Sex-Specific Changes in Neuronal Activity and Affective Behavior after Chronic Alcohol Consumption in CRF1-cre Transgenic Rats

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SGQ: conceptualization, formal analysis, investigation (behavioral and immunofluorescence experiments), data curation, writing (original draft and review & editing), visualization; **GMA**: conceptualization, investigation (behavioral and immunofluorescence experiments), writing (original draft) ; **MGC**: investigation (immunofluorescence experiments); **MWH:** investigation (immunofluorescence experiments)**; DPE:** investigation (immunofluorescence experiments) ;**MCR**: investigation (behavioral and immunofluorescence experiments); **ME:** investigation (behavioral experiments); **MAH**: conceptualization, writing (review & editing), supervision, funding acquisition

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ABSTRACT

Alcohol use disorders are complex conditions characterized in part by excessive ethanol (EtOH) drinking and withdrawal-induced malaise. The corticotropin releasing factor-1 receptor (CRF1) has been implicated in EtOH drinking, affective states, and pain sensitivity; often in a sex-dependent manner. The studies presented here investigate the associations between baseline behavior, chronic intermittent EtOH intake, and withdrawal-induced affective behavior in male and female CRF1:cre: ^{td}Tomato rats. There were no sex differences in basal affective state or thermal sensitivity, as measured by the splash test, novelty suppressed feeding test, and Hargreaves, respectively. However, female rats displayed increased mechanical sensitivity, measured by the Von Frey test. Following baseline testing, rats underwent voluntary EtOH or water drinking under intermittent access conditions. Female rats consumed significantly more EtOH, but only during the first week. There were no group effects of EtOH on behavioral tests, but all of the rats demonstrated increased malaise upon repeated testing. There were significant individual differences where affective behavior positively correlated with negative affect in both sexes. There were also significant correlations between EtOH intake and CRF1-cFos co-expression in the infralimbic cortex and basolateral amygdala. EtOH decreased CRF1+ expression in the lateral amygdala, but there were no significant group effects of EtOH on cFos or CRF1-cFos co-expression in the medial prefrontal cortex or amygdala. Together, these results illustrate the complex interplay between affect, pain sensitivity, EtOH drinking, and the role of CRF1 containing neurons in mediating these behaviors.

Keywords: alcohol, anxiety, pain, corticotropin releasing factor receptor 1, amygdala, prefrontal cortex

1. INTRODUCTION

In 2020, 28.3 million Americans reported having an alcohol use disorder (AUD)(SAMHSA, 2021). AUDs are generally characterized by excessive drinking and negative affective states upon drinking cessation(Koob and Le Moal, 2005). These states can promote dependence as individuals drink to alleviate withdrawal symptoms like anxiety and anhedonia(Howe et al., 2021; Martinotti et al., 2008). However, anxiety also often precedes alcohol use and is linked to a more rapid progression of AUD in both humans(Boschloo et al., 2013; Kushner et al., 2011; Sartor et al., 2007; Sloan et al., 2003) and rodents(Hayton et al., 2012; Pelloux et al., 2015; Spanagel et al., 1995).

Numerous studies have investigated the co-occurrence of AUDs and mood disorders; however, our understanding of this relationship remains incomplete as both are influenced by a variety of other factors including sex. For example, rats bred for high anxiety or depressive phenotypes drink more alcohol (EtOH) than their low anxiety- and low-depressive phenotypic counterparts; but differences are only present in females(Izidio and Ramos, 2007; Vengeliene et al., 2005). Other studies found that anxiety-like behavior predicts EtOH intake in male, but not female, rats(McNamara and Ito, 2021). In humans, AUD is more prevalent among men(Grant et al., 2015); however, alcohol consumption and AUDs in women are increasing at a faster rate than men(Grant et al., 2017) and the sex-related differences are narrowing(White et al., 2015). Additionally, mood disorders are more common in women(Franceschini and Fattore, 2021; Salk et al., 2017) and women are more likely to develop comorbid anxiety and AUD(Kessler et al., 2005).

One major target of AUD is the pro-stress corticotropin releasing factor (CRF) system. While hypothalamic release of CRF activates the hypothalamic-pituitary-adrenal axis, extrahypothalamic CRF acts as an important neuromodulator through actions at its two G-protein coupled receptors, CRF1 and CRF2. In humans, polymorphisms in the gene encoding CRF1 are associated with AUD and AUD-related behaviors such as binge drinking, EtOH intake, and intoxication(Chen et al., 2010; Treutlein et al., 2006). Additionally, polymorphisms in CRF1 are also associated with negative affective behaviors such as post-traumatic stress disorder, panic disorder, depression, suicidality, and loneliness(Bradley et al., 2008; Chou et al., 2014; Grabe et al., 2010; Keck et al., 2008; Kranzler et al., 2011; Wasserman et al., 2009; Weber et al., 2016). In rodents, systemically-administered CRF1 antagonists decrease anxiety-like behavior, EtOH intake, EtOH-induced negative affect and EtOH-induced hyperalgesia(Breese et al., 2005; Edwards et al., 2012; Lowery et al., 2010; Overstreet et al., 2005; Sparta et al., 2008; Valdez et al., 2002; Wang et al., 2011). Alcohol-preferring rats have increased CRF1 levels in multiple regions of the brain, including cortex and amygdala(Hansson et al., 2006).

The prefrontal cortex and amygdala are two regions extensively involved in both affective behavior and AUDs(Abernathy et al., 2010; Agoglia and Herman, 2018; Centanni et al., 2019; Egli et al., 2012; Hare and Duman, 2020; Jacobs and Moghaddam, 2021; Kenwood et al., 2022; Lu and Richardson, 2014; McCool, 2021; Melkumyan and Silberman, 2022; Neugebauer et al., 2020; Roberto et al., 2021; Smith

and Torregrossa, 2021; Veinante et al., 2013; Zhang et al., 2021). Both regions have an abundance of CRF1-containing cells (Agoglia et al., 2020; Calakos et al., 2017; Conte et al., 2019; Gilpin et al., 2015; Patel et al., 2022; Robinson et al., 2019; Weera et al., 2022). However, the specific contributions of these cells to AUD and associated negative affect remains unclear. The present study uses behavioral testing, voluntary EtOH drinking, and immunofluorescence in a CRF1 transgenic rat line to investigate the interaction between EtOH drinking and affective behavior in a sex-specific manner.

2. METHODS

2.1 Subjects

32 adult male and female (n=16/sex) CRF1:cre: ^{td}Tomato rats(Weera et al., 2022) (8-11 weeks old, bred in-house) were housed in temperature and humidity-controlled rooms with 12h light/dark cycle (7:00 am lights ON). Rats had *ad libitum* access to food and water unless otherwise stated. Rats were handled before behavioral testing intake began. All animal protocols were approved by the Animal Care and Use Committee at the University of North Carolina at Chapel Hill.

2.2 Splash Test:

Rats were allowed to habituate in the behavioral testing room for 1h prior to testing. Rats were sprayed with 10% w/v sucrose solution, placed into a plexiglass/textured flooring behavioral testing chamber [50cm(*l*)×50cm(*w*)×38cm(*h*)] and recorded for 10min in red light conditions, as previously reported(Quadir et al., 2022). Latency to groom and total time spent grooming were scored by an experimenter blind to experimental group using the Behavioral Observation Research Interactive Software (BORIS) program(Friard and Gamba, 2016).One subject was excluded from analysis due to equipment malfunction during testing.

2.3 Mechanical Sensitivity Testing (Von Frey):

Rats were brought into behavioral testing room 1h prior to testing, and were habituated for 15min to a stainless-steel table [(78cm(*l*)×32cm(*w*)×19.5cm(*h*)], with a perforated sheet containing staggered holes (0.28cm diameter). Plastic filaments of increasing force (Bioseb EB2-VFF) were applied perpendicularly to hind paws until bending, and nocifensive responses defined as shaking, licking, or withdrawing the paw were recorded, Each paw was prodded 3 times/filament, beginning with the 2g filament and increasing until the rat exhibited a nocifensive response during at least 2 trials. Withdrawal thresholds for each hind paw were then averaged for each animal.

2.4 Novelty Suppressed Feeding (NSF):

Rats were given 1 Froot Loop in the homecage 48h prior to testing to prevent neophobia. 24h later, food was removed from the cage. On test day, rats were habituated to behavioral testing room for 1h prior to testing. During testing, rats were individually placed into a brightly-lit (150 lux) behavioral testing chamber [50cm(*l*)×76cm(*w*)×40cm(*h*)] containing one Froot Loop on filter paper in the center. Latency to eat was recorded, consistent with previous work(Quadir et al., 2022). After feeding initiation (or after 10min), rats were removed from the chamber and returned to the homecage where post-test consumption of pre-weighed Froot loops was recorded for 10min.

2.5 Thermal Sensitivity Testing (Hargreaves Test)

Rats were assessed for thermal sensitivity using the Plantar Analgesia Meter (IITC). Rats were brought into the testing room 1h prior to testing and habituated to the heated glass (temperature: 32C) apparatus for 20min. Infrared light (artificial intensity: 40) was focused onto each paw and withdrawal latency was recorded. A maximum cutoff of 20s was used to prevent tissue damage. Each paw was tested twice, unless latencies

differed by more than 1s, then test was repeated. Latencies were averaged per paw then per animal, consistent with our previous studies(Quadir et al., 2022).

2.6 Intermittent Access to Two Bottle Choice (IA2BC)

Intermittent access two bottle choice alcohol drinking was performed as previously described (Quadir et al., 2022; Quadir et al., 2020b; Quadir et al., 2021b; Wise, 1973). After 4d acclimatization to water bottle drinking, one bottle containing 20% EtOH and one bottle of water for 24h were placed on cages on alternating days. Water drinking rats had bottles changed every 24h to ensure comparable treatment. Bottle placement was alternated to eliminate side preference. Ethanol for voluntary drinking experiments (20% v/v) was prepared by diluting 95% ethanol (Pharmco Products Inc) in tap water.

2.7 Immunohistochemistry

24h after the last alcohol presentation, rats were anesthetized with isoflurane and perfused transcardially using phosphate-buffered saline (PBS) followed by 4% paraformaldehyde. Brains were post-fixed in 4% PFA at 4°C overnight and then transferred to 30% sucrose in PBS at 4°C until brains sank. Brains were serially sectioned at 40 µm and stored long-term in cryoprotectant (30% sucrose, 30% ethylene glycol, 0.01% sodium azide in PBS) at 4°C.

4-6 sections per region were used. These 40 µm sections underwent PBS washes and were incubated in 50% methanol for 30 minutes. Slices were blocked in 3% hydrogen peroxide for 10min then 3% normal goat serum, 1% bovine serum albumin, 0.3% Triton X in PBS for 1h (with PBS washes in-between). Slices were incubated at 4°C for 48h with rabbit anti-cFos primary antibody (1:3000, Millipore Sigma ABE457) and mouse anti-RFP primary antibody (1:500, Invitrogen MA5-15257) in blocking solution. Slices were then washed with 0.1% Tween-20 in Tris-buffered saline (TNT) before 30min incubation in TNB blocking buffer (Perkin-Elmer FP1012). Next, slices were incubated with goat anti-rabbit horseradish peroxidase (1:400, Abcam ab6721) and goat anti-mouse 555 (1:800, Invitrogen A2123) in TNB buffer for 2h before TNT washes. Lastly, slices were incubated in tyramide conjugated fluorescein (Akoya Biosciences, NEL741001KT, 1:50) for 10 min. Following TNT washes, slices were mounted and sealed with mounting medium (Vector Laboratories H1500, Burlingame, CA). Slides were stored at 4ºC until imaging with the Keyence BZ-X800 fluorescence microscope.

Imaging and quantification were performed by an experimenter blind to the condition and sex of each animal. Images were taken at 20X and stitched together using the BZ-X800 Analyzer program. One hemisphere was selected at random for quantification, where the area of interest was outlined and counted manually using the FIJI multi-point counter tool(Schindelin et al., 2012). Cell counts were averaged per animal.

2.8 Statistical Analysis

All statistical analysis was performed using Graphpad Prism 8.0 (San Diego, CA). In all studies, the threshold for significance was set to $p<0.05$. Data was assessed for normality using the Shapiro–Wilk test or D'Agostino & Pearson. For data that did not follow a normal distribution, nonparametric tests were used.

Baseline behavioral tests were analyzed using an unpaired Student's t test (parametric data with equal sample variances), Welch's t test (parametric data with unequal sample variances), or a Mann-Whitney U test (nonparametric data). For cumulative incidence graphs, Kaplan-Meier survival curves were generated and were followed by Mantel-Cox log-rank test (followed by Bonferonni when applicable). Fluid intake and preference were analyzed by a mixed model 2-way ANOVA, using session as a within-subjects factor and sex as a between-subjects factor. Cumulative intake data were analyzed using an unpaired Student's t test (equal sample variances) or an unpaired Welch's t test (unequal sample variances). When behavioral tests were repeated (following drinking), data was analyzed using a 3-way mixed ANOVA (using time as a within-subjects factor while EtOH and sex were analyzed as between-subjects factors). Correlational analyses were performed using Pearson's correlation (parametric data) or Spearman's correlation (nonparametric data). To compare correlations between males and females, Fisher's r to z transformation was used:

Fisher's Z: $z_r=0.5\ln((1+r)/(1-r))$ where r = Pearson's r (or Spearman's r_s)

Z_r from males and females (denoted z_m and z_f, respectively) was used to compute an observed z value, z_{obs} with the following calculation:

$$z_{obs}=\frac{z_m-z_f}{\mathrm{sqrt}(1/(N_m-3)+1/(N_f-3))}$$ where N = number of males (N_m) or females (N_f).

In each set of correlations, the same analysis (Pearson's or Spearman's) was used for both sexes to calculate a z_{obs}.

Immunofluorescence data was analyzed by a 2-way ANOVA using sex and EtOH as between-subjects factors.

3. RESULTS

3.1 Experimental Timeline

Rats were single-housed 1wk before testing. Baseline behavioral tests were conducted on separate but consecutive days (**Fig 1A)**. Rats were then split into 2 groups (EtOH and Water). After 3wks of drinking, testing began.in a pseudo-randomized subject order to minimize effects of circadian cycle and withdrawal time. After the last behavioral test, rats underwent a final EtOH drinking session before tissue collection.

3.2 Baseline Affective Behavior

The Von Frey and Hargreaves tests were used to examine sex differences in mechanical and thermal sensitivity respectively. In the Von Frey test, females exhibited decreased withdrawal thresholds, suggesting increased mechanical sensitivity (**Fig 1B**; Mann-Whitney test, $p<0.0001$). There were no sex differences in latency to withdrawal in the Hargreaves test, suggesting similar thermal sensitivity. (**Fig 1C;** unpaired Welch's t-test, $t(23.55)=0.5586$, $p=0.5817$).

The splash test was used to examine sex differences in affective behavior. There were no sex differences in latency to groom (**Fig 1D**; Mann-Whitney Test, $p=0.5140$). As several animals never initiated grooming (n=2 for males, n=5 for females; rats assigned a value of 600s), these data were also examined with a Kaplan-Meier survival curve and compared using the Mantel-Cox log-rank test (**Fig S1A**). This analysis demonstrates that even when accounting for the rats that never groomed (rather than artificially assigning them a maximum value or excluding them from studies), there was no sex difference in grooming initiation (**Fig S1A;** Mantel-Cox log-rank test: $\chi^2=1.430$, df=1, $p=0.2318$). There was also no sex difference in total time spent grooming (**Fig 1E;** Mann-Whitney test, $p=0.8901$).

The NSF was also used to examine affective behavior, and there was no effect of sex on latency to eat (**Fig 1F;** Mann-Whitney test, $p=0.7520$) or cumulative occurrence of feeding initiation (**Fig S1C**; Mantel-Cox log-rank test, $\chi^2=0.3366$, df=1, $p=0.5618$). In order to control for motivation to eat, post-test home-cage consumption was monitored and there was no effect of sex (**Fig 1G**; unpaired Student's t-test, $t(30)=0.02634$, $p=0.9792$). Together, these tests suggest there are no sex differences in basal affective behavior.

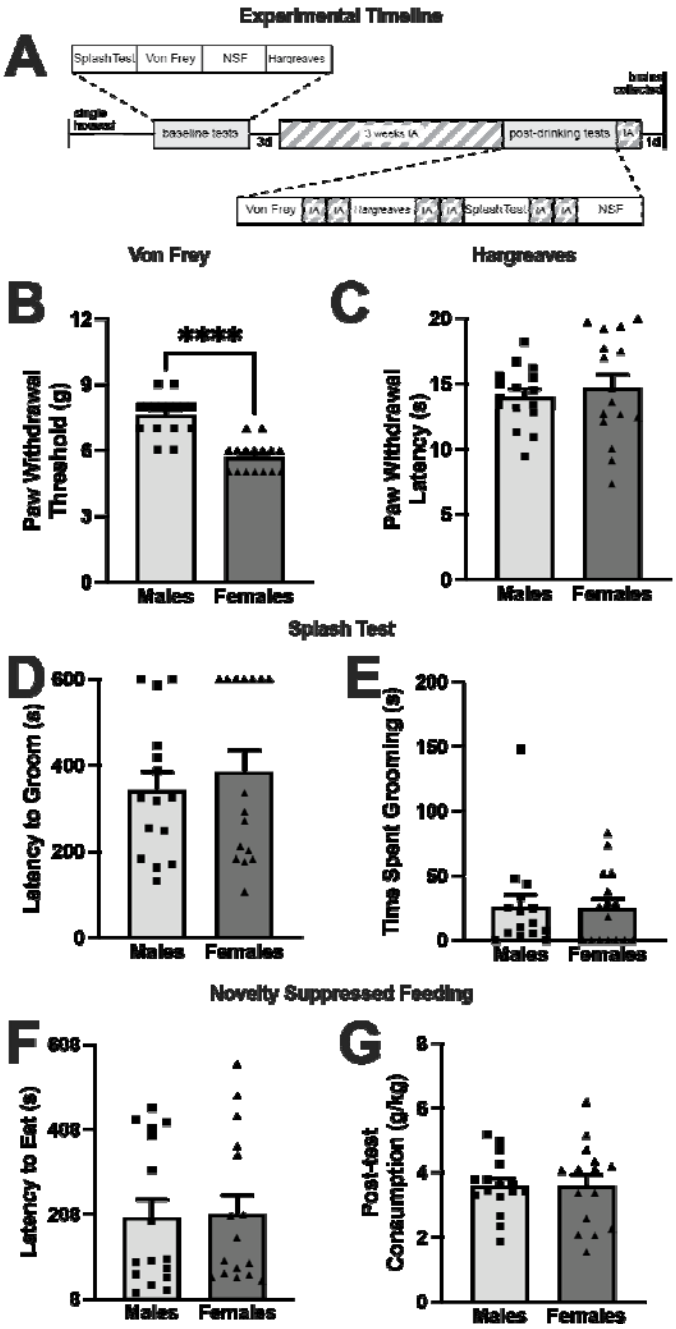

Figure 1.

Experimental timeline and baseline behavior. **A**) Timeline of entire experiment. **B**) Mechanical sensitivity in Von Frey test, as measured by paw withdrawal threshold. **C**) Thermal sensitivity in the Hargreaves test, as measured by latency to withdraw. **D)** Anxiety-like behavior in the splash test, as measured by latency to groom. **E)** Depressive-like behavior in the splash test, as measured by time spent grooming. **F**) Anxiety-like behavior in the novelty suppressed feeding test, as measured by latency to eat. **G**) Motivation to eat in the novelty suppressed feeding test, as measured by post-test consumption

**** $p<0.0001$ (Mann-Whitney test)

3.3 Voluntary EtOH Drinking

After assessing baseline affective behavior, rats were divided into two groups, one of which drank EtOH on alternating days. There were no significant sex differences in overall EtOH intake (**Fig. 2A**; 2-way ANOVA; Sex□×□Time: $F(15,207)=1.352$, $p=0.1740$; Sex: $F(1,14)=1.187$, $p=0.2943$; Time: $F(15,207)=1.612$, $p=0.0726$); however, females consumed significantly more EtOH in the first week of drinking (**Fig 2B**; unpaired Student's t test: $t(14)=3.442$, $p=0.0040$). There were no sex differences in water intake overall (**Fig. 2C**; 2-way ANOVA; Sex□×□Time: $F(15,205)=1.026$, $p=0.4302$; Sex: $F(1,14)=1.804$, $p=0.2006$; Time: $F(15,205)=2.882$, $p=0.004$) or during the first week (**Fig 2D**; unpaired Student's t test, $t(14)=1.739$, $p=0.1039$), although water intake in both sexes decreased over time. While EtOH preference increased over time in a non-sex-dependent manner (**Fig. 2E**; 2-way ANOVA; Sex□×□Time: $F(15,204)=1.344$, $p=0.1784$; Sex: $F(1,14)$□=□0.001386, $p=0.9708$; Time: $F(15,204)=3.382$, $p<0.0001$), females exhibited a stronger preference for EtOH during the first week (**Fig 2F;** unpaired Student's t test, $t(14)=2.192$, $p=0.0458$). Additionally, females consumed significantly more total fluid compared to males overall (**Fig 2G**; 2-way ANOVA; Sex□×□Time: $F(15,206)=0.7308$, $p=0.7516$; Sex: $F(1,14)=5.320$, $p=0.0369$; Time: $F(15,206)=1.269$, $p=0.2240$) and during the first week (**Fig 2H;** Welch's t test, $t(9.424)=2.299$, $p=0.0459$).

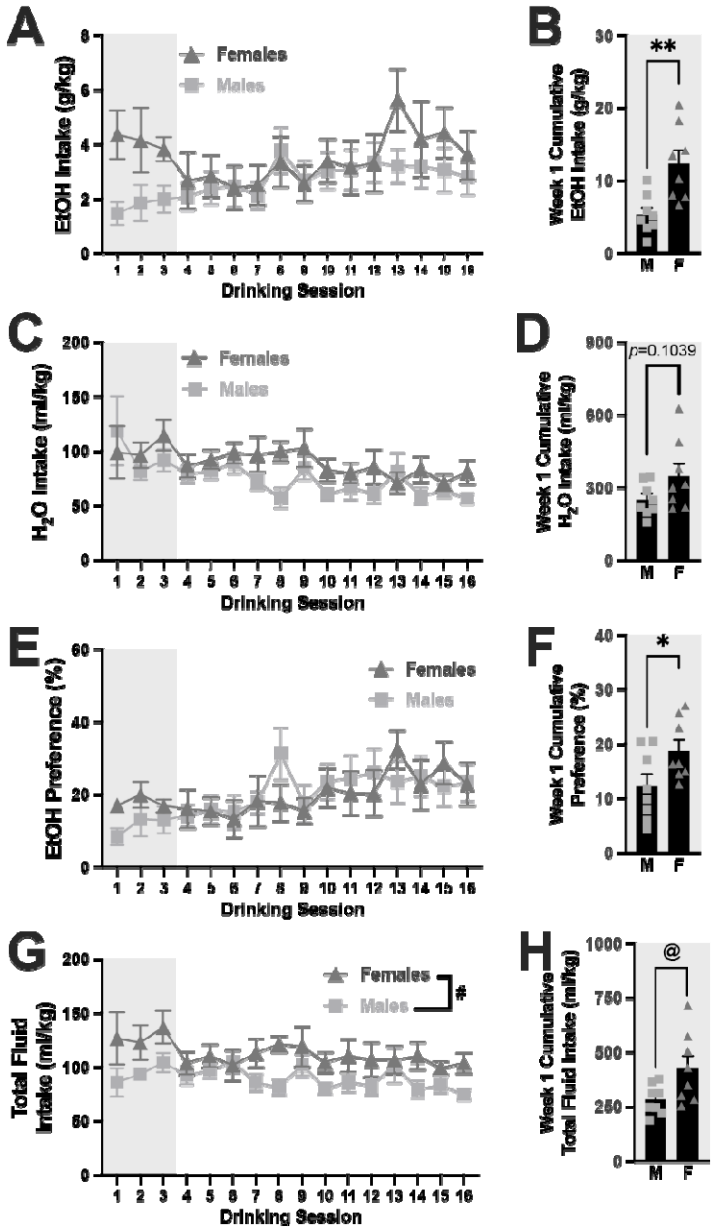

Figure 2.
Voluntary EtOH drinking under an intermittent access paradigm. **A**) EtOH intake over time. **B**) Cumulative EtOH intake during the first week of EtOH access. **C**) Water intake over time. **D)** Cumulative water intake during the first week of EtOH access. **E**) EtOH preference over time. **F**) Cumulative EtOH preference during the first week of EtOH access. **G**) Total fluid intake over time. **H**) Cumulative total fluid intake during the first week of EtOH access.

** $p<0.01$, *$p<0.01$ (unpaired Student's t test); # $p<0.05$ (2-way ANOVA, Main Effect of Sex); @ $p<0.05$ (unpaired Welch's t test)

3.4 Relationship Between Baseline Behavior and Initial EtOH Intake

Behavioral measures were correlated with first day EtOH intake. There was a trend where the rats with lower withdrawal thresholds drank more EtOH (**Fig S2A**; Pearson's correlation; males and together: r(14)= -0.4742, *p*=0.0635; males: r(6)=0.5673, *p*=0.1425; females: r(6)=-0.2699, *p*=0.5179). Interestingly, there was no association between initial EtOH intake and basal thermal withdrawal (**Fig S2B;** Pearson's correlation: males and together: r(14)= 0.1836, I=0.4962; males r(6)=0.3344, *p*=0.4162; females: r(6)=0.1388 *p*=0.7431), or basal latency to groom (**Fig S2C**; Spearman's Correlation; males and females together: $r_s(14)=0.3991$, *p*=0.1266; males: $r_s(6)=0.03593$, *p*=0.9434; females: $r_s(6)=0.4637$, *p*=0.2619) and time spent grooming (**Fig S2D**; Spearman's Correlation; males and females together: $r_s(14)=-0.2088$, *p*=0.4355 males: $r_s(6)=0.3234$, *p*=0.4317; females: $r_s(6)=-0.4364$, *p*=0.3036) in the splash test. There was also no significant relationship between initial EtOH intake and latency to eat (**Fig S2E**; Spearman's Correlation; males and females together: $r_s(14)=0.1177$, *p*=0.6631; males: $r_s(6)=0.3571$; *p*=0.3894; females: $r_s(6)=-0.2857$, *p*=0.5008) or post-test consumption (**Fig S2F**; Pearson's correlation: males and together: r(14)=0.1751, *p*=0.5167; males: r(6)=0.4654, *p*=0.2451; females: r(6)=-0.01082, *p*=0.9797) in the NSF test. Together, these analyses demonstrate that baseline affective state is not associated with basal pain sensitivity or affective behavior.

3.5 EtOH Drinking and Associated Pain Sensitivity

After 3 weeks of drinking, paw withdrawal threshold decreased over time and there were main effects of EtOH and sex and a sex by time interaction (**Fig. 3A**; 3-way ANOVA; Sex × Time × EtOH: F(1,28)=0.000, *p*>0.999; Time × EtOH: F(1,28)=1.674, *p*=0.2062; Sex × EtOH: F(1,28)=0.04308, *p*=0.8371; Sex × Time: F(1,28)=5.628, *p*=0.0248; Time: F(1,28)=39.12, *p*<0.0001, Sex: F(1,28)=4.308, *p*=0.0472; EtOH: F(1,28)=26.92; *p*<0.0001). However, paw withdrawal threshold was not correlated with previous day (**Fig 3B**; Pearson's correlation; males and females: r(14)=0.3155, p=0.2340; males: r(6)=0.04155, p=0.9222; females: r(6)=0.6262, p=0.0967) or subsequent day EtOH intake (**Fig 3C**; Pearson's correlation; males and females: r(14)=0.2660, *p*=0.3193, males: r(6)=0.03441, *p*=0.9355; females: r(6)=0.5708, *p*=0.1395); nor did testing influence EtOH consumption (**Fig S3A**; 2-way ANOVA; Sex × Day: F(1,14)=0.1799, *p*=0.6779; Sex: F(1,14)=0.01875, *p*=0.8930; Day: F(1,14)=1.244, *p*=0.2835).

The effect of chronic EtOH drinking on thermal sensitivity was examined next. There were no significant effects of time, EtOH, or sex on the latency to withdraw (**Fig. 3D**; 3-way ANOVA; Sex × Time × EtOH: F(1,28)=0.5697, *p*=0.4567; Time × EtOH: F(1,28)=3.394, *p*=0.0760; Sex × EtOH: F(1,28)=0.2055, *p*=0.6538; Sex × Time: F(1,28)=0.2997, *p*=0.5884; EtOH: F(1,28)=1.367; *p*=0.2521; Sex: F(1,28)=3.529 *p*=0.0707; Time: F(1,28)=1.224, *p*=0.2780). However, withdrawal latency correlated with both previous day (**Fig 3E**; Pearson's correlation; males and females: r(14)=0.2846, p=0.2853; males: r(6)=0.9043, *p*=0.0020; females: r(6)=-0.1378, *p*=0.7488) and subsequent day (**Fig 3F**; Pearson's correlation; males and females together: r(14)=-0.03709, *p*=0.8915; males: r(6)=0.8494, *p*=0.0075; females: r(6)=-0.6227, *p*=0.0991) EtOH intake in males, but not females. Indeed, both of these correlations were

statistically different (**Fig 3E**: Fisher's r to z transformation; z_{obs}=2.5836, *p*=0.0098; **Fig 3F:** Fisher's r to z transformation; z_{obs}=3.1360, *p*=0.0017). Lastly, there was no effect of Hargreaves testing on subsequent EtOH intake, (**Fig S3B**; 2-way ANOVA; Sex × Day: F(1,14)=0.01586, *p*=0.9016; Sex: F(1,14)=5.719e005, *p*=0.9941; Day: F(1,14)=0.2869, *p*=0.6006). These findings suggest that there are sex differences in the bidirectional relationship between individual EtOH intake and thermal sensitivity despite the lack of group effects.

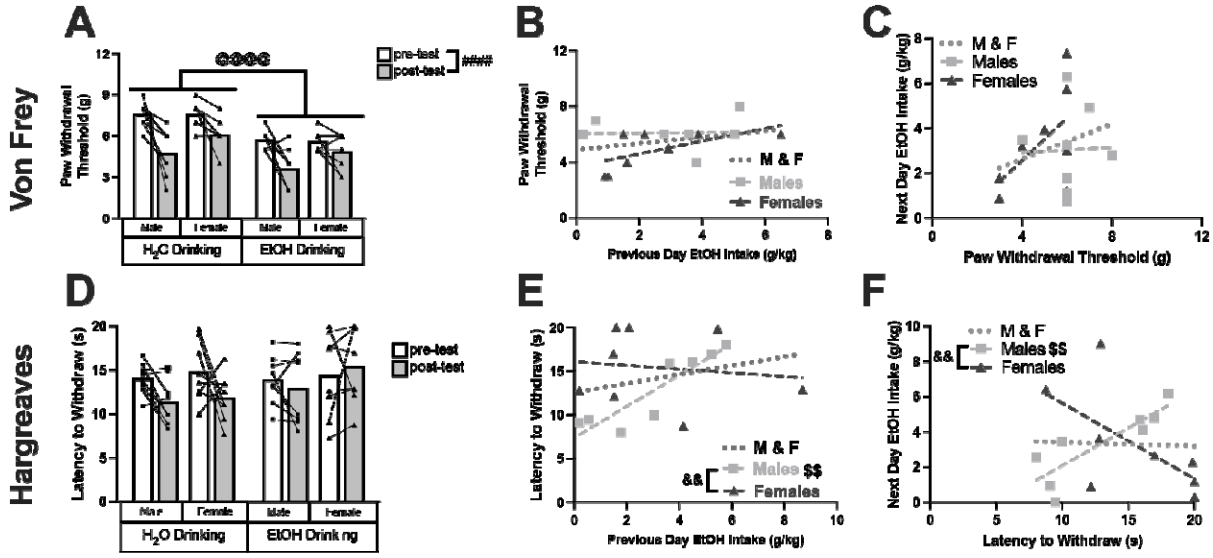

Figure 3.

Mechanical and thermal sensitivity following chronic EtOH drinking. The Von Frey test was repeated and mechanical sensitivity (**A**) was correlated with previous day EtOH intake(**B**) and subsequent EtOH intake (**C**). The Hargreaves test was also repeated and thermal sensitivity (**D**) was correlated with previous day EtOH intake (**E**) and subsequent EtOH intake (**F**).

p<0.0001 (3-way ANOVA, Main Effect of Time); @@@@ *p*<0.0001 (3 way ANOVA, Main effect of EtOH); $$ *p*<0.01 (Pearson's correlation); && *p*<0.01 (Fisher's r to z transformation)

3.6 EtOH Drinking and Associated Affective States

After 5 weeks of voluntary drinking, there was no effect of EtOH drinking on latency to groom in the splash test (**Fig 4A**; 3-way ANOVA; Sex × Time × EtOH: $F(1,27)=0.03307$, $p=0.8570$; Time × EtOH: $F(1,27)=0.3165$, $p=0.5784$; Sex × EtOH: $F(1,27)=4.164$, $p=0.0512$; Sex × Time: $F(1,27)=2.776$, $p=0.1073$; EtOH: $F(1,27)=0.6438$; $p=0.5923$; Sex: $F(1,27)=3.782$, $p=0.0623$; Time: $F(1,27)=0.9851$, $p=0.3298$). To account for animals that failed to groom during the 10 min test period, the cumulative incidence of grooming was plotted as previously described. Due to statistical limitations preventing within-group comparisons, baseline and post-test cumulative incidences of grooming were plotted on separate graphs. There were no differences in cumulative incidence of grooming during the baseline test (**Fig S4A;** Mantel-Cox log-rank test; $\chi^2=4.469$, df=3, $p=0.2151$). However, there was an effect during the post-test (**Fig S4B**; Mantel-Cox log-rank test; $\chi^2=6.968$, df=3, $p=0.0467$), specifically where there was an increased cumulative incidence of grooming in EtOH-drinking males compared to EtOH-drinking females ($\alpha_{Bonferroni}=0.0125$; $\chi^2=7.255$, df=1, $p=0.0071$). This suggests there are sex differences in anxiety-like behavior in rats with a history of EtOH drinking. Latency to groom was correlated significantly with previous day EtOH intake when sexes were combined (**Fig 4B**; Pearson's correlation; males and females together: $r(14)=0.6108$, $p=0.0120$), but not when separated (Pearson's correlation; males: $r(6)=0.4120$, $p=0.3105$; females: $r(6)=0.6172$, $p=0.1030$). Latency to groom was not associated with subsequent drinking, (**Fig 4C**; Pearson's correlation; males and females together: $r(13)=0.2592$, $p=0.3508$; males: $r(6)=0.4774$, $p=0.2269$; females: $r(5)=0.1107$, $p=0.3132$). nor did testing affect EtOH intake (**Fig S4C**; 2-way ANOVA; Sex × Day: $F(1,13)=1.958$, $p=0.1852$; Sex: $F(1,13)=1.883$, $p=0.1932$; Day: $F(1,13)=1.989$, $p=0.1819$).

Time spent grooming was also used to measure affective behavior, where there was a main effect of time, but not sex or EtOH (**Fig 4D**; 3-way ANOVA; Sex × Time × EtOH: $F(1,27)=2.150$, $p=0.1551$; Time × EtOH: $F(1,27)=0.1993$, $p=0.6589$; Sex × EtOH: $F(1,27)=1.272$, $p=0.2694$; Sex × Time: $F(1,27)=0.07479$, $p=0.7866$; EtOH: $F(1,27)=0.1483e\text{-}007$; $p=0.9997$; Sex: $F(1,27)=0.03729$, $p=0.8483$; Time: $F(1,27)=4.743$, $p=0.0383$). EtOH intake prior to the test was inversely correlated with time spent grooming, an effect driven primarily by females (**Fig 4E**; Spearman's correlation; $r_s(14)=-0.5859$, $p=0.0193$; males: $r_s(6)=-0.8456$, $p=0.0179$; females: $r_s(6)=0.07143$, $p=0.0179$). There was no association between total time spent grooming and subsequent EtOH intake (**Fig 4F**; Spearman's correlation; males and females: $r_s(13)=-0.02951$, $p=0.9180$; males: $r_s(6)=0.09524$, $p=0.8401$; females: $r_s(5)=-0.1782$, $p=0.7182$).

Next, the NSF test was repeated, and all rats increased latency to eat (**Fig. 4G**; 3-way ANOVA; Sex × Time × EtOH: $F(1,28)=0.09325$, $p=0.7623$; Time × EtOH: $F(1,28)=0.01534$, $p=0.9023$; Sex × EtOH: $F(1,28)=0.9187$, $p=0.3460$; Sex × Time: $F(1,28)=1.512$, $p=0.2291$; EtOH: $F(1,28)=0.3362$; $p=0.5667$; Sex: $F(1,28)=0.4367$, $p=0.5141$; Time: $F(1,28)=7.069$, $p=0.0128$; these data did not follow a normal distribution and the ANOVA was run on log-transformed data). There were no

differences in cumulative incidence of feeding initiating during baseline (**Fig S4D**; Mantel-Cox log-rank test; χ^2=0.5295, df=3, *p*=0.9124) or after chronic drinking (**Fig S4E**; Mantel-Cox log-rank test; χ^2=3.717, df=3, *p*=0.2937), suggesting that while rats decreased their anxiety-like behavior in the NSF, there are no sex differences between groups at each timepoint. Latency to eat correlated with previous day EtOH intake, driven primarily by males, (**Fig 4H**; Spearman's correlation; males and females together: $r_s(14)=0.5647$, p=0.0248; males: $r_s(6)=0.7381$, *p*=0.0458; females: $r_s(6)=0.4286$, *p*=0.2992) but did not correlate with subsequent EtOH intake (**Fig 4I**, Spearman's correlation; males and females: $r_s(14)=0.1912$, *p*=0.4769; males: $r_s(6)=0.6667$, *p*=0.0831; females: $r_s(6)=0.-0.1985$, *p*=0.4769). Similar to the Splash test, there was no effect of testing on EtOH intake in either sex (**Fig S4F**; 2-way ANOVA; Sex□×□Day: *F*(1,13)=0.5462, *p*=0.4721; Sex: F(1,14)=1.901, *p*=0.3442; Day: F(1,14)=1.901, *p*=0.1896).

Post-test home-cage consumption was monitored as a measure of motivation to eat. All rats ate significantly less in their home-cage compared to baseline (**Fig. 4J**; 3-way ANOVA; Sex × Time × EtOH: F(1,28)=0.2294, *p*=0.6357; Time × EtOH: F(1,28)=0.1.494, *p*=0.2317; Sex × EtOH: F(1,28)=0.8227, *p*=0.3727; Sex × Time: F(1,28)=0.001506, *p*=0.9693; EtOH: F(1,28)=1.248; *p*=0.2734; Sex: F(1,28)=0.006310, *p*=0.9372; Time: F(1,28)=29.96, *p*<0.0001). Post-test consumption did not correlate with previous day (**Fig 4K**; Pearson's correlation; males and females: r(14)=0.4202, *p*=0.1051; males: r(6)=0.1630, *p*=0.6997; females: r(6)=-0.6833, *p*=0.0617) or subsequent EtOH intake (**Fig 4L**; Pearson's correlation; males and females: r(14)=0.1875, *p*=0.4867; males: r(6)=-0.1263, *p*=0.7657; females: r(6)=0.4296, *p*=0.2881).

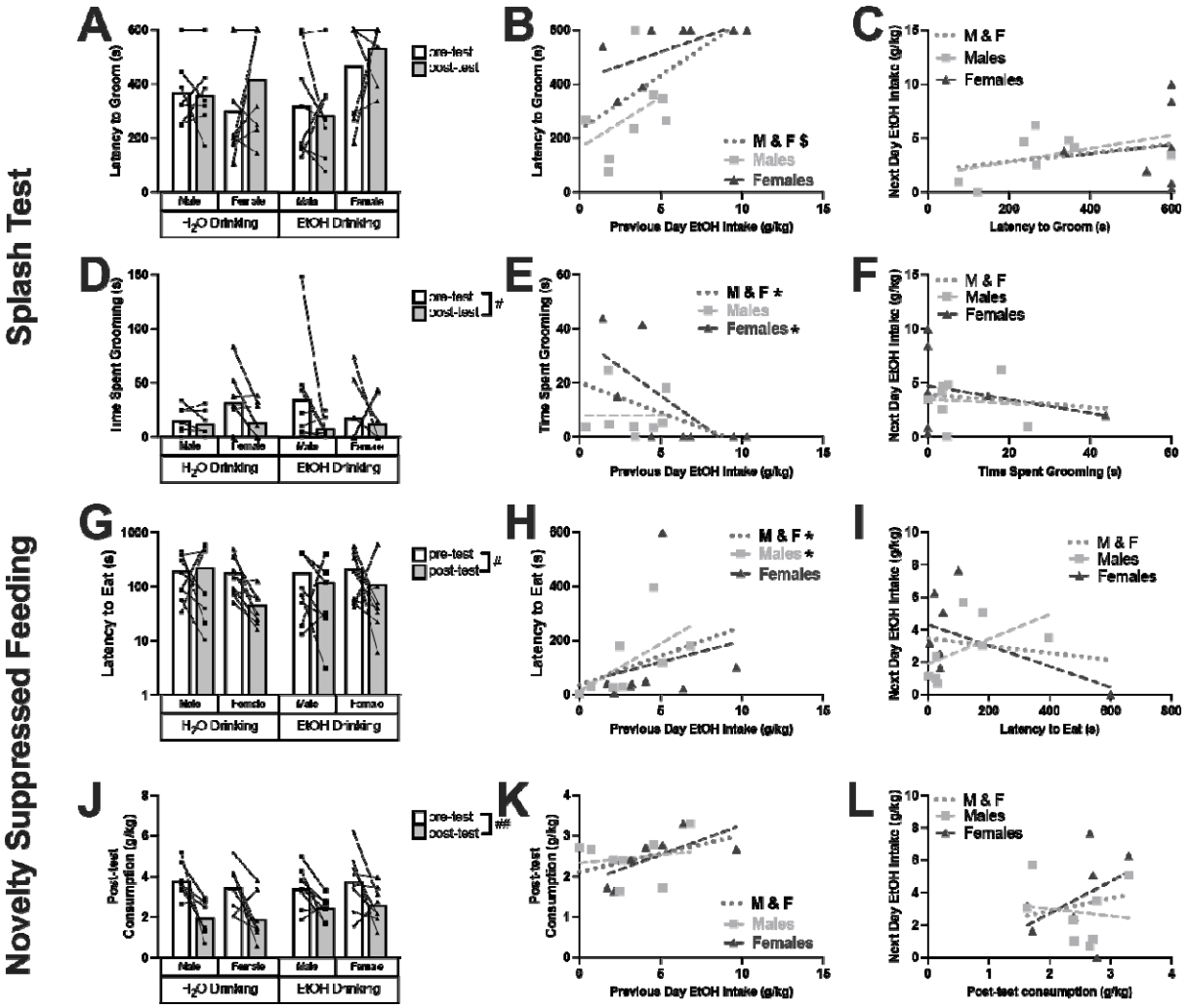

Figure 4.

Anxiety- and depressive-like behavior following chronic EtOH drinking. The splash test was repeated and anxiety-like behavior (**A**) was correlated with previous day EtOH intake (**B**) or subsequent EtOH intake (**C**). Depressive-like behavior in the splash test (**D**) was correlated with previous day EtOH intake (**E**) or subsequent EtOH intake (**F**). The Novelty Suppressed Feeding Test was also repeated. Anxiety-like behavior (**G**) was correlated with previous day EtOH intake (**H**) or subsequent EtOH intake (**I**). Motivation to eat (**J**) was correlated with previous day EtOH intake (**K**) or subsequent EtOH intake (**L**).

# $p<0.05$, ## $p<0.01$ (3-way ANOVA, Main Effect of Time); $ $p<0.05$ (Pearson's correlation); * $p<0.05$ (Spearman's correlation)

3.7 EtOH drinking and cFos in the Prefrontal Cortex

In the prelimbic cortex (PrL, **Fig 5A**) and there was no effect of sex or EtOH on CRF1+ expression (**Fig 5B**; 2-way ANOVA; Sex × EtOH: F(1,28)=0.0005307, *p*=0.9818; EtOH: F(1,28)=0.002376, *p*=0.9615; Sex: F(1,28)=0.1528, *p*=0.6988) . However, there was a trend toward decreased cFos expression in EtOH-drinking rats (**Fig 5C**; 2-way ANOVA; Sex × EtOH: F(1,28)=0.0003888, *p*=0.9844; EtOH: F(1,28)=3.972, *p*=0.0561; Sex: F(1,28)=2.112 *p*=0.1573). There was no effect of sex or EtOH on total double-labeled cells (**Fig 5D;** 2-way ANOVA; Sex × EtOH: F(1,28)=0.1194, *p*=0.7323; EtOH: F(1,28)=1.108, *p*=0.3015; Sex: F(1,28)=2.725 *p*=0.1100) or double-labeled cells as a function of total CRF1+ cells (%CRF1+) and while there was no significant effect of sex or EtOH (**Fig 5E**; 2-way ANOVA; Sex × EtOH: F(1,28)=0.1477, *p*=0.7037; EtOH: F(1,28)=2.757, *p*=0.1080; Sex: F(1,28)=4.015 *p*=0.0549), there was a trend toward sex differences.

EtOH intake did not correlate with CRF1+ expression (**Fig S5A**; Spearman's correlation; males and females: $r_s(14)=-0.1735$, *p*=0.5193; males: $r_s(6)=-0.5476$, *p*=0.1710; females: $r_s(6)=0.09524$, *p*=0.8401), cFos+ cells (**Fig S5B**; Pearson's correlation; males and females: r(14)=-0.1997, *p*=0.4582; males: r(6)=-0.25643, *p*=0.5271; females: r(6)=-0.3325, *p*=0.4210), total double-labeled cells (**Fig S5C;** Pearson's correlation; males and females: r(14)=-0.07430, *p*=0.7842; males: r(6)=-0.2966, *p*=0.4757; females: r(6)=-0.02222, *p*=0.9583) or double-labeled cells when expressed as %CRF1+ cells (**Fig S5D**; Pearson's correlation; males and females: r(14)=-0.01427, p=0.9582; males: r(6)=-0.1604, p=0.7043; females: r(6)=-0.09422, p=0.8244). Together, these findings suggest EtOH mediates PrL activity, but not in a dose-dependent manner.

In the infralimbic cortex (IL, **Fig 5F**), there no effect of sex or EtOH on CRF1+ cells (**Fig 5G**; 2-way ANOVA; Sex × EtOH: F(1,28)=0.7886, *p*=0.3821; EtOH: F(1,28)=0.01444, *p*=0.9052; Sex: F(1,28)=0.8904, *p*=0.3534). However, females had increased cFos+ expression (**Fig 5H**; 2-way ANOVA; Sex × EtOH: F(1,28)=0.000825, *p*=0.9773; EtOH: F(1,28)=1.639, *p*=0.2110; Sex: F(1,28)=7.233, *p*=0.0119). There were no significant differences in total double-labeled cells (**Fig 5I**; 2-way ANOVA; Sex × EtOH: F(1,28)=2.961, *p*=0.0963; EtOH: F(1,28)=2.026, *p*=0.1657; Sex: F(1,28)=1.307, *p*=0.2626), but the sex difference seen in **Fig 5H** persisted when presented as %CRF1+ cells (**Fig 5J;** 2-way ANOVA; Sex × EtOH: F(1,28)=0.7972, *p*=0.3795; EtOH: F(1,28)=1.242, *p*=0.2746; Sex: F(1,28)=7.356, *p*=0.0113). CRF1+ expression correlated directly with EtOH intake in females, but inversely correlated with EtOH intake in males (**Fig 5K**; Pearson's correlation; males and females: r(14)=-0.07998, *p*=0.7684; males: r(6)=-0.7277, *p*=0.0407; females: r(6)=0.8307, *p*=0.0106; Fisher's r to z transformation; z_{obs}=-3.3429, p=0.0008). cFos+ expression correlated inversely with EtOH intake in males only (**Fig 5L**; Pearson's correlation; males and females: r(14)=-0.1695, *p*=0.5403; males: r(6)=-0.7775, *p*=0.0231; females: r(6)=0.2192, *p*=0.6022; Fisher's r to z transformation; z_{obs}=-1.19950, *p*=0.0460). Consistent with the correlations seen thus far, total double-labeled cells correlated inversely with EtOH intake in males only (**Fig 5M**; Pearson's correlation; males and females: r(14)=-0.3485, *p*=0.1858; males: r(6)=-0.5444, *p*=0.1630; females: r(6)=-0.4090, *p*=0.3144; Fisher's r to z transformation; z_{obs}=-2.6132, *p*=0.0090). Finally, %CRF1+ cells did not correlate with

EtOH intake on the last session (**Fig 5N**; Pearson's correlation; males and females: r(14)=-0.3485, *p*=0.1858; males: r(6)=-0.5444, *p*=0.1630; females: r(6)=-0.4090, *p*=0.3144). Taken together, these findings suggest sex- and dose-dependent effects of EtOH on IL CRF1+ and cFos containing cells.

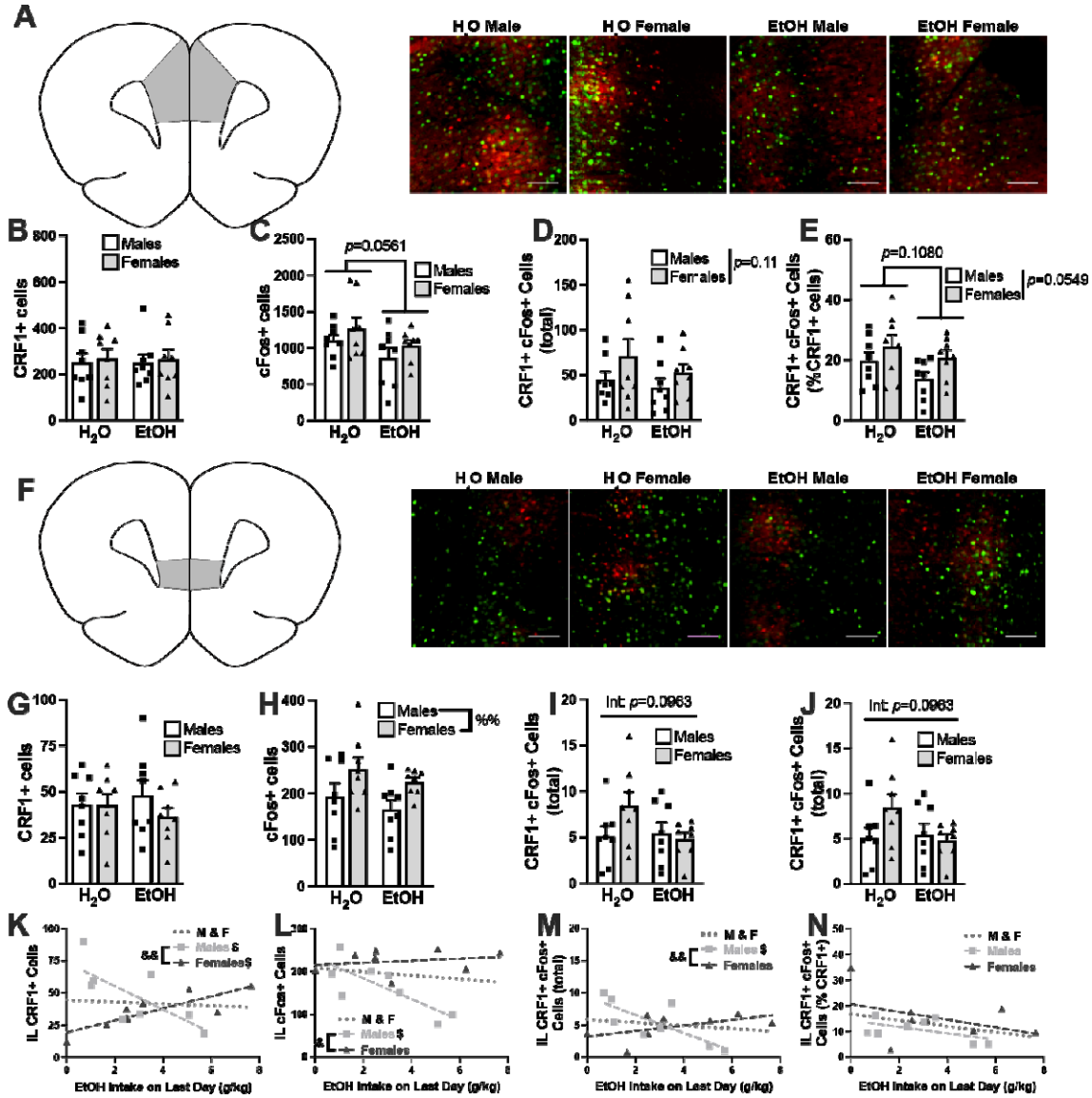

Figure 5.

Prefrontal correlates of EtOH Drinking. Slices containing both the prelimbic and infralimbic cortices (PrL and IL, respectively) were stained for RFP (as a measure of CRF1+ neurons, shown in red) and cFos (as a measure of cellular activity, shown in green). PrL representative images are shown in **(A)**. PrL CRF1+, cFos, and total double labeled cell counts are shown in **B-D** respectively. **E**) Double labeled cells were also expressed as % total CRF1+ cells. IL staining was also quantified, with representative images shown in **(F)**. IL CRF1+, cFos, and total double labeled cell counts are shown in (**G-I)** respectively. **J)** Double labeled cells were also expressed as % total CRF1+ cells. We also correlated EtOH intake on the last day with IL labeling of CRF1+ cells **(K),** cFos+ cells **(L),** total double labeled cells **(M)** and double labeled cells as %CRF1+ **(N)**.

Scale bars= 100µm; %% $p<0.01$ (2-way ANOVA; Main effect of Sex); $ $p<0.05$ (Pearson's correlation); && $p<0.01$ (Fisher's r to z transformation)

3.8 EtOH Drinking and cFos in the amygdala

CRF1 and cFos expression were examined in the amygdala, specifically the central amygdala (CeA), lateral amygdala (LA), and basolateral amygdala (BLA). Beginning with the CeA (**Fig 6A**), there were statistically no effects of EtOH or sex on CRF1+ expression (**Fig 6B**; 2-way ANOVA; Sex × EtOH: $F(1,27)=0.1812$, $p=0.6737$; EtOH: $F(1,27)=0.6537$, $p=0.4259$; Sex: $F(1,27)=3.432$, $p=0.0749$). Additionally, there was no effect of sex or EtOH on cFos+ (**Fig 6C**; 2-way ANOVA; Sex × EtOH: $F(1,27)=0.2769$, $p=0.6031$; EtOH: $F(1,27)=2.729$, $p=0.1101$; Sex: $F(1,27)=0.02006$, $p=0.8884$) or total double-labeled cells (**Fig 6D**; 2-way ANOVA; Sex × EtOH: $F(1,27)=0.1210$, $p=0.7307$; EtOH: $F(1,27)=1.872$, $p=0.1825$ Sex: $F(1,27)=1.953$, $p=0.1736$). Despite no effects on total co-expression, there was a trend toward correlation between EtOH and %CRF1+ cells (**Fig 6E**; 2-way ANOVA; Sex × EtOH: $F(1,27)=0.04185$, $p=0.8394$; EtOH: $F(1,27)=3.871$, $p=0.0595$; Sex: $F(1,27)=2.065$, $p=0.1622$). EtOH intake was not correlated with CRF1+ (**Fig S5E**; Pearson's correlation; males and females: $r(14)=0.3287$, $p=0.2139$; males: $r(6)=0.5681$, $p=0.1418$; females: $r(6)=0.3301$, $p=0.4245$), cFos+ (**Fig S5F**; Pearson's correlation; males and females: $r(14)=0.4026$, $p=0.1221$; males: $r(6)=0.3614$, $p=0.3789$; females: $r(6)=0.5234$, $p=0.1831$), total double-labeled cells (**Fig S5G**; Pearson's correlation; males and females: $r(14)=0.3907$, $p=0.1346$; males: $r(6)=0.6851$, $p=0.0608$; females: $r(6)=0.3078$, $p=0.4582$) or %CRF1+ cells (**Fig S5H**; Pearson's correlation; males and females: $r(14)=-0.06656$, $p=0.8065$; males: $r(6)=0.1472$, $p=0.7279$; females: $r(6)=-0.5870$, $p=0.1261$). Taken together, these data suggest that EtOH intake does not induce activation of the CeA stress circuitry, nor is activity within this region directly correlated with individual EtOH intake in males or females.

In the LA (**Fig 6F),** there was a main effect of EtOH on CRF1+ cells, where EtOH drinking rats had decreased CRF1+ expression (**Fig 6G**; 2-way ANOVA; Sex × EtOH: $F(1,28)=0.1328$ $p=0.7182$; EtOH: $F(1,28)=5.466$; $p=0.0268$; Sex: $F(1,28)=1.975$, $p=0.1709$). However, there was no effect of EtOH on cFos+ (**Fig 6H**; 2-way ANOVA; Sex × EtOH: $F(1,28)=0.2883$, $p=0.5383$; EtOH: $F(1,28)=0.1083$; $p=0.7445$; Sex: $F(1,28)=0.1293$, $p=0.7219$), total double-labeled cells (**Fig 6I;** 2-way ANOVA; Sex × EtOH: $F(1,28)=0.04207$, $p=0.8390$; EtOH: $F(1,28)=2.810$, $p=0.1048$; Sex: $F(1,28)=0.2382$, $p=0.6293$) or %CRF1+ (**Fig 6J;** 2-way ANOVA; Sex × EtOH: $F(1,28)=1.790$, $p=0.1918$; EtOH: $F(1,28)=0.1045$, $p=0.7489$; Sex: $F(1,28)=0.05208$, $p=0.8211$). There were no significant correlations between EtOH intake and CRF1+ (**Fig S6I**; Pearson's correlation; males and females: $r(14)=-0.3468$, $p=0.1881$; males: $r(6)=-0.2797$, $p=0.5023$; females: $r(6)=-0.4123$, $p=0.3101$), cFos+ (**Fig S6J**; Pearson's correlation; males and females: $r(14)=0.1378$, $p=0.6109$; males: $r(6)=-0.1201$, $p=0.7770$; females: $r(6)=-0.2698$, $p=0.5182$), total double-labeled cells (**Fig S6K**; Spearman's correlation; males and females: $r_s(14)=-0.5191$, $p=0.5526$; males: $r_s(6)=-0.4853$, $p=0.2143$; females: $r_s(6)=0.2299$, $p=0.5810$) or %CRF1+ (**Fig S6L**; Spearman's correlation; males and females: $r_s(14)=-0.06744$, $p=0.8028$; males: $r_s(6)=-0.4059$, $p=0.3155$; females: $r_s(6)=0.1649$, $p=0.5952$). Together, the results of the lateral

amygdala experiments indicate that while EtOH decreases LA CRF1+ expression, it is independent of EtOH intake levels.

The last region of interest was the BLA (**Fig 6K**). While there was a trend toward decreased CRF1+ expression as a result of EtOH drinking, it did not reach statistical significance (**Fig 6L**; 2-way ANOVA; Sex × EtOH: $F(1,28)=0.005907$, $p=0.9393$; EtOH: $F(1,28)=3.470$, $p=0.0730$; Sex: $F(1,28)=0.2340$, $p=0.6323$). Females in the water group had decreased cFos compared to males, but females in the EtOH group had increased cFos compared to the males (**Fig 6M**). Despite this observation, the interaction for EtOH x Sex did not reach statistical significance (Fig 6M; 2-way ANOVA; Sex × EtOH: $F(1,28)=2.844$, $p=0.1028$; EtOH: $F(1,28)=2.372$, $p=0.1347$; Sex: $F(1,28)=0.1038$, $p=0.7497$). There were no effects of EtOH or sex when examined as total double-labeled cells (Fig 6N; 2-way ANOVA; Sex × EtOH: $F(1,28)=0.06711$, $p=0.7975$; EtOH: $F(1,28)=0.7639$, $p=0.3895$; Sex: $F(1,28)=0.05866$, $p=0.8104$) or as %CRF1+ (**Fig 6O**; 2-way ANOVA; Sex × EtOH: $F(1,28)=0.07703$, $p=0.7834$; EtOH: $F(1,28)=0.003430$, $p=0.9537$; Sex: $F(1,28)=1.004$, $p=0.3250$). While EtOH intake did not correlate significantly with CRF1 expression, there was a trend toward an inverse correlation in females (**Fig 6P**; Spearman's correlation; males and females: $r_s(14)=-0.3912$, $p=0.1351$; males: $r_s(6)=-0.2143$, $p=0.6191$; females: $r_s(6)=-0.6905$, $p=0.0694$). There was no significant correlation between EtOH intake and cFos expression (**Fig 6Q**; Spearman's correlation; males and females: $r_s(14)=0.1763$, $p=0.5122$; males: $r_s(6)=-0.1667$, $p=0.7033$; females: $r_s(6)=0.3571$, $p=0.3894$). However, there was an inverse correlation between EtOH intake and total double-labeled cells (**Fig 6R;** Spearman's correlation; males and females: $r_s(14)=-0.7026$, $p=0.0032$, males: $r_s(6)=-0.5389$, $p=0.1769$; females: $r_s(6)=-0.7425$, $p=0.0419$), but not %CRF1+ (**Fig 6S**; Spearman's correlation; males and females: $r_s(14)=-0.4150$, $p=0.1106$, males: $r_s(6)=-0.1905$, $p=0.6646$; females: $r_s(6)=-0.4286$, $p=0.2992$). Collectively, these data suggest that CRF1-containing cells in the BLA are involved in mediating voluntary EtOH intake.

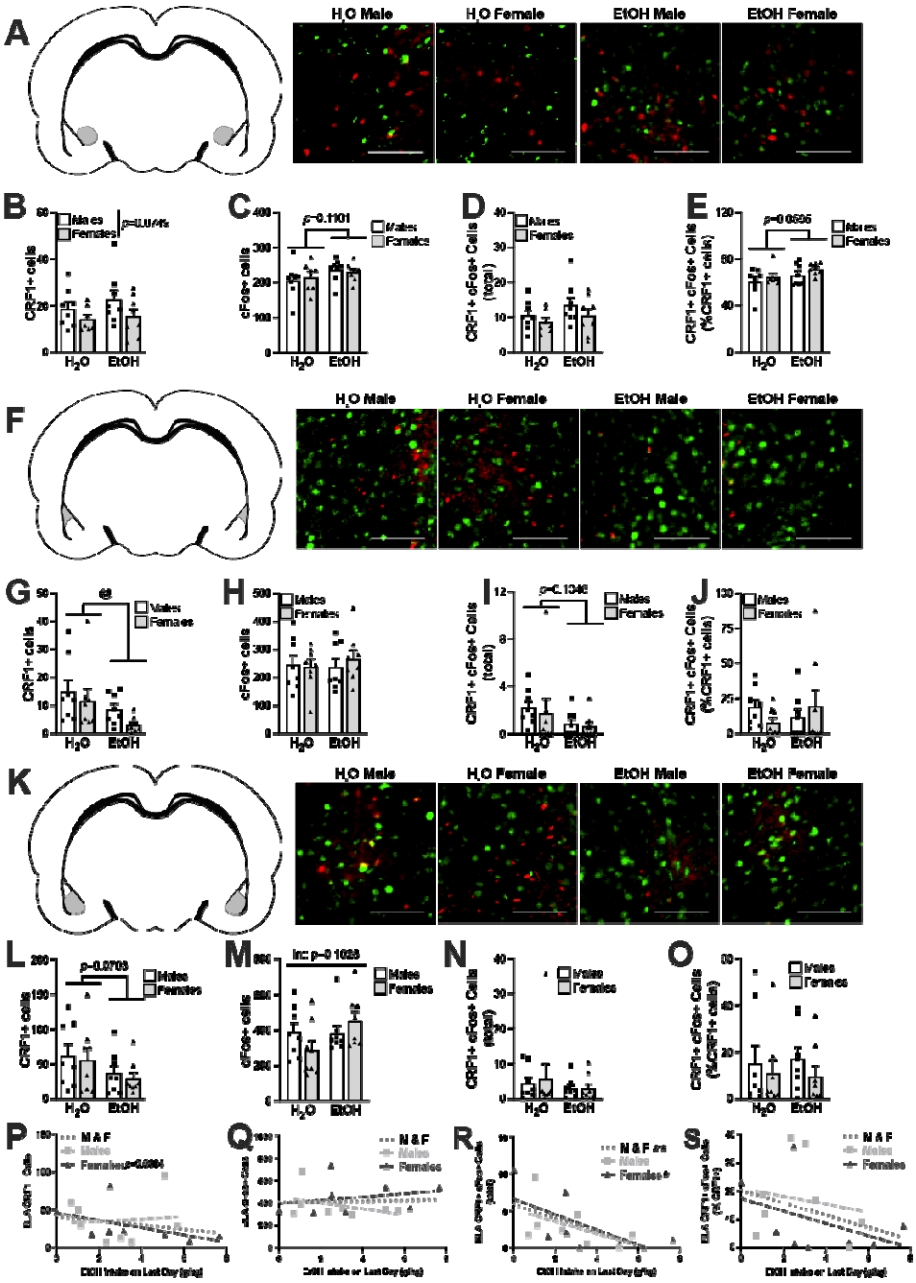

Figure 6.

Amygdalar correlates of EtOH Drinking. Slices containing both the central amygdala (CeA), lateral amygdala (LA), and basolateral amygdala (BLA) were stained for RFP (as a measure of CRF1+ neurons, shown in red) and cFos (as a measure of cellular activity, shown in green). **A)** Representative images for the CeA. CeA CRF1+, cFos, and total double labeled cell counts are shown in **B-D** respectively. **E)** Double labeled cells were also expressed as % total CRF1+ cells.Staining in the LA was also quantified, with representative images shown in **(F)**. LA CRF1+, cFos, and total double labeled cell counts for infralimbic cortex are shown in **G-I** respectively. **J)** Double labeled cells were also expressed as % total CRF1+ cells. The BLA was also quantified, with

representative images shown in **(K)**. BLA CRF1+, cFos, and total double labeled cell counts for infralimbic cortex are shown in (**L-N)** respectively. **O)** Double labeled cells were also expressed as % total CRF1+ cells. We also correlated EtOH intake on the last day with CRF1+ cells **(P),** cFos+ cells **(Q),** total double labeled cells **(R)** and double labeled cells as %CRF1+ **(S)** in the BLA.

Scale bars= 100µm; @ $p<0.05$ (2-way ANOVA (Main Effect of EtOH); ****** $p<0.01$, *****$p<0.05$ (Spearman's correlation)

4. DISCUSSION

The current study set out to examine the dynamic relationship between affective state, pain sensitivity, and alcohol (EtOH) intake in a sex-specific manner in CRF1:cre: ^{td}Tomato rats. While there were no baseline sex differences in negative affect or thermal stimuli, females were more sensitive to mechanical stimuli and consumed significantly more EtOH during the first week of drinking. After 3 weeks of chronic drinking, all rats displayed increased malaise-like behavior and previous day EtOH intake directly correlated with negative affect in both sexes. EtOH intake associated inversely with thermal sensitivity in males only. EtOH intake also produced sex- and region-dependent effects on neuronal activity in the prefrontal cortex and amygdala. While a history of EtOH drinking did not affect cFos or cFos-CRF1 co-expression in either region, CRF1+ expression was decreased in the lateral amygdala of EtOH drinking rats. Additionally, there were sex-dependent opposing correlations between EtOH intake and CRF1-containing cells in the infralimbic cortex, where EtOH intake and CRF1 expression were directly correlated in males, but inversely correlated in females. Lastly, CRF1-cFos co-expression was inversely correlated with EtOH intake in the basolateral amygdala of both males and females. These findings provide new insight into individual- and sex-specific differences in EtOH intake and its association with affective state, pain sensitivity, and CRF1-specific neuronal activation in key regions of the brain.

Our first goal was to characterize pain sensitivity and affect in male and female CRF1:cre: ^{td}Tomato rats. Consistent with some, but not all reports, female rats were more sensitive to mechanical stimuli (measured by Von Frey)(Araya et al., 2020; Gogulski and Craft, 2022; Mapplebeck et al., 2018) but these sex differences were specific to mechanical sensitivity. Pain sensitivity in male and female CRF1:cre: ^{td}Tomato rats was previously examined, however, the study was not appropriately powered to detect sex effects(Weera et al., 2022). In contrast to our previous work, we did not see any basal sex differences in either the splash or NSF test(Quadir et al., 2022). While these observations may be confounded by the rats that never groomed in the splash test, subsequent analysis of cumulative incidence of grooming found no statistically significant sex difference in grooming initiation. Future studies will employ additional behavioral tests to investigate behavioral sex differences.

After baseline testing, rats were allowed to drink alcohol under an intermittent access paradigm, which produces high levels of EtOH intake in multiple rat strains (Carnicella et al., 2014; Simms et al., 2008; Wise, 1973). This study revealed that male and female CRF1:cre: ^{td}Tomato rats drink 2-4g/kg EtOH with an approximate 15-30% EtOH preference, consistent with studies in Wistar rats(Adermark et al., 2011; Albrechet-Souza et al., 2020; Cippitelli et al., 2012; Kimbrough et al., 2017; Moench and Logrip, 2021; Momeni and Roman, 2014; Scott et al., 2020). While there were no sex

differences in overall intake, females drank significantly more EtOH during the first week. Studies comparing male and female EtOH intake under intermittent access conditions in Wistar rats are limited and inconclusive: some researchers found females drink more EtOH whereas others found no sex differences(Albrechet-Souza et al., 2020; Priddy et al., 2017). Additional research exploring sex differences in early drinking behavior are warranted.

The impact of chronic EtOH drinking changes on pain sensitivity and negative affect was examined. In three of the four behavioral tests (with the Hargreaves test of thermal sensitivity being the exception), rats exhibited increased discomfort compared to baseline levels. Specifically, the present study found increased malaise in both male and female rats in the Von Frey (decreased withdrawal thresholds, suggesting increased mechanical sensitivity), splash test (decreased time spent grooming, suggestive of increased anhedonia), and NSF (increased latency to groom and decreased post-test consumption, suggestive of decreased motivated behavior). This is relatively consistent with our previous study in Sprague Dawley rats, using only repeated Splash test where male rats displayed decreased time grooming after chronic drinking(Quadir et al., 2022). While our current findings indicate increased malaise regardless of sex, strain differences may account for this minor discrepancy. Because this occurred in both water-drinking and EtOH-drinking rats and it has now occurred across strains, we hypothesize it is due to the stress of single-housing. Indeed, prolonged social isolation during adulthood increases pain and negative affect in both humans and animals(Baker et al., 2022; Bannon et al., 2021; Blass et al., 1987; Mazur et al., 2017; Santini et al., 2020; Taheri Zadeh et al., 2021; Tuboly et al., 2009; Yamada et al., 2021). The effect of EtOH drinking on pain sensitivity and affective behavior was examined next. While it initially appears that EtOH drinking reduced mechanical sensitivity, this effect was present during both the baseline and post-drinking tests and is thus likely driven by pre-existing individual differences in mechanical sensitivity. Notably, there were no group effects of EtOH drinking on pain sensitivity or affective behavior. While previous studies have seen effects of EtOH on pain sensitivity and affect(Quadir et al., 2021b) (Gong et al., 2017; Overstreet et al., 2005; Van Skike et al., 2015; Xu et al., 2021), others studies have not (Bloch et al., 2020; McNamara and Ito, 2021; Pirino et al., 2022) One potential explanation is that rats in the present study only drank for 3-4 weeks before behavioral testing and thus did not consume enough EtOH to produce behavioral deficits. Studies in Wistar rats report escalated intake after ~20 EtOH drinking sessions(Carnicella et al., 2009; George et al., 2012; Kimbrough et al., 2017); rats in this study only drank for 16 sessions. Others have found that various aspects of EtOH withdrawal (including increased anxiety-like behavior, seizures, and EtOH intake during withdrawal) increase with repeated EtOH withdrawal cycles (Becker, 1996; Holter et al., 1998; Lopez and Becker, 2005; Overstreet et al., 2004; Rimondini et

al., 2003; Zhang et al., 2007). Another possibility is that the environment was too anxiogenic, creating a ceiling effect of anxiety- as suggested by a recent study of testing outside the homecage(Neira et al., 2022). Future studies will investigate negative affect as it occurs in the home cage, as it may be more indicative of withdrawal behaviors.

While EtOH drinking did not induce group differences in pain sensitivity or negative affect, we observed direct correlations between EtOH intake with these behaviors. Specifically, there was a bidirectional inverse relationship between EtOH intake and thermal sensitivity, where male rats that consumed more EtOH were less sensitive to thermal stimuli and male rats with increased thermal sensitivity consumed less EtOH on the next day. These findings are in accordance with a previous study reporting that EtOH withdrawal increases nociceptive threshold(Schunck et al., 2015). However, it is important to note that many other studies have demonstrated EtOH-induced decreases in pain threshold (Avegno et al., 2018; Kang et al., 2019; Kononoff et al., 2018; Quadir et al., 2020a; Quadir et al., 2021a; Quadir et al., 2021b; Walcott et al., 2018; You et al., 2020). Our findings suggest male CRF1:cre: ^{td}Tomato rats may be resistant to the analgesic properties of EtOH; however, further studies will test thermal sensitivity during intoxication to directly address this speculation.

In addition to pain sensitivity, individual differences in EtOH intake and affect were examined. In contrast to previous work in Sprague-Dawley rats(Quadir et al., 2022), there were direct correlations between EtOH intake and subsequent affective behavior in both the splash test and NSF. These findings mirror studies that demonstrate increased fear-potentiated startle in various genetic lines of alcohol preferring rats (McKinzie et al., 2000) and mice (Barrenha and Chester, 2007). While all of these correlations held true when both sexes were analyzed together, analyzing the sexes separately revealed that the EtOH intake associations with anxiety-like behavior in the NSF were primarily driven by males whereas EtOH intake associations with anhedonia in the Splash test were primarily driven by females. These findings suggest test-specific contributions of sex in EtOH-induced affective behavior. In rodents, increased anxiety-like behavior is associated with increased EtOH intake in some studies(Hayton et al., 2012; Pelloux et al., 2015; Spanagel et al., 1995) but not in others(Correia et al., 2009; Da Silva et al., 2004; Henniger et al., 2002; McNamara and Ito, 2021). While we found associations between EtOH intake prior to the behavioral test, we did not find associations between behavior and subsequent drinking.

In addition to examining behaviors following chronic EtOH drinking in the CRF1:cre: ^{td}Tomato rats, the effects of chronic drinking on the prefrontal cortex (PFC) and amygdala were investigated. In our studies, EtOH intake and CRF1+ expression in the infralimbic cortex (IL) were directly correlated in female, but inversely correlated in male,

rats. A previous found no effect of drinking on CRF1 mRNA levels in either IL or PrL(Ketchesin et al., 2016), however this study used a different drinking paradigm and only examined group differences (not individual correlations)(Ketchesin et al., 2016). In this study, EtOH intake in male rats did not correlate with PrL cFos levels but correlated inversely with IL cFos levels. This contradicts a previous study that found a direct correlation between EtOH intake and PFC cFos levels, although their grouping of PrL and IL may contribute to the discrepancy(George et al., 2012).

Three subregions of the amygdala were studied: the central (CeA), lateral (LA), and basolateral amygdalae (BLA). While it did not reach statistical significance, there was a trend (p=0.0595) toward EtOH-induced activation of CRF1+ cells in the CeA, suggesting activation of the CRF1 system by EtOH. Consistent with this finding, others demonstrated that CRF1 antagonists administered directly into the CeA reduce EtOH intake (Gondre-Lewis et al., 2016; Lowery-Gionta et al., 2012). Additionally, there was no effect of chronic EtOH drinking on CRF1 expression in the CeA, consistent with previous studies showing no effect of voluntary EtOH intake or EtOH vapor on CRF1 mRNA levels in CeA(Ketchesin et al., 2016; Sommer et al., 2008). In this study, EtOH decreased CRF1 expression in the LA in both sexes. This group has previously reported that EtOH drinking increased excitability of CRF1-containing neurons in the LA of mice (Agoglia et al., 2020) but the functional effect in CRF1-cre rats remains to be investigated. Lastly, the BLA was examined, where there were negative correlations between EtOH intake and CRF1-cFos co-expression but no group effects of EtOH on CRF1 or cFos expression, consistent with previous work reporting no effect of voluntary drinking on CRF1 mRNA levels in mouse BLA(Ketchesin et al., 2016). Future studies will manipulate CRF1-containing neurons in these areas and investigate their role in drinking.

Together, our results illustrate the complex interplay between affective state, pain sensitivity, alcohol drinking, and the role of CRF1 containing neurons in mediating these behaviors. Moreover, these data highlight the importance of examining individual differences in addition to group averages when investigating AUD and affective states.

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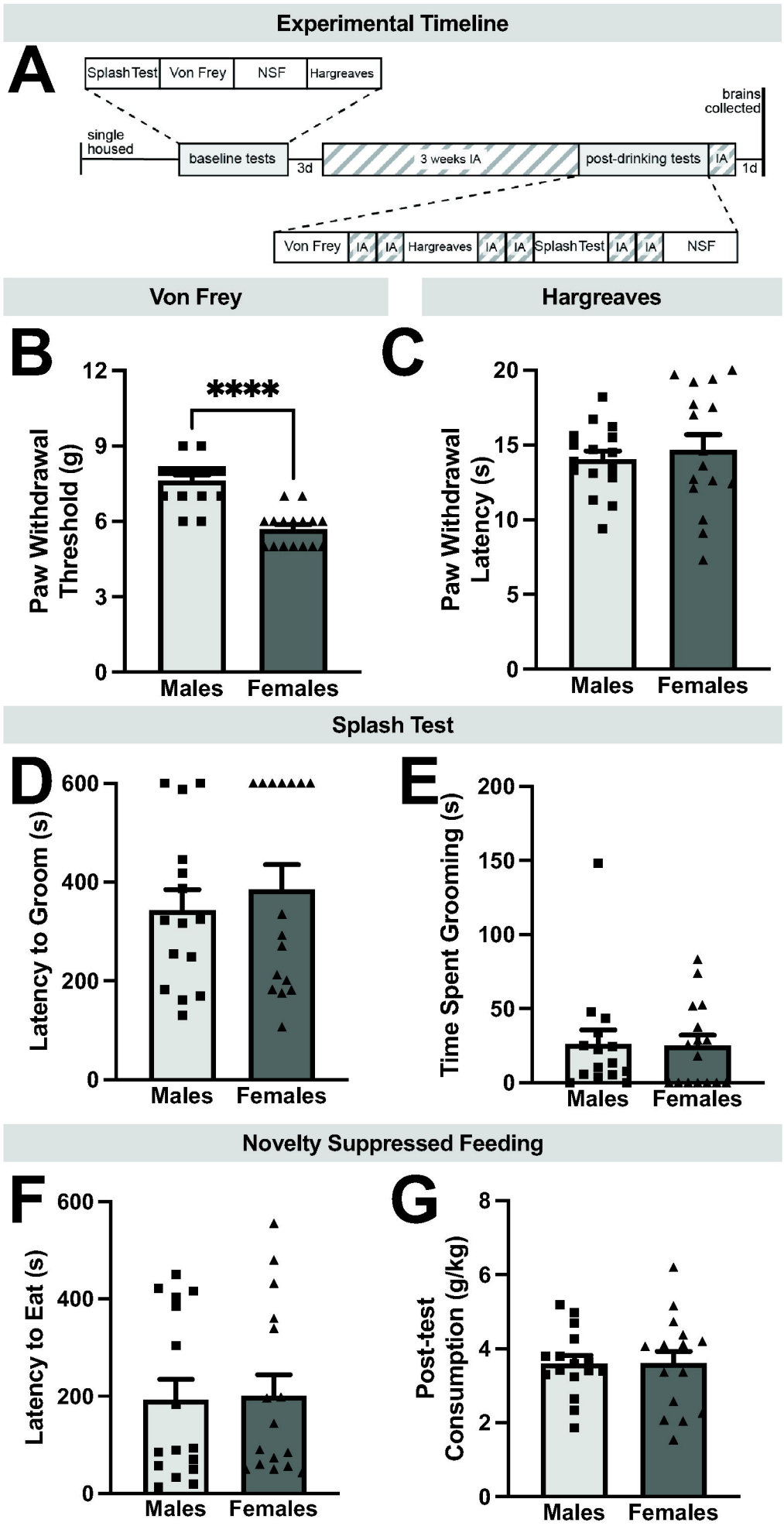
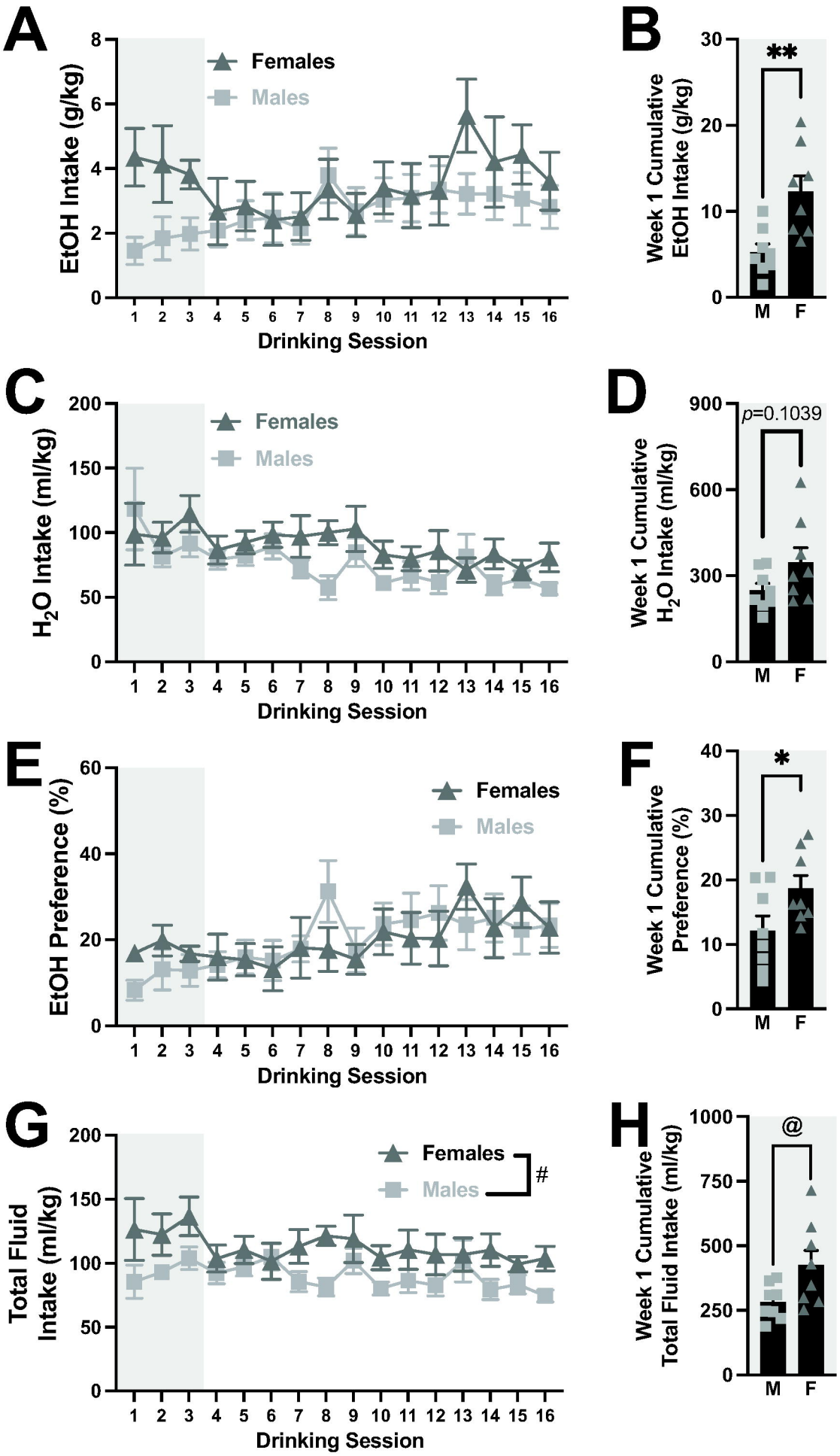
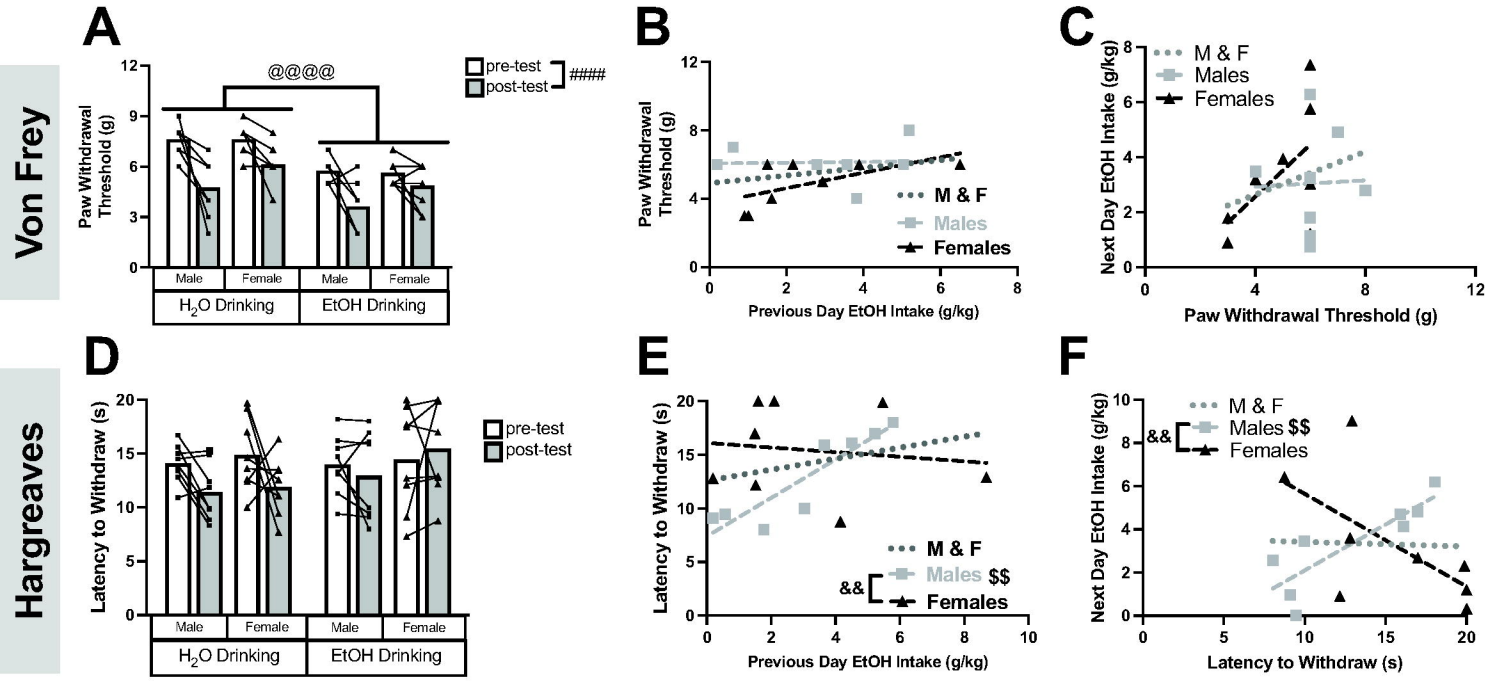
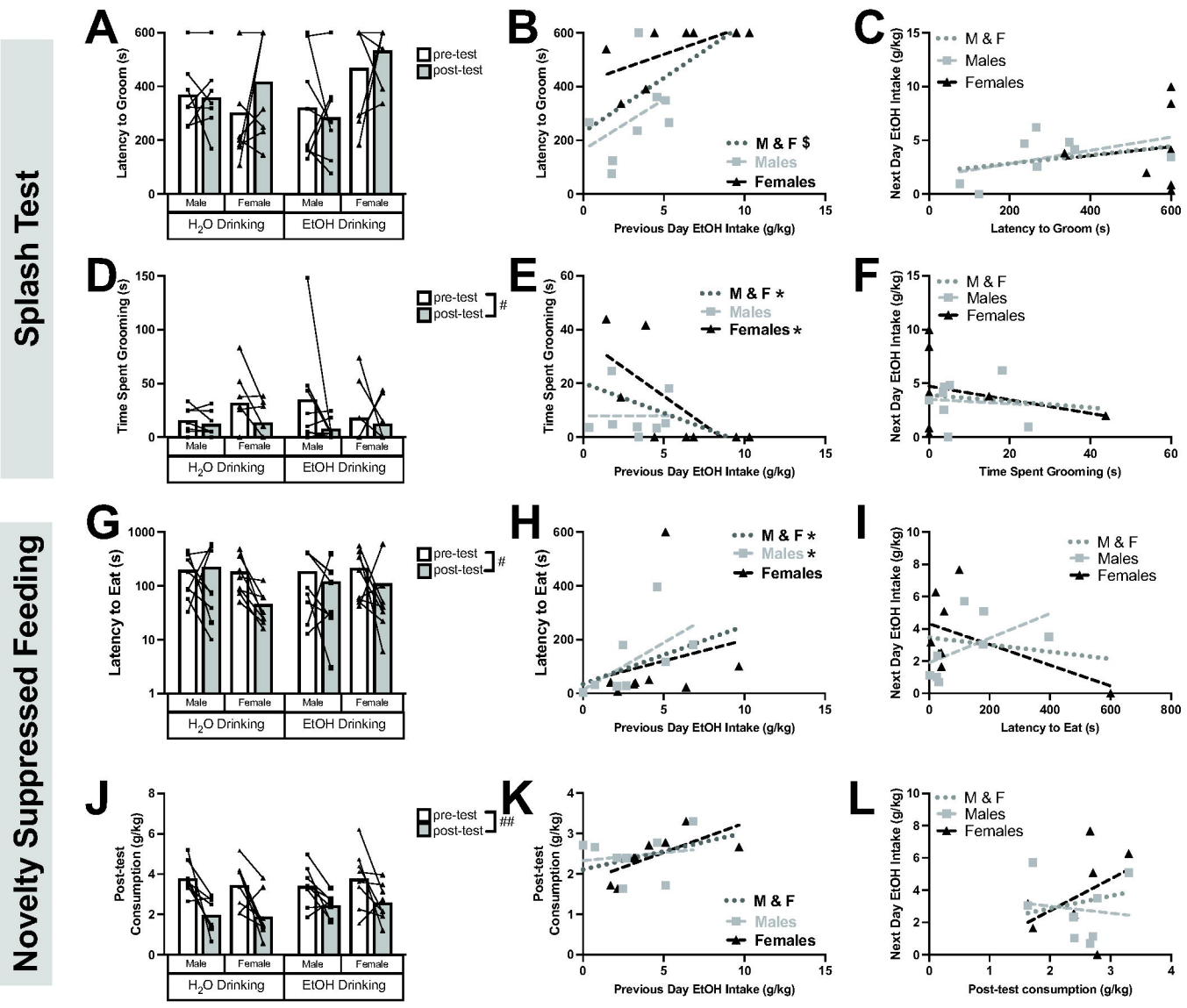
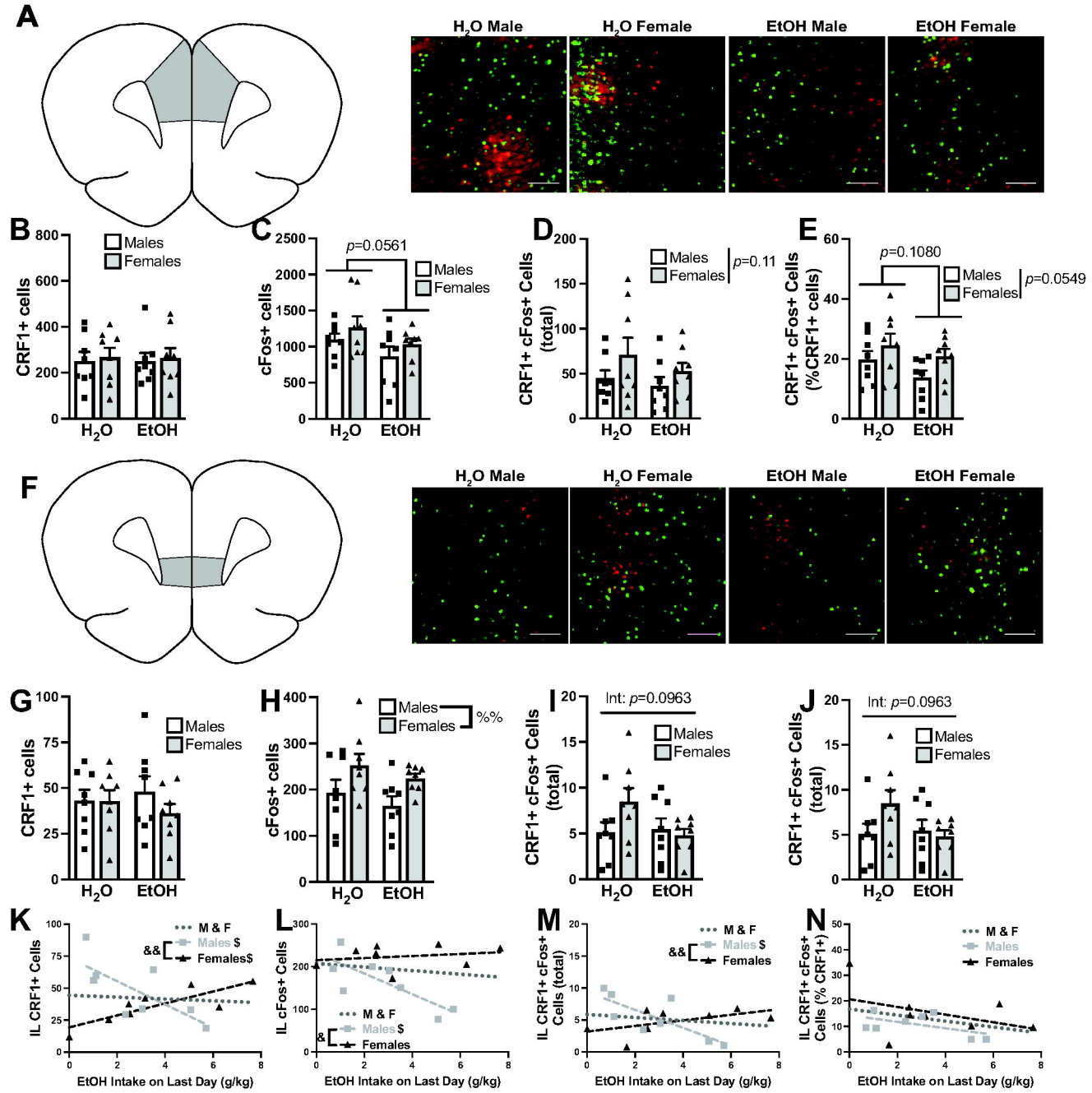
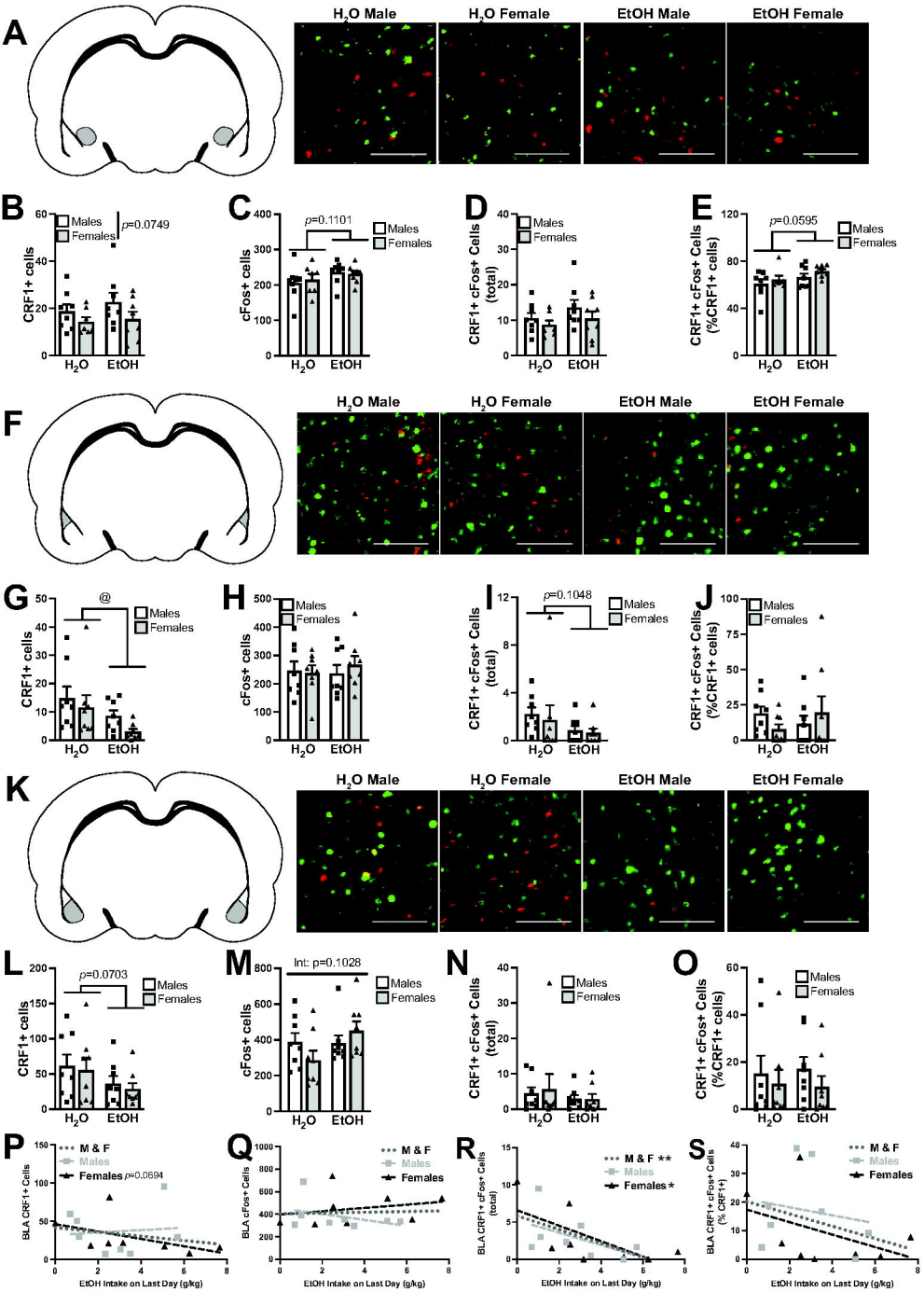

Experimental Timeline
A
Splash Test
Von Frey
NSF
Hargreaves
single housed
baseline tests
3d
3 weeks IA
post-drinking tests
IA
1d
brains collected
Von Frey
IA
IA
Hargreaves
IA
IA
Splash Test
IA
IA
NSF
Von Frey
Hargreaves
B
Paw Withdrawal Threshold (g)
****
Males
Females
C
Paw Withdrawal Latency (s)
Males
Females
Splash Test
D
Latency to Groom (s)
Males
Females
E
Time Spent Grooming (s)
Males
Females
Novelty Suppressed Feeding
F
Latency to Eat (s)
Males
Females
G
Post-test Consumption (g/kg)
Males
Females


A
EtOH Intake (g/kg)
Females
Males
Drinking Session
B
Week 1 Cumulative EtOH Intake (g/kg)
**
M
F
C
H2O Intake (ml/kg)
Females
Males
Drinking Session
D
Week 1 Cumulative H2O Intake (ml/kg)
p=0.1039
M
F
E
EtOH Preference (%)
Females
Males
Drinking Session
F
Week 1 Cumulative Preference (%)
*
M
F
G
Total Fluid Intake (ml/kg)
Females
Males
#
Drinking Session
H
Week 1 Cumulative Total Fluid Intake (ml/kg)
@
M
F


Von Frey
Hargreaves
A
@@@@
pre-test
post-test
####
Paw Withdrawal Threshold (g)
Male
Female
Male
Female
H2O Drinking
EtOH Drinking
B
Paw Withdrawal Threshold (g)
M & F
Males
Females
Previous Day EtOH Intake (g/kg)
C
Next Day EtOH Intake (g/kg)
M & F
Males
Females
Paw Withdrawal Threshold (g)
D
Latency to Withdraw (s)
pre-test
post-test
Male
Female
Male
Female
H2O Drinking
EtOH Drinking
E
Latency to Withdraw (s)
M & F
Males $$
&&
Females
Previous Day EtOH Intake (g/kg)
F
Next Day EtOH Intake (g/kg)
M & F
Males $$
&&
Females
Latency to Withdraw (s)


Splash Test
Novelty Suppressed Feeding
A
Latency to Groom (s)
pre-test
post-test
Male
Female
Male
Female
H2O Drinking
EtOH Drinking
B
Latency to Groom (s)
M & F $
Males
Females
Previous Day EtOH Intake (g/kg)
C
Next Day EtOH Intake (g/kg)
M & F
Males
Females
Latency to Groom (s)
D
Time Spent Grooming (s)
pre-test
post-test
#
E
Time Spent Grooming (s)
M & F *
Males
Females *
Previous Day EtOH Intake (g/kg)
F
Next Day EtOH Intake (g/kg)
M & F
Males
Females
Time Spent Grooming (s)
G
Latency to Eat (s)
pre-test
post-test
#
H
Latency to Eat (s)
M & F *
Males *
Females
Previous Day EtOH Intake (g/kg)
I
Next Day EtOH Intake (g/kg)
M & F
Males
Females
Latency to Eat (s)
J
Post-test Consumption (g/kg)
pre-test
post-test
##
K
Post-test Consumption (g/kg)
M & F
Males
Females
Previous Day EtOH Intake (g/kg)
L
Next Day EtOH Intake (g/kg)
M & F
Males
Females
Post-test consumption (g/kg)


A
H2O Male
H2O Female
EtOH Male
EtOH Female
B
CRF1+ cells
Males
Females
H2O
EtOH
C
cFos+ cells
p=0.0561
D
CRF1+ cFos+ Cells (total)
p=0.11
E
CRF1+ cFos+ Cells (%CRF1+ cells)
p=0.1080
p=0.0549
F
G
H
%%
I
Int: p=0.0963
J
K
M & F
Males $
Females$
&&
IL CRF1+ Cells
EtOH Intake on Last Day (g/kg)
L
IL cFos+ Cells
&
M
IL CRF1+ cFos+ Cells (total)
N
IL CRF1+ cFos+ Cells (% CRF1+)


A
H2O Male
H2O Female
EtOH Male
EtOH Female
B
Males
Females
p=0.0749
CRF1+ cells
H2O
EtOH
C
p=0.1101
cFos+ cells
D
CRF1+ cFos+ Cells (total)
E
p=0.0595
CRF1+ cFos+ Cells (%CRF1+ cells)
F
G
@
H
I
p=0.1048
J
K
L
p=0.0703
M
Int: p=0.1028
N
O
P
M & F
Males
Females p=0.0694
BLA CRF1+ Cells
EtOH Intake on Last Day (g/kg)
Q
BLA cFos+ Cells
R
M & F **
Females *
BLA CRF1+ cFos+ Cells (total)
S
BLA CRF1+ cFos+ Cells (% CRF1+)