001$ ) time points; the piriform/apiriform cortex ( $F_{4,65}=38.55$ ,  $p<0.0001$ ) at the T0 ( $p<0.0001$ ), T2 ( $p<0.0001$ ), and T7 ( $p<0.0001$ ) time points; the posterolateral cortical amygdaloid nucleus ( $F_{4,65}=20.52$ ,  $p<0.0001$ ) at the T0 ( $p<0.0001$ ), T2 ( $p<0.0001$ ), and T7 ( $p<0.0001$ ) time points; the somatosensory cortex ( $F_{4,65}=8.506$ ,  $p<0.0001$ ) at T2 ( $p<0.0001$ ); and the visual cortex ( $F_{4,65}=5.681$ ,  $p=0.0006$ ) at T2 ( $p=0.0020$ ) and T7 ( $p=0.0033$ ).

### **Vimentin Immunoreactivity Was Dramatically Increased Throughout the Hippocampus in Ethanol-Treated Rats**

Following a detailed survey of vimentin+IR across cortical and limbic regions, the hippocampus and six additional regions (below) where immunoreactivity appeared visually distinct were chosen for further analysis. Vimentin immunoreactivity (vimentin+IR) was apparent in multiple hippocampal regions, with the highest average expression at the T7 time point. Five subregions of the hippocampus were analyzed separately as shown in Figure 4C. Vimentin+IR was significantly elevated above controls in the CA1 band (Figure 4A;  $F_{3,50}=247.9$ ,  $p<0.0001$ ) at T2 ( $p<0.0001$ ), T7 ( $p<0.0001$ ), and T14 ( $p<0.0001$ ); the CA2/CA3 bands ( $F_{3,50}=112.4$ ,  $p<0.0001$ ) at T2 ( $p<0.0001$ ), T7 ( $p<0.0001$ ), and T14 ( $p<0.0001$ ); the molecular layer ( $F_{3,50}=141.8$ ,  $p<0.0001$ ) at T2 ( $p<0.0001$ ), T7 ( $p<0.0001$ ), and T14 ( $p<0.0001$ ); and the hilus ( $F_{3,50}=32.46$ ,  $p<0.0001$ ) at T2 ( $p<0.0001$ ), T7 ( $p<0.0001$ ), and T14 ( $p<0.0001$ ). Vimentin+IR was largely absent from controls except for one region: the granule cell layer of the dentate gyrus. As a result, in this region controls were not collapsed, and are included in the analysis and graph (Figure 4B). Two-way ANOVA found a significant effect of ethanol treatment ( $F_{1,48}=12.44$ ,  $p=0.0009$ ) and time-point ( $F_{2,48}=9.601$ ,  $p=0.0003$ ), with Bonferroni's multiple comparisons indicating a statistically significant increase in vimentin+IR of ethanol-treated animals over controls at the T14 time point ( $p=0.0203$ ).

### **Ethanol Treatment Resulted in Increased Vimentin+IR in Cortical and Limbic Regions**

Outside of the hippocampus, vimentin+IR appeared distinct in the somatosensory cortex, insular cortex, piriform/apiriform cortex, basolateral amygdala, peri/entorhinal cortex, and auditory cortex. Each region was found to have statistically significant elevated vimentin+IR over controls in at least one of the three time points (Figure 5A). Vimentin+IR was significantly elevated at the following time points in each brain region: the auditory cortex ( $F_{3,50}=20.49$ ,  $p<0.0001$ ) at T2 ( $p=0.0001$ ) and T7 ( $p<0.0001$ ); the basolateral amygdala ( $F_{3,50}=26.22$ ,  $p<0.0001$ ) at T2 ( $p=0.0001$ ) and T7 ( $p<0.0001$ ); the insular cortex ( $F_{3,50}=20.36$ ,  $p<0.0001$ ) at T2 ( $p=0.0002$ ) and T7 ( $p<0.0001$ ); the peri/entorhinal cortex ( $F_{3,50}=19.74$ ,  $p<0.0001$ ) at T2 ( $p<0.0001$ ) and T7 ( $p<0.0001$ ); the piriform/apiriform cortex ( $F_{3,50}=22.94$ ,  $p<0.0001$ ) at T2 ( $p<0.0001$ ) and T7 ( $p<0.0001$ ); and the somatosensory cortex ( $F_{3,50}=17.60$ ,  $p<0.0001$ ) at T2 ( $p=0.0030$ ) and at T7 ( $p<0.0001$ ).

### **Vimentin+ Cells are Astrocytes**

To verify that vimentin+ cells were astrocytes, multiple fluorescent immunohistochemistry was carried out for vimentin plus GFAP (a marker for mature astrocytes) and vimentin plus Iba-1, a marker for microglia (Ahmed et al., 2007). When examined by eye, vimentin



appeared to co-label with GFAP (Figure 6A–C, E), but not Iba-1 (Figure 6D), throughout the brain. This is consistent with the large majority of the recent literature (Potokar et al., 2020) despite earlier findings of vimentin present in microglia (Graeber et al., 1988). The vimentin signal also appeared to be stronger in astrocytes in the hippocampus than in the entorhinal cortex or the basolateral amygdala. Therefore, the coloc tool on Imaris software was employed to examine the colocalization of vimentin and GFAP across these regions. One-way ANOVA of Manders Correlation Coefficients indicated that the fraction of GFAP that colocalized with vimentin was indeed different among the three brain regions analyzed ( $F_{2,21}=24.39$ ,  $p<0.0001$ ). Tukey's multiple comparisons test revealed that colocalization of GFAP with vimentin was higher in the hippocampus compared to the entorhinal cortex ( $p<0.0001$ ) or BLA ( $p<0.0001$ ; Figure 6F).

## Discussion

While animal models have been used to great effect to elucidate specific regions and time courses of alcohol-related neurodegeneration, most studies use only males. Therefore, even if both males and females are used, as is slowly becoming the expectation, the regions and time points chosen for analysis are often biased by previous studies which omitted females. Given the context of increasing alcohol use among women, the need to understand how alcohol may uniquely affect women versus men is long overdue (White, 2020). Therefore, this study sought to focus only on female rats and characterize, in detail, the presence of two markers of alcohol-induced damage throughout the brain at multiple time points. To do so, FJB staining, a reliable and highly sensitive method of labeling degenerating neurons (Schmued & Hopkins, 2000) was quantified throughout cortical and limbic regions. The brains of female rats exposed to ethanol contained FJB+ cells in multiple cortical and limbic regions previously unreported in males. Next, vimentin, an intermediate filament protein upregulated in reactive glial cells in several neurodegenerative diseases (Chen et al., 2022; Yamada et al., 1992) was examined as another metric of potential damage. Vimentin+ cells co-labeled with the astrocyte marker GFAP (Figure 6) and did not share any overlap with microglia marker, Iba-1, suggesting that these cells were indeed reactive astrocytes. Additionally, vimentin+ reactive astrocytes appeared at an earlier time point in ethanol-exposed females compared to what has been observed in males (see Figure 7 for a summary of regions where FJB and vimentin+ cells were observed, yellow highlights indicate regions that may be unique to females). Thus, these findings suggest that alcohol induced neurodegeneration could occur earlier and in different regions compared to males. These data have important implications for our understanding of how alcohol affects the female brain and ultimately how these changes underlie the development of AUDs.

A thorough survey throughout the brain was undertaken to identify possible regional differences in FJB staining following alcohol administration. Although this approach required extensive effort, it eliminated the bias of only looking at regions where damage had been reported in males. The FJB method for detecting neuronal degeneration compares favorably with more traditional methods such as silver stain (Anderson et al., 2005; Kelso et al., 2011) but appears to label a later phase of cell death (Poirier et al., 2000). FJB+ cells were significantly elevated in the same brain regions previously reported in males (Corso et al., 1998; Crews et al., 2000; Hayes et al., 2013; Kelso et al., 2011; Obernier,

Bouldin, et al., 2002; Walker et al., 1980) including the insular cortex, piriform cortex, peri/entorhinal cortex, and the dentate gyrus granule cell layer. Several new regions of degeneration emerged: the posterolateral cortical amygdaloid nucleus and the cingulate/retrosplenial, motor, parietal, somatosensory, and visual cortices, though reactive microglia - which suggested some level of perturbation - were observed by us in some of these regions in males (Nixon et al., 2008). Like in males (Kelso et al., 2011), and similar to other indicators of degeneration such as amino cupric silver stain (Obernier, White, et al., 2002), FJB+ cells were highest in most brain regions immediately following the end of binge ethanol administration, with degeneration continuing at lower but still detectable levels during abstinence from ethanol at T2 and T7. At T14 almost no FJB+ cells were found in any brain regions. Though the number of FJB+ cells appears to fluctuate across time points (e.g., Figure 3), T2 does not differ significantly from T0 or T7. Furthermore, any perceived difference may not be meaningful given the high variability in cell death markers historically noted in this model, though this point is only based on observations from male rats (see Crews et al., 2000; Corso et al., 1998; Collins et al., 1996 and especially Kelso et al., 2011). Cell death observed at the T2 or T7 time points theoretically could be a response to reactive microglia rather than alcohol itself (Crews & Nixon, 2009). However, our recent work examining microglia in female rats is highly similar to our past work in males (Morris, Kelso, et al., 2010; Peng et al., 2017): a modest microglial reaction occurs, based on Iba1+ cell morphology and cytokine expression in hippocampal and entorhinal cortex homogenates (Melbourne et al., *in preparation*). Furthermore, previous reports in males have identified the method of cell death in this model as necrotic through careful ultrastructural examination, and a lack of TUNEL staining, an indicator of apoptosis (Morris, Eaves, et al., 2010; Obernier, Bouldin, et al., 2002). A lack of TUNEL staining has been confirmed with female rats as well (Maynard et al., 2018). Therefore, while cell death appears in female rats at similar time points as in males following binge ethanol exposure, there appear to be some regional differences.

Additional regions of cell death in females compared to males could underlie certain vulnerabilities to AUD in women. These previously unreported areas containing FJB+ cells were widespread in corticolimbic regions in female rats. Although some studies fail to find any sex differences when comparing the brain volumes of men and women with AUD (Demirakca et al., 2011; Mechtcheriakov et al., 2007), those that do typically note differences in the cortex, particularly in cortical thickness (Momenan et al., 2012; Thayer et al., 2016). As shown in this study (Figure 3), disparities in cortical thickness could be explained by cell death in more cortical sub-regions in females compared to males. Furthermore, some of the cortical subregions observed in this study to contain FJB+ cells may also be involved in the progression and outcomes of AUD. For instance, the cingulate cortex is involved in reward-based decision-making (Bush et al., 2002), and size reductions in this region are observed in an animal model of AUD (Zhao et al., 2021). In those with AUD, reduced cingulate connectivity occurs, and serves as a marker of future relapse (Zakiniiez et al., 2017). Deficits in the parietal cortex may be responsible for decreased performance in visuospatial tasks in both men and women with AUD (Sullivan et al., 2000, 2002). And impairments in visual learning commonly observed in those with AUD (Stavro et al., 2013) may relate to neuronal cell loss or altered cellular metabolism in the visual

cortex (Bagga et al., 2014). The presence of cell death in these additional brain regions in females may therefore facilitate the development of AUD and/or impact maintaining abstinence and recovery.

Vimentin reactivity in this study with females was more regionally and temporally widespread compared to previous reports using male rats. Vimentin immunoreactivity in female rats was noted in all brain regions previously reported in males (piriform/apiriform cortex, peri/entorhinal cortex, insular cortex, and hippocampus), and three previously unreported regions: somatosensory cortex, basolateral amygdala, and auditory cortex (Figure 5). Vimentin immunoreactivity was highest at T7, similar to peak reactivity in males (Kelso et al., 2011). However, most cortical regions and all hippocampal regions had significantly increased vimentin expression after only two days of abstinence following ethanol exposure compared to four days in males. Additionally, in all hippocampal regions, the timeline of immunoreactivity persisted compared to males, with significant vimentin immunoreactivity at the T14 time point. These findings complement other reports examining sex differences in astrocyte reactivity following ethanol exposure. In adult mice after chronic ethanol exposure, GFAP density is increased in the hippocampus of females but not males (Wilhelm et al., 2016). These authors also noted increased *Tnf* mRNA expression in astrocyte cultures from females but not males following ethanol exposure, which suggests that these astrocytes may be of a more pro-inflammatory phenotype. In contrast, more modest exposure in adolescent rats increased GFAP immunoreactivity following 30 days abstinence in the hippocampus of both females and males (Nwachukwu et al., 2022). Sex differences were suggested as there were fewer GFAP+ cells in the CA2/3 and dentate gyrus of females, although these studies did not use stereology and the reduced number of cells may simply reflect that females are physically smaller than males. Similarly, in a mouse model of chronic alcohol exposure decreased GFAP expression occurred in the hippocampus of females but not males, although only the dentate gyrus was examined (McGrath et al., 2017). Further evidence for sex differences is observed in other brain regions. Ethanol exposure blunts GFAP immunoreactivity in male mice, but not females in the BLA (Brewton et al., 2023). Gene expression is altered in a sexually dimorphic manner in response to mouse ethanol exposure, with skewed enrichment of reactive astrocyte genes in the medial prefrontal cortex of females (Wilhelm et al., 2015). In ethanol dependent mice, reductions in GFAP immunoreactivity are found the nucleus accumbens, suggesting altered astrocyte function (Giacometti et al., 2020). Overall, these differences suggest that vimentin+IR and therefore astrocyte reactivity may occur differently in females compared to males following ethanol administration, although effects are variable and may be influenced by the animal model of alcohol use, time points chosen, and the regions examined.

The dramatic upregulation of vimentin observed in female rats may be important for helping astrocytes restore brain homeostasis altered during alcohol use. Normally, vimentin is not expressed by many astrocytes in the adult brain, and usually only appears in astrocytes before cortical myelination (Dahl, 1981; Sancho-Tello et al., 1995) or in radial glial stem cells (Schnitzer et al., 1981). Only under pathological conditions like heavy alcohol use is vimentin expressed at high levels in mature astrocytes. Vimentin is required for the formation of other intermediate filaments like synemin (Jing et al., 2007), and possibly GFAP as well (Galou et al., 1996). These intermediate filaments, including vimentin,

are involved in several processes which may help reactive astrocytes respond to their environment. Upregulation of intermediate filaments aids in vesicular mobility, potentially allowing astrocytes to more efficiently release factors in response to the acute stages of injury (Potokar et al., 2010). Intermediate filament upregulation also allows astrocytes to more efficiently deliver MHC class II molecules to the cell surface, which may allow astrocytes to better respond to neuroimmune insults (Vardjan et al., 2012). In line with these roles, glial scar formation following brain lesion (Pekny et al., 1999) and neurological recovery following stroke (Liu et al., 2014) are both impaired when intermediate filaments are knocked out in animal models. Interestingly, when the degree of colocalization of GFAP with vimentin was quantified in several brain regions, GFAP positive astrocytes correlated more strongly with vimentin-labeled cells in the hilus of the hippocampus (an area in the vicinity of both robust cell death, and the neurogenic dentate gyrus granule cell layer) than in the entorhinal cortex or basolateral amygdala (Figure 6). This area of adult neurogenesis is visible in control images of the dentate gyrus (see insert, Figure 4) as quiescent neural progenitor cells label positive for vimentin (Encinas et al., 2006). Upregulation of vimentin may therefore be a mechanism for reactive astrocytes to respond to an altered environment quickly and effectively.

Gonadal hormones could contribute to some of the potential sex differences in vimentin immunoreactivity exhibited by the female rats in this study. Astrocytes express both estrogen receptor beta and alpha (Azcoitia et al., 1999; Pawlak et al., 2005). They are also highly steroidogenic and capable of producing progesterone, testosterone, and estradiol (Zwain & Yen, 1999). Consequently, estrogen can impact multiple measures of astrocyte reactivity. In studies of animal models of ischemia, estrogen treatment reduces both astrocyte proliferation and number in the vicinity of the injury (Garcia-Estrada et al., 1993; García-Estrada et al., 1999). However, GFAP immunoreactivity can vary based on the estrous cycle (Arias et al., 2009), with ischemia-induced increases in GFAP greater in female mice in the diestrus period compared to males (Cordeau et al., 2008). In this study, vimentin immunoreactivity varied substantially among subjects, with some ethanol-treated rats exhibiting very high amounts of vimentin expression and other rats very little. We did not assess for the estrous cycle in these rats, so the variability in stages of the estrous cycle across rats theoretically could have contributed to this differential expression of vimentin. However, there is growing evidence that the estrous cycle contributes little to variability of behavior in rodents (Dayton et al., 2016; Levy et al., 2023), though it is well known that estrogen has anti-inflammatory and neuroprotective roles in neurodegenerative conditions (Crespo-Castrillo & Arevalo, 2020). Furthermore, variability in vimentin+IR was also very high among males subjects (Hayes et al., 2013; Kelso et al., 2011), so the effects of estrogen may be modest. To fully resolve this question, it might be of interest to measure the estrous cycle in future studies.

Astrocyte reactivity has significant functional implications (Escartin et al., 2019). In many neurodegenerative conditions, astrocyte homeostatic functions such as neurotransmitter and ion buffering, gliotransmitter release, cytokine, and growth factor release, phagocytosis, and production or detoxification of reactive oxygen species are altered (Escartin et al., 2019, 2021). These changes may be either enhanced or reduced, and importantly are disease/insult-specific (Escartin et al., 2019). While some groups have examined the role of astrocytes or astrocytic glutamate transporters in addiction behaviors (Alhaddad et al.,

2022; Bull et al., 2014; Nwachukwu et al., 2021; Rao et al., 2015), how astrocytes alter glutamate homeostasis following alcohol dependence is only beginning to be examined (Adermark & Bowers, 2016; Miguel-Hidalgo, 2021). In ethanol preferring rats, increased glutamine synthetase-positive astrocytes are observed after chronic alcohol drinking (Miguel-Hidalgo, 2006), which may contribute to the known glutamate dysregulation that occurs with alcohol dependence (Adermark & Bowers, 2016). Thus, reactive astrocytes are likely redirected from homeostatic activities like glutamate uptake, potentially altering glutamatergic transmission and synaptic plasticity (Ayers-Ringler et al., 2016; Mulholland et al., 2009; Smith, 1997).

An intriguing story is emerging on the large functional diversity in astrocyte reactivity (Liddelow & Barres, 2017). Our assessment of key cytokines and growth factors supports a more anti-inflammatory or pro-regeneration environment in both males (Peng et al., 2017; Peng & Nixon, 2020) and females (Melbourne et al., in prep). Work in rats, however, is in direct contrast to recent work in C57Bl/6J mice that find a transcriptomic signature of an acute injury phenotype (Holloway et al., 2023). C57Bl/6J mice have a mutation in the *nnt* gene that renders them more susceptible to oxidative stress and inflammation. Indeed, this mutation may explain why C57Bl/6J mice consistently have pro-inflammatory effects to ethanol (Kane & Drew, 2016; Qin et al., 2008), while most rats studies do not (Bell-Temin et al., 2013; Gano et al., 2016; Marshall et al., 2013; Peng et al., 2017; Peng & Nixon, 2020; Zahr et al., 2010). The possibility that reactive astrocytes after alcohol dependence may have a beneficial phenotype is supported by the absence of significant astrocyte proliferation (Adermark & Bowers, 2016; Nixon & Crews, 2004) or scarring, both of which occur in conditions with more significant cell death events such as TBI or ischemia (Burda et al., 2016; Pekny & Nilsson, 2005)

This study sought to fill in an important gap in the literature and examine how excessive alcohol exposure may uniquely affect the female brain by examining multiple brain regions at several time points following ethanol exposure. A direct comparison of males and females was not made in the interest of reducing the use of animals by not duplicating past work, as these studies could be conducted identically to past work in males with overlapping personnel. Thus, when comparing to past work by ourselves and others (Crews et al., 2000; Kelso et al., 2011; Walker et al., 1980) these data suggest that alcohol induced neurodegeneration both earlier and later and in different regions compared to males. Neuronal degeneration appeared in additional brain regions compared to past studies with males. In female rats, vimentin+ astrocytes appeared at an earlier and later time point, and in some previously undescribed regions. This may indicate that the female brain is more vulnerable to alcohol-induced degeneration. This study provides a strong foundation to better understand the potentially sex-specific mechanisms that underlie alcohol-induced brain damage in AUDs. Understanding how alcohol use affects men and women differently will be key to designing better more targeted therapeutic strategies for AUDs.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Data Availability Statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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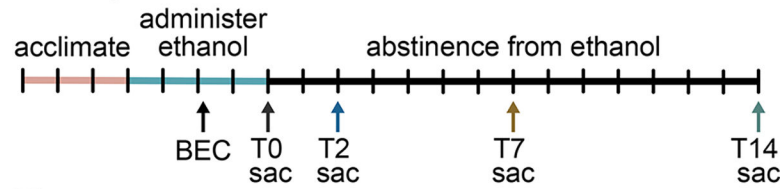
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## A Experimental Timeline



## B Intoxication Scale

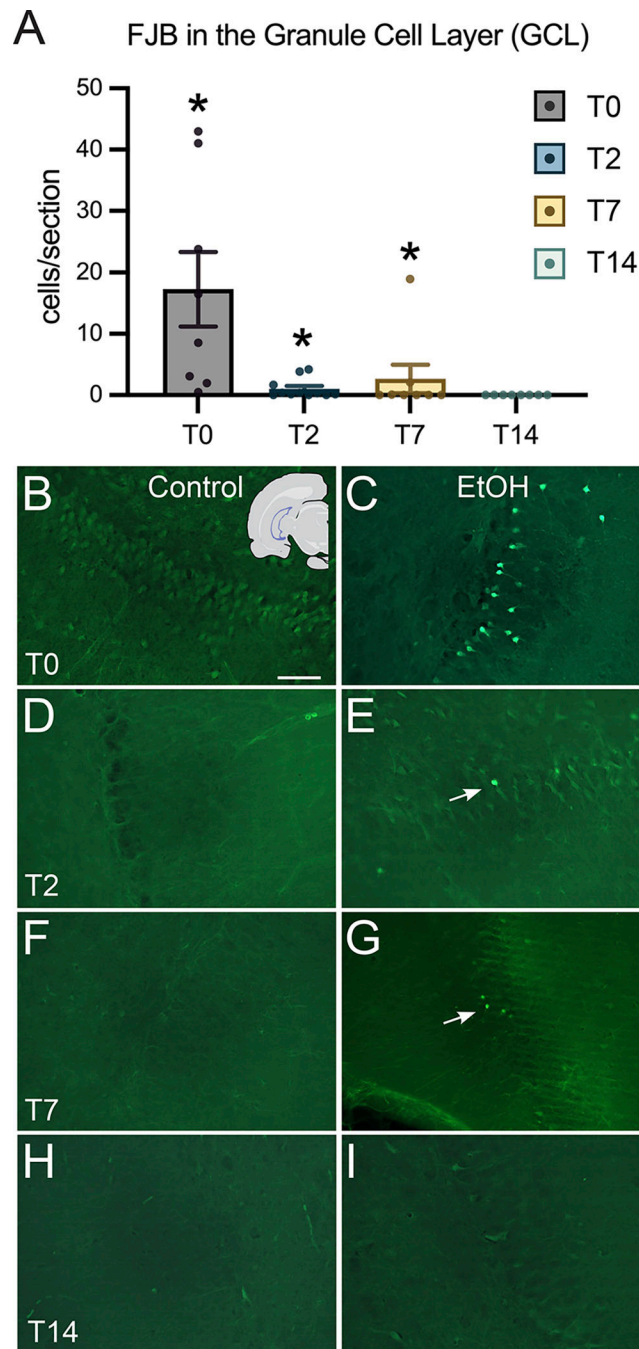
Score	Behavioral Indications	EtOH Dose
0	Normal rat	5 g/kg
1	Hypoactive, mild ataxia	4 g/kg
2	Ataxia (elevated abdomen)	3 g/kg
3	Delayed righting reflex Severe ataxia (drags abdomen)	2 g/kg
4	Loss of righting reflex	1 g/kg
5	Loss of eye-blink reflex	0 g/kg

## C Withdrawal Severity Scale

Score	Behavioral Indications
1	Hyperactivity
1.4	Tail tremor
1.6	Tail spasm
2.0	Caudal tremor
2.4	Splayed limbs
2.6	General tremor
3.0	Head tremor
3.4	Wet dog shake
3.6	Chattering teeth
3.8	Spontaneous convulsion

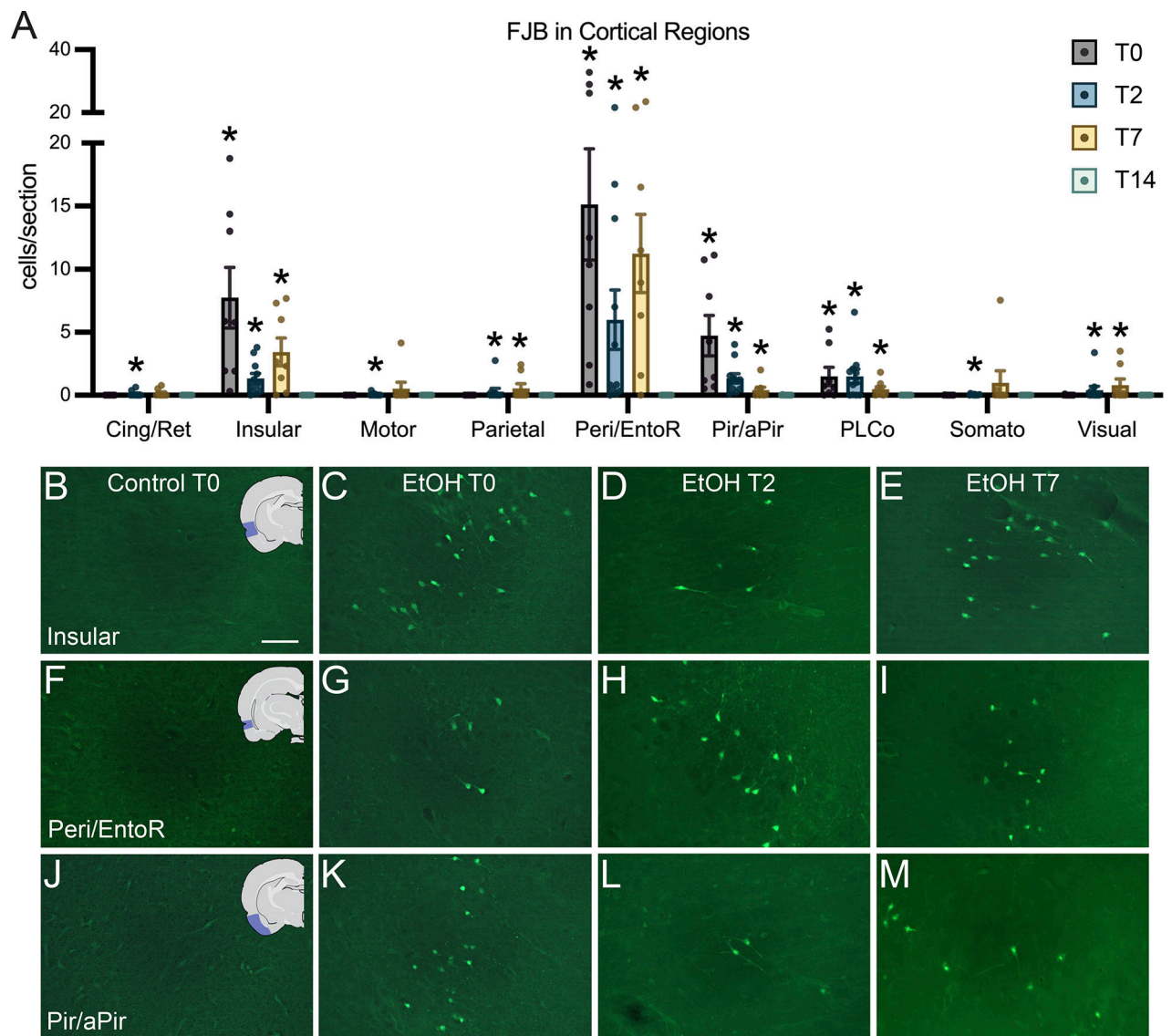
### Figure 1:

(A) Animals were acclimated to the vivarium, then administered ethanol three times a day for four days, with BECs taken after the 7<sup>th</sup> dose. Animals were sacrificed (sac) at one of four time points, either after the end of ethanol administration (T0), or at two (T2), seven (T7), or fourteen (T14) days of abstinence from ethanol. (B) Intoxication behavior scale and each corresponding ethanol dose. (C) Withdrawal severity scale used from 10 – 27 hours after the last dose of ethanol.

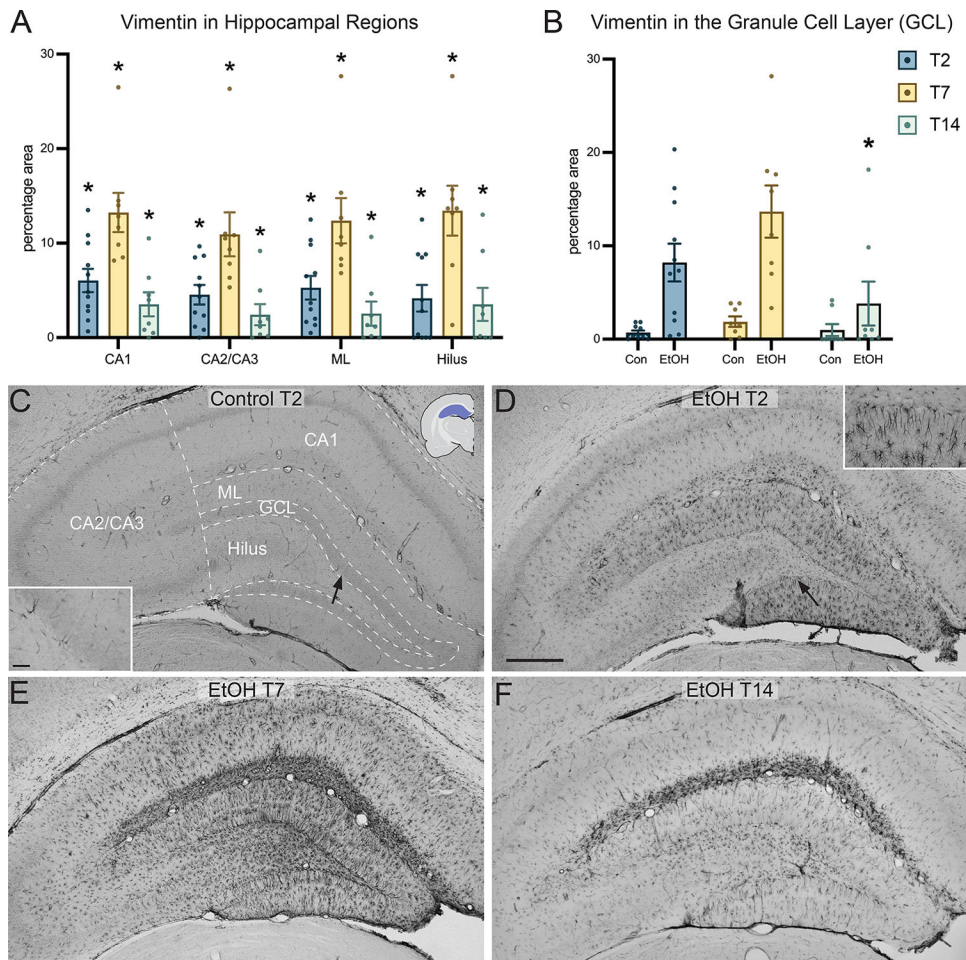


**Figure 2:** FluoroJade-B dye labeling of degenerating neurons in the granule cell layer of the dentate gyrus. (A) Quantification of FJB cells per section at each of the four time points in the dentate gyrus. (B-I) Representative images of the granule cell layer from control and ethanol treated rats are shown for each time point. Scale bar = 50 $\mu$ m; \* $p < 0.05$ .

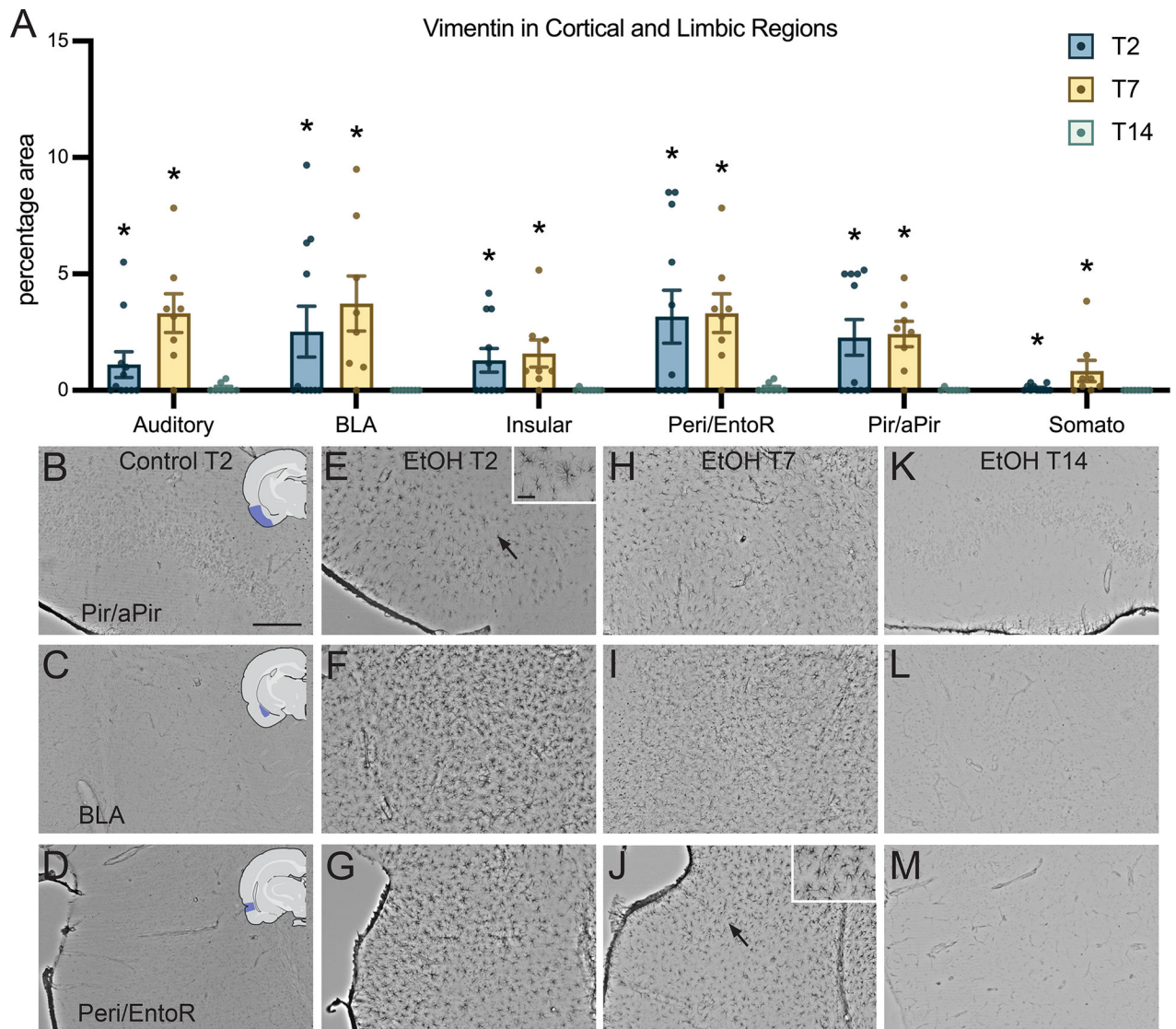




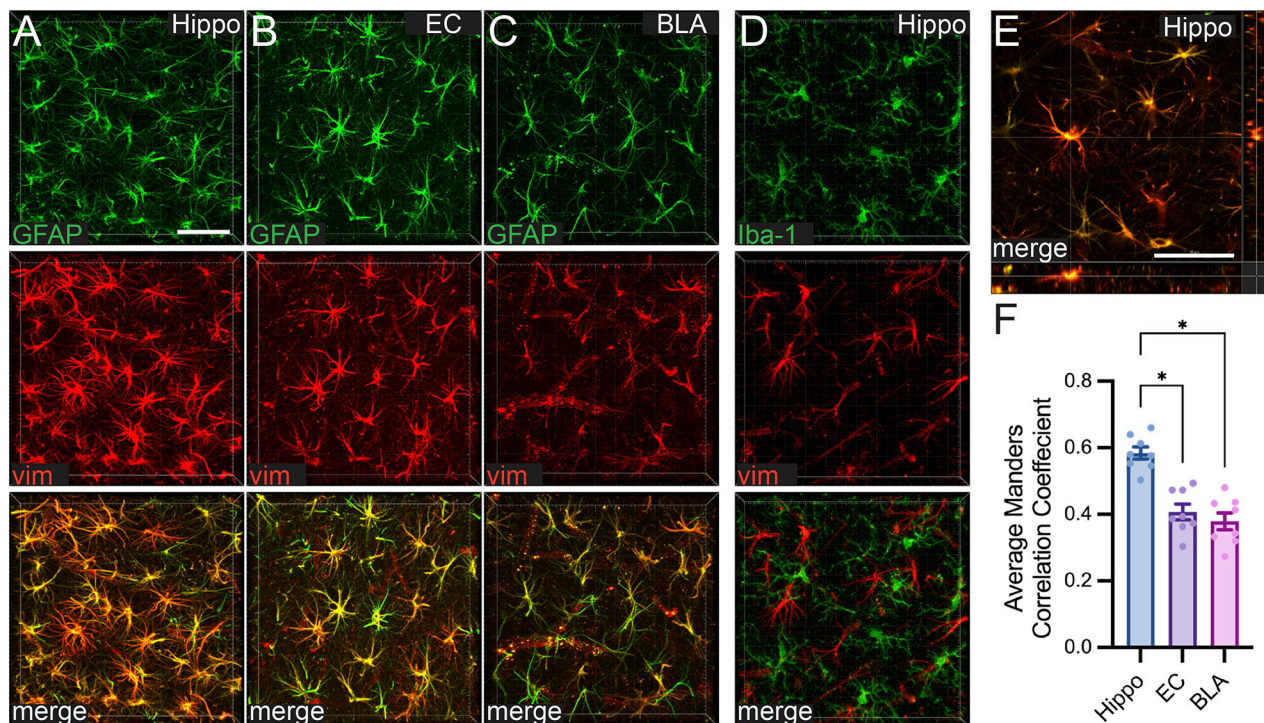
**Figure 3:** FluoroJade-B dye labeling of degenerating neurons in cortical regions. (A) Quantification of FJB cells per section in regions where FJB was significantly elevated above controls: cingulate/retrosplenial cortex (Cing/Ret), insular cortex, motor cortex, parietal cortex, combined perirhinal and entorhinal cortex (Peri/EntoR), combined piriform and apiriform cortex (Pir/aPir), posterolateral cortical amygdaloid nucleus (PLCo), somatosensory cortex, and visual cortex. (B-M) Representative images in control rats at T0 and ethanol treated rats at T0, T2, and T7 of abstinence from ethanol in the insular cortex, peri/entorhinal cortex and piriform/apiriform cortices. Scale bar = 50 $\mu$ m; \* $p < 0.05$ .



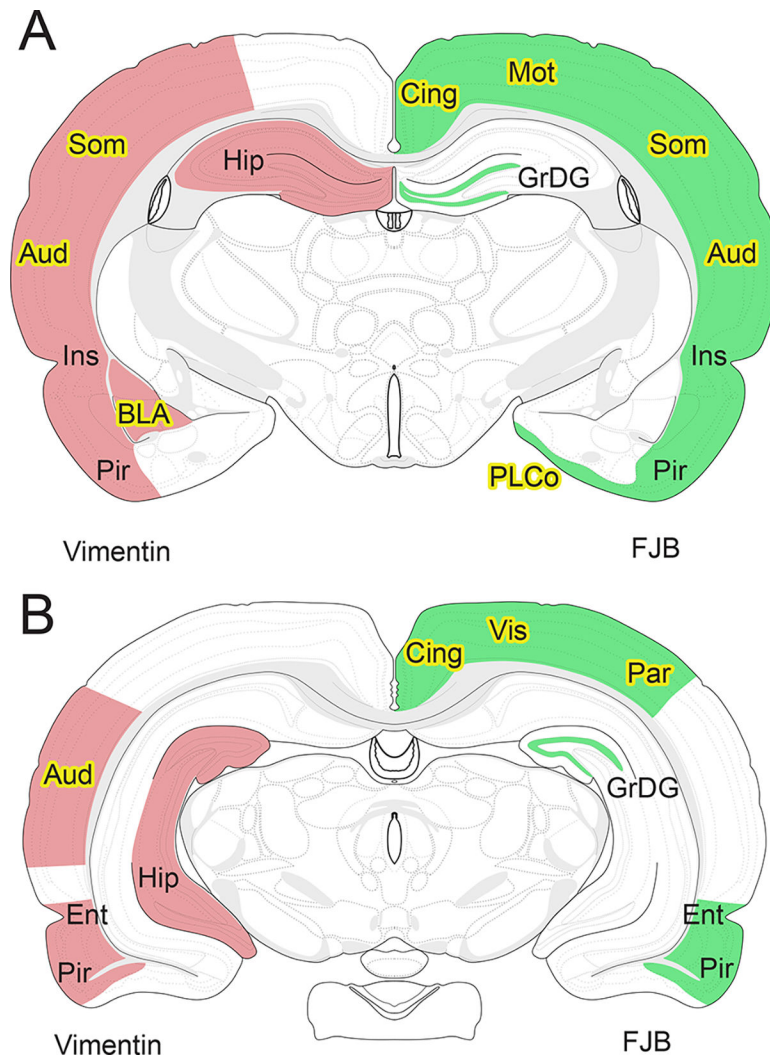
**Figure 4:** Vimentin immunoreactivity in the hippocampus. (A) Quantification of vimentin immunoreactivity in different hippocampal regions, the CA1 band, CA2/CA3 bands, molecular layer (ML) and the hilus at the T2, T7, and T14 time points. (B) Due to higher baseline immunoreactivity of neural stem/progenitor cells in the granule cell layer of the hippocampus, vimentin immunoreactivity was measured separately at the T2, T7, and T14 time points. (C) Representative 4x image of a control treated rat hippocampus at the T2 time point with hippocampal subregions delineated. (D-F) Representative images of the hippocampus of ethanol treated rats at T2, T7, and T14 time points. Both inserts are taken in the dentate gyrus, location is indicated by an arrow. Scale bar = 500  $\mu$ m; insert scale bar = 50  $\mu$ m; \*  $p < 0.05$ .



**Figure 5:** Vimentin immunoreactivity in cortical regions. (A) Quantification of vimentin immunoreactivity at T2, T7, and T14 in cortical regions: auditory cortex, the basolateral amygdala (BLA), the insular cortex, the peri/entorhinal cortex (Peri/EntoR), the piriform/apiriform cortex (Pir/aPir), and the somatosensory cortex (Somato). (B-M) Representative images of vimentin in control treated rats at T2, and ethanol treated rats at T2, T7 and T14 in the piriform/apiriform cortex, basolateral amygdala, and peri/entorhinal cortex. Scale bar = 250  $\mu$ m; insert scale bar = 50  $\mu$ m; \*  $p < 0.05$ .



**Figure 6:** Z-stack images of vimentin colabeled with markers for astrocytes (A-C) but not microglia (D). (A-C) Representative images of a GFAP (green)/vimentin (red) colabel (merge image on the bottom) taken in the hippocampus (Hippo), entorhinal cortex (EC), and basolateral amygdala (BLA) of an ethanol treated rat at T7. (D) Representative 3D rendered z-stack images of an Iba-1 (green)/vimentin (red) colabel (merge image on the bottom) taken in the hippocampus of an ethanol treated rat at T7. (E) Orthogonal view of the merged GFAP/vimentin colabel in B to show vimentin and GFAP labeling the same cell. (F) The overlap of GFAP with vimentin quantified using the average Manders Correlation Coefficient in the three brain regions examined. Both scale bars= 50µm; \*  $p < 0.05$ .



**Figure 7:**

A comparison of brain regions damaged by alcohol in female versus male rats after alcohol dependence in a 4-day binge model. Regions with statistically significant increases in vimentin (pink) or FluoroJade-B (green) over controls in ethanol treated female rats. Yellow highlights indicate regions where statistically significant vimentin staining or FluoroJade-B was not previously reported in males. (A) Coronal view of the rat brain at  $-3.00\text{mm}$  Bregma and at (B)  $-5.00\text{mm}$  Bregma. Label abbreviations are as follows. GrDG: granule cell layer of the dentate gyrus, Ins: insular cortex, Pir: piriform/apiriform cortex, BLA: basolateral amygdala, Hip: hippocampus, Cing: cingulate/retrosplenial cortex, Mot: motor cortex, Som: somatosensory cortex, Aud: auditory cortex, PLCo: posterolateral cortical amygdaloid nucleus, Vis: visual cortex, Par: parietal cortex, Ent: peri/entorhinal cortex. Comparison is to male data from: Collins et al., 1996; Crews et al., 2000; Hayes et al., 2013; Kelso et al., 2011; Obernier, Bouldin, et al., 2002).

**Table 1.**

Alcohol model subject data

Time point	Subjects	BEC (mg/dL)	Dose (g/kg/day)	Intoxication Score	Mean WD	Peak WD
<b>T0</b>	n=8	395.7±70.5	9.6±1.2	1.8±0.4 <sup>b</sup>	n/a	n/a
<b>T2</b>	n=11	412.6±62.4	8.5±0.8 <sup>a</sup>	1.5±0.4	2.0±1.0 <sup>a</sup>	2.5±1.0
<b>T7</b>	n=8	396.7±68.2	10.2±1.0	1.6±0.3	1.0±0.7	3.3±0.4
<b>T7 (co-label only)</b>	n=8	362.2±45.1	9.9±1.2	1.5±0.2	0.7±0.5 <sup>c</sup>	2.7±1.1
<b>T14</b>	n=8	320.2±128	10.2±1.5	1.3±0.3	0.5±0.5	2.6±1.3

<sup>a</sup> p < 0.05 T2 vs. T7/T14;<sup>b</sup> p < 0.05 T0 vs. T14;<sup>c</sup> p < 0.05 T2 vs. T7 co-label