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Noradrenergic tone mediates marble burying behavior after chronic stress and ethanol

Carolina R. den Hartog¹, Katrina L. Blandino¹, McKenzie L. Nash¹, Emily R. Sjogren¹, Michael A. Grampetro¹, David E. Moorman², Elena M. Vazey¹

¹Department of Biology, University of Massachusetts Amherst, MA, 01003, USA

²Department of Psychological and Brain Sciences, University of Massachusetts Amherst, MA, 01003, USA

Abstract

Rationale—Stress plays a major role in the development of alcohol use disorder (AUD) – a history of chronic stress contributes to alcohol misuse, and withdrawal from alcohol elevates stress, perpetuating cycles of problematic drinking. Recent studies have shown that, in male mice, repeated chronic intermittent ethanol (CIE) and stress elevates alcohol use above either manipulation alone, and impacts cognitive functions such as behavioral flexibility.

Objective—Here we investigated the impact of CIE and stress on anxiety in both sexes, and whether the norepinephrine (NE) system via locus coeruleus, which is implicated in both stress and alcohol motivation, is involved.

Results—Male and female mice received multiple cycles of CIE and/or repeated forced swim stress (FSS), producing elevated drinking in both sexes. CIE/FSS treatment increased anxiety, which was blocked by treatment with the α_1 -AR inverse agonist prazosin. In contrast, administration of the corticotropin releasing factor receptor antagonist CP376395 into locus coeruleus did not reduce CIE/FSS elevated anxiety. We also observed sex differences in behavioral responses to a history of CIE or FSS alone as well as differential behavioral consequences of prazosin treatment.

Conclusions—These data indicate that NE contributes to the development of anxiety following a history of alcohol and/or stress, and that the influence of both treatment history and NE signaling is sex dependent. These results argue for further investigation of the NE system in relation to disrupted behavior following chronic alcohol and stress, and support the assertion that treatments may differ across sex based on differential neural system engagement.

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Corresponding Author: Elena Vazey PhD, Department of Biology, 611 North Pleasant St, Amherst MA 01003, USA, evazey@umass.edu, Ph: 413-545-8659, Fax: 413-545-3243.

Author Contributions

CdH, DEM and EMV designed the experiments and wrote the paper. CdH, KLB, MLN, ERS, MAG and EMV performed experiments and analyzed the data.

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Keywords

Locus coeruleus; Prazosin; CRF; Anxiety; Alcohol; Sex differences; Noradrenaline

INTRODUCTION

Alcohol use disorder (AUD), characterized by excessive and uncontrollable drinking, is one of the leading global causes of disease burden (Alcohol and Drug Use 2018). Stress plays a critical role in the progression from initial alcohol use to dependence and loss of control over drinking (Becker et al. 2011; Breese et al. 2011). Individual stress sensitivity can augment the rewarding properties of alcohol that may increase motivation to drink (Blaine et al. 2016). Neuroadaptations caused by chronic alcohol underlie the development of tolerance and dependence, and are associated the negative symptoms during withdrawal that contribute to alcohol craving and increase the risk of relapse (Becker 2012; Blaine et al. 2016; Edwards et al. 2013). AUD is highly comorbid with other psychiatric disorders including depression and anxiety, suggesting common biological pathways (Grant et al. 2015). While extensive work has uncovered some interactions between stress and alcohol, more work is required to elucidate the distinct neurochemical alterations associated with these factors (Becker 2017; Koob and Volkow 2016; Retson et al. 2016).

One potential target for the treatment AUD is the norepinephrine (NE) system. NE originating in brainstem nuclei including the locus coeruleus (LC) and nucleus solitarius (NTS), is distributed by widespread afferent networks to cortical and subcortical targets (Moore and Bloom 1979; Swanson and Hartman 1975). Central NE plays major roles in a variety of behaviors from arousal to cognition, and changes in NE signaling are associated with a many pathologies, particularly excessive stress and anxiety (Aston-Jones and Cohen 2005; Berridge and Waterhouse 2003; Daviu et al. 2019; Rinaman 2011; Tanaka et al. 1990). NE has been strongly associated with alcohol use, including regulation of motivation for alcohol as well as aspects of stress and anxiety resulting from withdrawal (reviewed in Vazey et al. 2018). Drugs that decrease NE tone reduce alcohol consumption in humans and animal models (Begleiter 1974; Fox et al. 2012; Froehlich et al. 2013; Gilpin and Koob 2010; Rasmussen et al. 2014). In particular, the α_1 -AR inverse agonist prazosin (Armstrong et al. 2020; Rossier et al. 1999) has been shown to decrease alcohol craving, anxiety, and negative affective states following stress exposure in early abstinent humans (Fox et al. 2012) as well as dependent or high-alcohol preferring rodents (Froehlich et al. 2015; Funk et al. 2016; Rasmussen et al. 2017). NE signaling may be an important nexus for alcohol and stress interactions in AUD. Neural activity in NE nuclei and NE dependent cognitive functions including attentional set shifting, are selectively disrupted by combining chronic ethanol with stress exposure (Rodberg et al. 2017). However, an outstanding question is what role NE signaling plays in the negative affective states associated with chronic alcohol.

Here we measured stress and alcohol interactions on anxiety-like behavior via marble-burying in male and female mice using CIE and/or repeated stress exposure, the combination of which has been previously shown to escalate drinking in males (Anderson et al. 2016; Lopez et al. 2016; Rodberg et al. 2017). To test if changes observed in marble burying

behavior were associated with alterations in NE tone, we treated mice with the systemic $\alpha 1$ -AR inverse agonist prazosin or LC-targeted cannula infusion of the corticotropin releasing factor receptor (CRF R1) antagonist CP376395 prior to marble burying. The stress related peptide CRF is known to be a potent driver of NE signaling (Valentino et al. 1983). Prazosin but not CP376395 decreased marble burying in mice with a history of chronic ethanol and stress. These findings further support a role of noradrenergic systems in mediating the anxiety-like/compulsive consequences of chronic alcohol and stress and highlight the importance and potential of this system as a target for therapeutic treatments.

METHODS

ANIMALS

Adult (8 week old) male and female C57BL/6J mice were single housed in a 12 hr:12 hr reverse light/dark cycle with *ad libitum* access to food, water, and enrichment unless otherwise specified. All procedures were performed in accordance with National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by the University of Massachusetts Amherst Institutional Animal Care and Use Committee.

SURGICAL PROCEDURE

Intra-LC antagonist animals received bilateral cannulae prior to the start of experiments. Mice were anesthetized using isoflurane and mounted to a stereotaxic frame using non-rupture ear bars (Kopf Instruments, Tujunga CA) while bilateral guide cannulae (33 gauge, 2.0 mm apart, 3.0 mm in length; Plastics One, Roanoke VA) directed towards LC (AP -5.4 , ML ± 1.0 , DV -2.4) were cemented in place. Internal cannulae were 0.8 mm longer than the guide cannulae to target LC at DV -3.2 mm. Mice recovered for at least 1 week before baseline drinking.

CHRONIC INTERMITTENT ETHANOL AND STRESS EXPOSURE

As previously described (Anderson et al. 2016; Lopez et al. 2016; Rodberg et al. 2017), mice underwent 4–5 weeks of baseline drinking in a limited access 1-bottle choice paradigm prior to vapor and stress exposure with 1 hr access to 15% ethanol (vol/vol) 3 hr after lights off (Fig. 1a). Mice then underwent weekly intermittent cycles (2–3) of ethanol vapor or air exposure (CIE or Air) interleaved with weeks of daily test drinking sessions 4 hr after forced swim stress or no stress (FSS or NS). Mice were assigned to one of four groups (Air/NS, Air/FSS, CIE/NS, and CIE/FSS; $n=26-33$ per group per sex) counterbalanced by ethanol consumption during baseline drinking. For analyses of drinking, average consumption during the last two weeks of baseline was used for baseline, and average consumption during test cycles 2–3 was used for test.

CIE vapor exposure was administered as previously described (Becker and Lopez 2004). CIE mice received pyrazole (1 mmol/kg) and a loading dose of ethanol (1.6 g/kg) i.p. prior to being passively exposed to four daily 16 hr sessions of ethanol vapor inhalation in plexiglass chambers. Control Air mice received pyrazole (1 mmol/kg). Blood samples were collected during each vapor cycle week from a male or female reporter mouse immediately following a 16 hr vapor exposure. Across all groups and all cycles CIE mice had blood

ethanol concentrations of 207 ± 23 g/dL in males and 267 ± 20 g/dL in females (unpaired t-test $t_{70}=1.87$, $p>0.05$). Blood samples were centrifuged at 10,000 *g* for 10 min at 4°C and plasma was run on an colorimetric alcohol oxidase assay to determine ethanol concentration (Prencipe et al. 1987). Stress exposure took place 4 hr prior to drinking on test weeks. FSS mice were placed in a 4 L beaker filled with water (24–26°C) for 10 min while NS mice remained in the colony room.

MARBLE BURYING TASK

Prior to pharmacological treatment, mice were habituated to a testing cage ($19.1 \times 29.2 \times 12.7$ cm) filled with 2.5 L of bedding for 15 min. Mice were returned to their homecage for 10–30 min while the bedding in their testing cage was leveled and 20 black marbles were arranged in a 4×5 grid. In the prazosin study, mice were injected with α 1-AR inverse agonist prazosin (0.75 mg/kg; s.c.; Tocris Bioscience, Bristol UK), or vehicle (water) immediately after habituation and tested for marble burying behavior 30 min later (males, $n=62$; females, $n=66$). In the intra-LC study, mice were infused via LC directed canula with the CRF-1 receptor antagonist CP376395 (CP; 0.3 μ g per hemisphere in 150 nl (total dose 0.6 μ g); Tocris Bioscience, Bristol UK), or aCSF at a rate of 75 nl/min in their homecage and then tested for marble burying 10 min later (males, $n=61$; females, $n=41$). CP dose was chosen a priori based on behavioral studies identifying impacts on stress induced alcohol intake and pharmacological studies establishing the ID₅₀ on CRF driven activity in LC (Chen et al. 2008; Hwa et al. 2016). For testing, mice were placed in their testing cage filled with marbles for 30 min. Habituation and testing were recorded, and videos were analyzed for locomotor activity using Any-Maze software (ANYmaze, Stoelting Co., Wood Dale, IL). Latency to bury marbles and number of buried marbles were scored by an experimenter blinded to all treatment conditions using a custom ImageJ macro. A marble was considered buried if $\frac{2}{3}$ of the surface was covered in bedding. Mice showing impaired gait or inactivity defined as the bottom 5th percentile of distance traveled in all animals were excluded from analyses (females, $n=6$ total from vehicle, aCSF and CP treatments; and males, $n=1$ from CP treatment).

HISTOLOGICAL VERIFICATION

Animals with LC cannulae were perfused with saline (0.9%) and neutral buffered formalin (10%). Brains were kept in formalin for 24 hr, then transferred to 20% sucrose-azide until fully saturated. Brains were flash frozen in isopentane and sectioned coronally at 30 μ m using a cryostat (Leica CM3050S, Germany). Serial sections were mounted onto slides (120 μ m between sections) and stained with neutral red (0.1%; (Acros Organics, Morris Plains, NJ). Images for verification of cannula placements were captured using StereoInvestigator software (MBF Biosciences, Williston, VT) at 10x magnification on a Zeiss AxioImager M2 upright microscope (Carl Zeiss AG, Germany) with Retiga 2000R CCD color camera (Teledyne Q-Imaging, Surrey, BC, Canada). All animals with bilateral cannula placements, each within 250 μ m of the LC were included in experimental data (Fig. 1b,c).

STATISTICAL ANALYSIS

For all analyses, $p < 0.05$ was used as the acceptable α level, and specific statistical comparisons are listed within the results. Data are reported as mean $\pm$ SEM value unless noted otherwise.

Changes between baseline average and weekly test cycles were analyzed using a mixed model ANOVA with cycle (baseline average and tests 1–3) as a repeated measure and history of chronic exposure as between-subjects factor followed by Dunnett's multiple comparison test. Group differences during weekly test cycles were analyzed using a mixed model ANOVA with test cycle as a repeated measure, and vapor and stress exposure as a between-subjects factor followed by Tukey's multiple comparisons test.

Female and male mice were classified as low, medium, or high drinkers by within sex ranking of their test average consumption (test 2–3). Mice with ethanol intake within the top tertile (males, >2.57 g/kg; females, >2.67 g/kg) or within the bottom tertile (males, <2.08 g/kg; females, <2.23 g/kg) were classified as high and low drinkers, respectively and the residual were medium drinkers.

Data from the marble burying studies were analyzed using a mixed model ANOVA with history, drug treatment, and sex as between-subjects factor followed by Tukey's multiple comparisons test. Pearson correlation was used to compare number of marbles buried and total distance traveled.

Statistical analyses were performed on GraphPad Prism version 8.00 (Graph-Pad Software, La Jolla, CA). All graphs were generated using GraphPad Prism, and figures were compiled in Adobe Illustrator CC (San Jose, CA).

RESULTS

Repeated cycles of chronic ethanol and stress increases ethanol intake in a sex-dependent manner

Prior to chronic vapor and stress exposure, mice from both the systemic prazosin and the intra-LC CP (cannula) study underwent 3–5 weeks of limited access (1 hr, 1-bottle) baseline drinking. There were no significant differences in drinking behavior in mice from each study therefore drinking data was analyzed collectively. Male and female mice initially consumed similar levels of ethanol during the first baseline week, with both sexes showing mild escalation and acclimation by week 4 (mixed-effects model REML, effect of week, $F(2.597, 728.0)=84.37$, $p<0.0001$). By the end of baseline acclimation females consumed significantly more ethanol than males (mixed-effects model REML, effect of sex, $F(1, 285)=25.80$, $p<0.0001$; week $\times$ sex interaction, $F(3, 841)=7.914$, $p<0.0001$). Combined chronic ethanol and stress increased ethanol drinking in both sexes (Fig. 2a,b). All cycles of CIE and FSS significantly increased ethanol intake (weekly test average) compared to baseline average (last two weeks of baseline drinking). This was seen in males as previously reported (mixed model ANOVA, effect of history, $F(3, 140)=20.62$, $p<0.0001$; effect of cycle, $F(2.852, 366)=62.34$, $p<0.0001$, cycle $\times$ history interaction, $F(9, 385)=7.232$, $p<0.0001$; Fig. 2a) and in females (mixed model ANOVA, effect of history, $F(3, 130)=7.22$,

$p < 0.001$; effect of cycle, $F(2.69, 336) = 21.6$, $p < 0.0001$, cycle $\times$ history interaction, $F(9, 375) = 2.44$, $p < 0.05$; Fig. 2b). Within the final test week of drinking we evaluated which factors facilitated the escalation in drinking across all groups in both sexes. Both CIE and FSS independently drove escalated drinking (mixed-effects 3 factor REML, effect of vapor, $F(1, 239) = 56.5$, $p < 0.0001$, effect of stress $F(1, 239) = 4.48$, $p = 0.035$). This analysis also revealed that there was a sex $\times$ stress interaction $F(1, 239) = 4.33$, $p = 0.039$) in that FSS impact on drinking was seen in males.

In addition to increasing average drinking, ethanol and stress exposure shifted the distribution of heavy drinkers in both sexes (males, $\chi^2(6, 188) = 93.28$, $p < 0.0001$, females, $\chi^2(6, 189) = 35.29$, $p < 0.0001$; Fig. 2c,d). We classified all animals as high, medium or low drinkers after CIE and FSS based on tertiles (see Methods). In the control Air/NS group, most male mice were classified as low drinkers with very few high drinkers (low, 71.4%; high, 2.4%). Most Air/NS females were also low drinkers with a small portion meeting criterion for high drinkers (low, 50.0%; high, 18.2%). A history of CIE/NS exposure increased the portion of high drinking animals in both males (low, 15.1%; high, 45.3%) and females (low, 13.7%; high, 49.0%). CIE/FSS exposure further increased the proportion of high drinkers in males (low, 4.4%; high, 77.8%) but not females (low, 21.3%; high, 53.2%).

We further investigated individual differences in baseline ethanol intake that may have impacted future alcohol-seeking behavior. CIE- and FSS-driven increases in drinking described above were evident at the individual level, with most animals in both sexes shifting below the identity line after ethanol and stress exposure regardless of baseline drinking (Fig. 2e,f). This shift below the identity line means that drinking after CIE and/or FSS (values on the x axis) was increased relative to drinking levels pre-CIE and/or FSS (values on the y axis). These data show, on a mouse-by-mouse basis, the impact of CIE and/or FSS on drinking, providing insight into individual differences in the effect of history as well as demonstrating the potent effect of these manipulations across individuals. In control Air/NS mice, test drinking was highly correlated with baseline drinking, indicating that intake remained stable and that individual differences were a strong determinant in intake across the study (Air/NS Pearson coefficient, males, $r = 0.64$, $p = 0.0002$; females, $r = 0.47$, $p = 0.0059$). In male Air/FSS mice drinking was no longer correlated with baseline intake. In animals exposed to CIE, increased drinking was still correlated with baseline drinking, showing that CIE enhanced drinking scales with individual intake propensity (CIE/NS, males, $r = 0.57$, $p = 0.0004$; females, $r = 0.48$, $p = 0.0054$). Despite changes in absolute consumption related to ethanol and stress exposure, female test drinking scaled relative to baseline drinking in mice exposed to either CIE or FSS, but not in the combined CIE/FSS group (Air/FSS, $r = 0.56$, $p = 0.0014$; CIE/FSS, $r = 0.15$, $p = 0.408$). The rightward shift regardless of baseline intake was also seen in male CIE/FSS mice (CIE/FSS $r = 0.10$, $p = 0.56$). Thus in males, stress exposure drove increases in individual drinking independent of baseline consumption. In females, baseline intake was predictive of future increases in consumption except for combined CIE and FSS exposure when intake increased independent of baseline consumption. Collectively, as expected, CIE and FSS were highly effective at increasing ethanol consumption on an individual and group level.

Increased marble burying behavior in mice with a history of chronic vapor and stress is inhibited by prazosin

We sought to establish the impact of ethanol and stress exposure on anxiety-like behavior using the marble burying task (MBT). CIE and FSS increased anxiety-like behavior as measured by MBT, with CIE groups showing the largest increases in marble burying within both males and females (Fig. 3). Although a significant amount of burying activity occurred during the first 10 minutes of the task, this behavior continued to escalate over time (Fig. 3a,b). We compared the number of marbles buried at 10 and 30 mins, as well as latency to bury the first marble. CIE and FSS exposure increased the number of marbles buried after 10 (3-way ANOVA, main effect of history, $F(3, 112)=4.727$, $p=0.004$; Fig. 3c) and 30 mins (3-way ANOVA, main effect of history, $F(3, 109)=3.836$, $p=0.012$) and decreased latency to bury across both sexes (3-way ANOVA, main effect of history, $F(3, 112)=3.507$, $p=0.018$; Fig. 3d). Posthoc analyses (two-stage step up corrected False Discovery Rate) confirmed that on the whole, CIE groups buried significantly more marbles than each Air group (Air/NS vs CIE/NS, Air/NS vs CIE/FSS, Air/FSS vs CIE/NS, Air/FSS vs CIE/FSS, $p<0.05$). There was no significant difference in marbles buried between Air/NS and Air/FSS or between CIE/NS and CIE/FSS in the prazosin experiment. These results show that the MBT identified changes in anxiety-like behavior in mice with a history of ethanol and stress exposure and that change was heavily driven by CIE history.

To determine whether NE tone contributed to this increase in anxiety-like behavior, we treated mice systemically with $\alpha 1$ -AR selective inverse agonist prazosin (0.75 mg/kg) 30 minutes prior to marble burying. Prazosin significantly ameliorated anxiety-like behavior in both sexes as shown by decreased number of marbles buried at 10 (3-way ANOVA drug x sex x history, main effect of prazosin, $F(1, 112)=14.50$, $p=0.0002$; Fig. 3c) and 30 mins (3-way ANOVA, main effect of prazosin, $F(1, 109)=4.135$, $p=0.044$) as well as increased latency to bury (3-way ANOVA, main effect of prazosin, $F(1, 112)=13.30$, $p=0.0004$; Fig. 3d). Thus marble burying behavior was increased in male and female mice with an ethanol and stress history, and this effect was reduced by disrupting NE signaling at $\alpha 1$ receptors. Posthoc analyses (two-stage step up corrected False Discovery Rate) identified that the effects of prazosin on the number of marbles buried were prominent within female Air/NS and CIE/NS mice and male CIE/FSS mice ($p<0.05$).

Since drugs that target NE can impact arousal, we analyzed total distance traveled during the MBT to see if treatment with prazosin impacted locomotor function. Prazosin treatment produced a consistent modest reduction in the total distance traveled compared to vehicle-treated controls (3-way ANOVA, main effect of prazosin, $F(1, 112)=27.99$, $p<0.0001$). To determine whether the effects of prazosin on MBT were related to changes in locomotor activity, we investigated the relationship between marble burying and distance traveled. There was no correlation between distance traveled and marbles buried in prazosin or vehicle treated animals (Pearson coefficient; $r=0.146$; $p=0.103$). Thus the effects of prazosin on arousal were independent from those seen on marble burying behavior.

Increased marble burying behavior in mice with a history of chronic vapor and stress is not affected by LC-targeted infusion of CRF-1 antagonist CP376395

CRF signaling is an important mediator of stress responses that can generate anxiety-like behavior via LC and influence LC-NE excitability in a sex-dependent manner (Curtis et al. 2006; McCall et al. 2015). We sought to determine whether NE-related changes in MBT established above were driven by changes in CRF input to LC. Consistent with results from our systemic prazosin study, we saw an increase in anxiety-like behavior in mice with a history of CIE and FSS exposure (Fig. 4 a,b) evidenced by increased marbles buried after 10 (3-way ANOVA, main effect of history, $F(3, 96)=10.49$, $p<0.0001$; Fig. 4c) and 30 mins (3-way ANOVA, main effect of history, $F(3, 96) = 2.764$, $p<0.05$), along with reduced latency to bury (3-way ANOVA, main effect of history, $F(3, 90)=6.509$, $p<0.0001$; Fig. 4d). Posthoc testing (two-stage step up corrected False Discovery Rate) confirmed that CIE/FSS groups buried significantly more marbles than all other groups including CIE/NS (Air/NS vs CIE/FSS, Air/FSS vs CIE/FSS and CIE/NS vs CIE/FSS, $p<0.05$) CIE/NS. CIE/NS animals buried more marbles than Air/NS but not other groups ($p<0.05$).

However, there was no effect of intra-LC CRF antagonism on the number of marbles buried at either 10 (3-way ANOVA, main effect of CP treatment, $F(1, 96)=0.036$, $p>0.05$; Fig. 4c) or 30 mins (3-way ANOVA, main effect of CP treatment, $F(1, 96)=0.557$, $p>0.05$), or in latency to bury (3-way ANOVA, main effect of CP treatment, $F(1, 90)=2.912$, $p>0.05$; Fig. 4d) regardless of history. Due to the role of LC-NE in arousal we also measured potential locomotor impact of intra-LC CP and found no effect (3-way ANOVA, main effect of CP treatment, $F(1, 90)=2.651$, $p>0.05$). These findings indicate that enhanced marble burying in mice with a history of ethanol and stress was not mediated by changes in CRF signaling in the LC.

DISCUSSION

Here we investigated the interaction of stress, alcohol, and sex on anxiety-like behavior, and its regulation by noradrenergic signaling. We focused on three interrelated questions. First, we asked how a history of chronic ethanol and/or stress impacted ethanol consumption in male and female mice, to parallel previous work with males (Anderson et al. 2016; Rodberg et al. 2017). Second, we asked whether a history of chronic ethanol and/or swim stress influenced anxiety via a noradrenergic mechanism. Third, we asked whether there were sex differences in the impact of chronic stress and/or ethanol on anxiety-like behavior and its noradrenergic regulation. Our results demonstrate sex differences in the impact of chronic stress and alcohol on ethanol consumption and anxiety as well as a differential response profile to $\alpha 1$ -AR modulation. Our data also show no impact of CRF receptor antagonism in the LC on stress/alcohol-elevated anxiety, indicating that these enhanced behavioral responses are driven by NE tone either via non-CRF modulation of LC neurons or through other noradrenergic nuclei such as the NTS.

Female mice consumed more ethanol than males during baseline, in line with previous reports (Eriksson and Pikkarainen 1968). Chronic ethanol exposure increased drinking in both sexes, but repeated swim stress exposure only exacerbated drinking in males. We did, however, observe a major disruption in drinking after CIE/FSS in both males and females

such that baseline drinking no longer predicted drinking post-CIE (Fig. 2e,f). The effects of CIE vapor exposure on drinking is well established in male rodents (Becker and Lopez 2004; Dhaher et al. 2008; Griffin et al. 2009) but reports from a limited number of female CIE vapor studies have been mixed. It appeared that female mice reached a higher blood ethanol concentration relative to males, which could potentially explain sex differences in both drinking and anxiety behaviors. However, analysis showed no significant sex differences in CIE blood ethanol concentration, arguing that inter-individual variability may account for the apparent differences more than systematic sex differences in ethanol vapor responses (see below for more discussion of individual differences).

A recent study reported no escalation in drinking in CIE-treated females (Jury et al. 2017). In that study, it was suggested that high levels of baseline drinking observed in females might prevent CIE exposure to further escalate drinking. One major difference between our findings and theirs is the duration of measurement - 1 hour in our study vs. 24 hours in theirs - suggesting that duration of ethanol access plays an important role in assessing motivation for ethanol. Another possible explanation is the use of extended vapor withdrawal periods in the present study when mice were exposed to either FSS or no stress. The differential impact of chronic stress on ethanol consumption supports the presence of sexually-dimorphic systems underlying stress contributions to alcohol use (Peltier et al. 2019). The stressor used (FSS) did not enhance drinking to the same degree in both sexes, and primarily impacted male drinking in our study. However previous studies in female mice have shown increased drinking after other types of stressors including chronic social stress and restraint stress (Caruso et al. 2018; Varlinskaya and Spear 2015). One important difference between our work and previous studies is the age of subjects - there appears to be a stronger effect of stress on drinking in adolescents. However, given that stress plays an important role in elevating drinking in both male and female humans and may even be a more significant contributor in females (Peltier et al. 2019), further investigation into stress influences in male vs. female drinking is needed.

One simple, but important observation from our drinking results came about by characterizing changes in drinking following CIE and/or FSS history on an individual mouse basis. As shown in Fig. 2, in addition to increasing relative drinking across the populations (Fig. 2a-d), a history of CIE and/or FSS results in increased drinking in most, but not all, mice relative to their baseline drinking levels. As with population results, the effects differ depending on history and sex. For example, in both CIE and CIE/FSS groups, most mice exhibited greater drinking during test vs. baseline conditions (shown as shifts in data points below the unity line in Fig. 2e,f) but at least some mice do not change (x and y values are similar). There were also some sex differences, with stress more potently affecting drinking in males vs. females. This raises the interesting question as to why some mice exhibit escalated drinking and some do not and highlights the importance of studying individual differences in both sexes. We also characterized the correlation between baseline vs. test drinking. Our results showed that that some chronic histories, such as CIE without FSS, scaled drinking across individuals such that high drinkers during baseline were high drinkers during test, only more so. In contrast, a history of CIE/FSS in both male and female mice resulted in no correlation between baseline and test drinking. This suggests that CIE/FSS potently disrupts drinking patterns such that mice do not simply show an additive

enhancement of drinking (as seen, for example with CIE alone). Instead, the combination of almost completely enhanced test drinking along with abolished correlation with prior drinking behavior indicates the profound influence of CIE/FSS to elevate drinking in almost all mice independent of their baseline drinking levels. The mechanisms underlying both individual differences as well as shifts in drinking patterns remain to be thoroughly elucidated but are critical, in combination with further investigation of sex differences, in understanding the impact of chronic alcohol and stress on alcohol use.

Anxiety, measured by marble-burying, was increased in both male and female mice with history of chronic ethanol and stress. These findings are consistent with previous reports of increased marble burying after ethanol administration in male mice (Jury et al. 2017; Pleil et al. 2015; Rose et al. 2016; Umathe et al. 2008). However, previous studies have also reported no increase in marble burying in females after CIE alone (Jury et al. 2017) indicating that more work is necessary to understand sex differences in the relationship between alcohol and stress. As noted above, procedural differences such as alternate week withdrawal periods may account for some disparities in results. The limited influence of chronic stress alone on marble burying is notable. Although CIE/NS and CIE/FSS-treated mice increased the number of buried marbles (and decreased latency to bury first marble), and in the CP study CIE/FSS significantly exacerbated marble burying over and above CIE alone, the Air/FSS mice showed no significant change from Air/NS controls. This may reflect the limited transfer of the chronic stressor (FSS) to general anxiety, particularly among females, in which FSS also did not increase drinking. Alternately, FSS treatment may not have produced sufficient anxiety in the absence of CIE to drive changes in marble-burying, suggesting that the impact of different stressors on general anxiety measures should be considered in future studies. However, the fact that chronic ethanol produced higher levels of anxiety-like behavior indicates the profound anxiogenic effect of chronic ethanol.

NE has been associated with anxiogenic aspects of chronic alcohol (Koob 2014). Here marble-burying was significantly impacted by treatment with the selective α_1 -AR inverse agonist prazosin, both in males and females (Fig. 3). In males, the strongest effects observed were on CIE/FSS elevated marble-burying, indicating that the anxiogenic influence of chronic ethanol and stress interactions is driven in part through noradrenergic mechanisms. Limited effects of prazosin were seen in non-CIE males, indicating that the effects of prazosin at the dose used here (0.75 mg/kg) did not produce an overall decrease in anxiety, but served a sex-specific role in reducing alcohol-related anxiety. Similar overall effects were observed in females, including an overall anxiolytic effect of prazosin and specific impact on CIE/NS and, to a lesser extent CIE/FSS. Unlike males, prazosin strongly reduced marble-burying in the Air/NS group, but not in the Air/FSS group highlighting a differential impact on alcohol and stress related marble burying between sexes. Differences in the effect of prazosin between males and females may result from prominent sex differences in the structure and function of noradrenergic nuclei (Bangasser et al. 2011; Mulvey et al. 2018; Pinos et al. 2001; Reyes et al. 2008) and underscore the need to incorporate both sexes in future investigations of the NE system in studies of alcohol and stress. In general, drugs such as prazosin targeting the α_1 -AR have anxiolytic effects on behavior (Rasmussen et al. 2017; Skelly and Weiner 2014). Specific anxiolytic impacts in our study argues against potential sedative effects of prazosin underlying reduced marble-burying. In combination with minor

(though significant) changes in locomotion and no correlation between changes in locomotion and marble-burying, these data support noradrenergic $\alpha 1$ -AR as a locus for ethanol-induced elevated anxiety. The fact that prazosin reduced ethanol-elevated marble-burying supports a particular role for this system in ethanol-induced stress, and supports further investigation as a potential future treatment, though as noted, differential effects across sex need to be strongly considered.

CRF plays an important modulatory role in LC function (Valentino and Wehby 1988). Our results indicate that this pathway does not directly regulate CIE/FSS driven increases in anxiety, at least as measured in the marble burying task. As a caveat, only one dose of CP376395 was tested. We chose our dose of 0.3 ug/hemisphere (total 0.6 ug) based on pharmacological studies of CP376392 efficacy in the LC, and prior behavioral studies showing that microinjections of this dose (0.6 ug) can decrease stress induced alcohol intake in other regions (Chen et al. 2008; Hwa et al. 2016). When delivered intravenously 0.1ug/10gm body weight of CP376392 halves the impact of exogenous CRF on LC activity. Thus 0.3ug directly administered to each LC should be sufficient to at least halve the impact of CRF on LC in mice. It is possible that a higher dose may have shown effects, though we also note that there are additional influences of CRF signaling in the LC such as those on respiratory function (Inceglu et al. 2016) that should be taken into consideration when considering higher doses.

As $\alpha 1$ -AR antagonism decreased marble burying, other hypotheses linking NE to elevated anxiety should be considered. Although early work indicated a less-prominent role for LC in anxiety, recent studies have shown a pronounced influence of LC signaling on anxiety-related behaviors (McCall et al. 2015), indicating that understanding LC contributions to the observed behavioral effects are a valid pursuit. Additionally, other noradrenergic nuclei such as NTS may play a critical role in regulating stress/alcohol associated increases in anxiety (Vazey et al. 2018). Future studies should consider the role of LC, or other noradrenergic nuclei, as a whole in these behaviors using, e.g., LC-neuronal modulation. Additionally, future studies should aim to incorporate alternate afferent regulation such as glutamate, dynorphin, orexin, or other peptide afferents, as well as NE signaling (α -1 or α -2) in relevant LC targets such as the medial prefrontal cortex or amygdala. The influence of norepinephrine on alcohol elevations in anxiety behaviors, as demonstrated here, strongly supports the need for future work to identify the specifics of neural networks underlying its role in these behaviors.

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References

- Alcohol and Drug Use Collaborators (2018) The global burden of disease attributable to alcohol and drug use in 195 countries and territories, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016. *Lancet Psychiatry* 5: 987–1012. [PubMed: 30392731]
- Anderson RI, Lopez MF, Becker HC (2016) Forced swim stress increases ethanol consumption in C57BL/6J mice with a history of chronic intermittent ethanol exposure. *Psychopharmacology (Berl)* 233: 2035–43. [PubMed: 26935824]
- Armstrong JF, Faccenda E, Harding SD, Pawson AJ, Southan C, Sharman JL, Campo B, Cavanagh DR, Alexander SPH, Davenport AP, Spedding M, Davies JA, Nc I (2020) The IUPHAR/BPS Guide to PHARMACOLOGY in 2020: extending immunopharmacology content and introducing the IUPHAR/MMV Guide to MALARIA PHARMACOLOGY. *Nucleic Acids Res* 48: D1006–D1021. [PubMed: 31691834]
- Aston-Jones G, Cohen JD (2005) An integrative theory of locus coeruleus-norepinephrine function: adaptive gain and optimal performance. *Annu Rev Neurosci* 28: 403–50. [PubMed: 16022602]
- Bangasser DA, Zhang X, Garachh V, Hanhauser E, Valentino RJ (2011) Sexual dimorphism in locus coeruleus dendritic morphology: a structural basis for sex differences in emotional arousal. *Physiol Behav* 103: 342–51. [PubMed: 21362438]
- Becker HC (2012) Effects of alcohol dependence and withdrawal on stress responsiveness and alcohol consumption. *Alcohol Res* 34: 448–58. [PubMed: 23584111]
- Becker HC (2017) Influence of stress associated with chronic alcohol exposure on drinking. *Neuropharmacology* 122: 115–126. [PubMed: 28431971]
- Becker HC, Lopez MF (2004) Increased ethanol drinking after repeated chronic ethanol exposure and withdrawal experience in C57BL/6 mice. *Alcohol Clin Exp Res* 28: 1829–38. [PubMed: 15608599]
- Becker HC, Lopez MF, Doremus-Fitzwater TL (2011) Effects of stress on alcohol drinking: a review of animal studies. *Psychopharmacology (Berl)* 218: 131–56. [PubMed: 21850445]
- Begleiter H (1974) Propranolol and alcohol consumption in the rat. *Am J Drug Alcohol Abuse* 1: 107–10. [PubMed: 4467717]
- Berridge CW, Waterhouse BD (2003) The locus coeruleus-noradrenergic system: modulation of behavioral state and state-dependent cognitive processes. *Brain Res Brain Res Rev* 42: 33–84. [PubMed: 12668290]
- Blaine SK, Milivojevic V, Fox H, Sinha R (2016) Alcohol Effects on Stress Pathways: Impact on Craving and Relapse Risk. *Can J Psychiatry* 61: 145–53. [PubMed: 27254089]
- Breese GR, Sinha R, Heilig M (2011) Chronic alcohol neuroadaptation and stress contribute to susceptibility for alcohol craving and relapse. *Pharmacol Ther* 129: 149–71. [PubMed: 20951730]
- Caruso MJ, Seemiller LR, Fetherston TB, Miller CN, Reiss DE, Cavigelli SA, Kamens HM (2018) Adolescent social stress increases anxiety-like behavior and ethanol consumption in adult male and female C57BL/6J mice. *Sci Rep* 8: 10040. [PubMed: 29968802]
- Chen YL, Obach RS, Braselton J, Corman ML, Forman J, Freeman J, Gallaschun RJ, Mansbach R, Schmidt AW, Sprouse JS, Tingley Jii FD, Winston E, Schulz DW (2008) 2-aryloxy-4-alkylaminopyridines: discovery of novel corticotropin-releasing factor 1 antagonists. *J Med Chem* 51: 1385–92. [PubMed: 18288792]
- Curtis AL, Bethea T, Valentino RJ (2006) Sexually dimorphic responses of the brain norepinephrine system to stress and corticotropin-releasing factor. *Neuropsychopharmacology* 31: 544–54. [PubMed: 16123744]
- Daviu N, Bruchas MR, Moghaddam B, Sandi C, Beyeler A (2019) Neurobiological links between stress and anxiety. *Neurobiol Stress* 11: 100191. [PubMed: 31467945]
- Dhaher R, Finn D, Snelling C, Hitzemann R (2008) Lesions of the extended amygdala in C57BL/6J mice do not block the intermittent ethanol vapor-induced increase in ethanol consumption. *Alcohol Clin Exp Res* 32: 197–208. [PubMed: 18162080]
- Edwards S, Baynes BB, Carmichael CY, Zamora-Martinez ER, Barrus M, Koob GF, Gilpin NW (2013) Traumatic stress reactivity promotes excessive alcohol drinking and alters the balance of prefrontal cortex-amygdala activity. *Transl Psychiatry* 3: e296. [PubMed: 23982628]

- Eriksson K, Pikkarainen PH (1968) Differences between the sexes in voluntary alcohol consumption and liver ADH-activity in inbred strains of mice. *Metabolism* 17: 1037–42. [PubMed: 5724168]
- Fox HC, Anderson GM, Tuit K, Hansen J, Kimmerling A, Siedlarz KM, Morgan PT, Sinha R (2012) Prazosin effects on stress- and cue-induced craving and stress response in alcohol-dependent individuals: preliminary findings. *Alcohol Clin Exp Res* 36: 351–60. [PubMed: 21919922]
- Franklin KBJ, Paxinos G (2008) *The Mouse Brain in Stereotaxic Coordinates*, Third edn. Academic Press
- Froehlich JC, Hausauer B, Fischer S, Wise B, Rasmussen DD (2015) Prazosin Reduces Alcohol Intake in an Animal Model of Alcohol Relapse. *Alcohol Clin Exp Res* 39: 1538–46. [PubMed: 26207767]
- Froehlich JC, Hausauer BJ, Federoff DL, Fischer SM, Rasmussen DD (2013) Prazosin reduces alcohol drinking throughout prolonged treatment and blocks the initiation of drinking in rats selectively bred for high alcohol intake. *Alcohol Clin Exp Res* 37: 1552–60. [PubMed: 23731093]
- Funk D, Coen K, Tamadon S, Li Z, Loughlin A, Le AD (2016) Effects of prazosin and doxazosin on yohimbine-induced reinstatement of alcohol seeking in rats. *Psychopharmacology (Berl)* 233: 2197–207. [PubMed: 27020784]
- Gilpin NW, Koob GF (2010) Effects of beta-adrenoceptor antagonists on alcohol drinking by alcohol-dependent rats. *Psychopharmacology (Berl)* 212: 431–9. [PubMed: 20676608]
- Grant BF, Goldstein RB, Saha TD, Chou SP, Jung J, Zhang H, Pickering RP, Ruan WJ, Smith SM, Huang B, Hasin DS (2015) Epidemiology of DSM-5 Alcohol Use Disorder: Results From the National Epidemiologic Survey on Alcohol and Related Conditions III. *JAMA Psychiatry* 72: 757–66. [PubMed: 26039070]
- Griffin WC 3rd, Lopez MF, Yanke AB, Middaugh LD, Becker HC (2009) Repeated cycles of chronic intermittent ethanol exposure in mice increases voluntary ethanol drinking and ethanol concentrations in the nucleus accumbens. *Psychopharmacology (Berl)* 201: 569–80. [PubMed: 18791704]
- Hwa LS, Holly EN, DeBold JF, Miczek KA (2016) Social stress-escalated intermittent alcohol drinking: modulation by CRF-R1 in the ventral tegmental area and accumbal dopamine in mice. *Psychopharmacology (Berl)* 233: 681–90. [PubMed: 26576941]
- Incheglu JM, Bicego KC, Gargaglioni LH (2016) Corticotropin-releasing factor in the locus coeruleus as a modulator of ventilation in rats. *Respir Physiol Neurobiol* 233: 73–80. [PubMed: 27543446]
- Jury NJ, DiBerto JF, Kash TL, Holmes A (2017) Sex differences in the behavioral sequelae of chronic ethanol exposure. *Alcohol* 58: 53–60. [PubMed: 27624846]
- Koob GF (2014) Neurocircuitry of alcohol addiction: synthesis from animal models. *Handb Clin Neurol* 125: 33–54. [PubMed: 25307567]
- Koob GF, Volkow ND (2016) Neurobiology of addiction: a neurocircuitry analysis. *Lancet Psychiatry* 3: 760–73. [PubMed: 27475769]
- Lopez MF, Anderson RI, Becker HC (2016) Effect of different stressors on voluntary ethanol intake in ethanol-dependent and nondependent C57BL/6J mice. *Alcohol* 51: 17–23. [PubMed: 26992696]
- McCall JG, Al-Hasani R, Siuda ER, Hong DY, Norris AJ, Ford CP, Bruchas MR (2015) CRH Engagement of the Locus Coeruleus Noradrenergic System Mediates Stress-Induced Anxiety. *Neuron* 87: 605–20. [PubMed: 26212712]
- Moore RY, Bloom FE (1979) Central catecholamine neuron systems: anatomy and physiology of the norepinephrine and epinephrine systems. *Annu Rev Neurosci* 2: 113–68. [PubMed: 231924]
- Mulvey B, Bhatti DL, Gyawali S, Lake AM, Kriaucionis S, Ford CP, Bruchas MR, Heintz N, Dougherty JD (2018) Molecular and Functional Sex Differences of Noradrenergic Neurons in the Mouse Locus Coeruleus. *Cell Rep* 23: 2225–2235. [PubMed: 29791834]
- Peltier MR, Verplaetse TL, Mineur YS, Petrakis IL, Cosgrove KP, Picciotto MR, McKee SA (2019) Sex differences in stress-related alcohol use. *Neurobiol Stress* 10: 100149. [PubMed: 30949562]
- Pinos H, Collado P, Rodriguez-Zafra M, Rodriguez C, Segovia S, Guillamon A (2001) The development of sex differences in the locus coeruleus of the rat. *Brain Res Bull* 56: 73–8. [PubMed: 11604252]
- Pleil KE, Lowery-Gionta EG, Crowley NA, Li C, Marcinkiewicz CA, Rose JH, McCall NM, Maldonado-Devincci AM, Morrow AL, Jones SR, Kash TL (2015) Effects of chronic ethanol

exposure on neuronal function in the prefrontal cortex and extended amygdala. *Neuropharmacology* 99: 735–49. [PubMed: 26188147]

Prencipe L, Iaccheri E, Manzati C (1987) Enzymic ethanol assay: a new colorimetric method based on measurement of hydrogen peroxide. *Clin Chem* 33: 486–9. [PubMed: 3549057]

Rasmussen DD, Beckwith LE, Kincaid CL, Froehlich JC (2014) Combining the alpha1 -adrenergic receptor antagonist, prazosin, with the beta-adrenergic receptor antagonist, propranolol, reduces alcohol drinking more effectively than either drug alone. *Alcohol Clin Exp Res* 38: 1532–9. [PubMed: 24891220]

Rasmussen DD, Kincaid CL, Froehlich JC (2017) Prazosin Prevents Increased Anxiety Behavior That Occurs in Response to Stress During Alcohol Deprivations. *Alcohol Alcohol* 52: 5–11. [PubMed: 27797712]

Retson TA, Sterling RC, Van Bockstaele EJ (2016) Alcohol-induced dysregulation of stress-related circuitry: The search for novel targets and implications for interventions across the sexes. *Prog Neuropsychopharmacol Biol Psychiatry* 65: 252–9. [PubMed: 26006055]

Reyes BA, Valentino RJ, Van Bockstaele EJ (2008) Stress-induced intracellular trafficking of corticotropin-releasing factor receptors in rat locus coeruleus neurons. *Endocrinology* 149: 122–30. [PubMed: 17947354]

Rinaman L (2011) Hindbrain noradrenergic A2 neurons: diverse roles in autonomic, endocrine, cognitive, and behavioral functions. *Am J Physiol Regul Integr Comp Physiol* 300: R222–35. [PubMed: 20962208]

Rodberg EM, den Hartog CR, Anderson RI, Becker HC, Moorman DE, Vazey EM (2017) Stress Facilitates the Development of Cognitive Dysfunction After Chronic Ethanol Exposure. *Alcohol Clin Exp Res* 41: 1574–1583. [PubMed: 28753742]

Rose JH, Karkhanis AN, Chen R, Gioia D, Lopez MF, Becker HC, McCool BA, Jones SR (2016) Supersensitive Kappa Opioid Receptors Promotes Ethanol Withdrawal-Related Behaviors and Reduce Dopamine Signaling in the Nucleus Accumbens. *Int J Neuropsychopharmacol* 19.

Rossier O, Abuin L, Fanelli F, Leonardi A, Cotecchia S (1999) Inverse agonism and neutral antagonism at alpha(1a)- and alpha(1b)-adrenergic receptor subtypes. *Mol Pharmacol* 56: 858–66. [PubMed: 10531388]

Skelly MJ, Weiner JL (2014) Chronic treatment with prazosin or duloxetine lessens concurrent anxiety-like behavior and alcohol intake: evidence of disrupted noradrenergic signaling in anxiety-related alcohol use. *Brain Behav* 4: 468–83. [PubMed: 25161814]

Swanson LW, Hartman BK (1975) The central adrenergic system. An immunofluorescence study of the location of cell bodies and their efferent connections in the rat utilizing dopamine-beta-hydroxylase as a marker. *J Comp Neurol* 163: 467–505. [PubMed: 1100685]

Tanaka M, Tsuda A, Yokoo H, Yoshida M, Ida Y, Nishimura H (1990) Involvement of the brain noradrenaline system in emotional changes caused by stress in rats. *Ann N Y Acad Sci* 597: 159–74. [PubMed: 1974753]

Umathe S, Bhutada P, Dixit P, Shende V (2008) Increased marble-burying behavior in ethanol-withdrawal state: modulation by gonadotropin-releasing hormone agonist. *Eur J Pharmacol* 587: 175–80. [PubMed: 18448097]

Valentino RJ, Foote SL, Aston-Jones G (1983) Corticotropin-releasing factor activates noradrenergic neurons of the locus coeruleus. *Brain Res* 270: 363–7. [PubMed: 6603889]

Valentino RJ, Wehby RG (1988) Corticotropin-releasing factor: evidence for a neurotransmitter role in the locus ceruleus during hemodynamic stress. *Neuroendocrinology* 48: 674–7. [PubMed: 2908000]

Varlinskaya EI, Spear LP (2015) Social consequences of ethanol: Impact of age, stress, and prior history of ethanol exposure. *Physiol Behav* 148: 145–50. [PubMed: 25431835]

Vazey EM, den Hartog CR, Moorman DE (2018) Central Noradrenergic Interactions with Alcohol and Regulation of Alcohol-Related Behaviors. *Handb Exp Pharmacol* 248: 239–260. [PubMed: 29687164]

Fig. 1. Study design. (a) Timeline of experimental procedures for systemic prazosin (left panel) and intra-LC CP (right panel) studies. Each box represents 1 week. Mice first underwent 4 weeks of baseline drinking before being assigned to ethanol and stress exposure groups (Air/NS, Air/FSS, CIE/NS, and CIE/FSS). (b) Cannula placements from intra-LC CP experiments in mice color coded by exposure history (adapted from (Franklin and Paxinos 2008)). Abbreviations: CIE, chronic intermittent ethanol; FSS, forced swim test; LC, locus coeruleus; NS, no stress; CP, CP376395; Veh, vehicle; aCSF, artificial cerebral spinal fluid

Fig. 2.

Repeated cycles of chronic intermittent ethanol and stress increases drinking in males and females. Ethanol consumption (g/kg) during 1h access to 15% ethanol (v/v) in mice with a history of repeated cycles of CIE and/or FSS exposure significantly increased ethanol consumption in male and female mice. Weekly average ethanol consumption (g/kg) per session in male (a) and female (b) mice treated with 2–3 cycles of CIE and FSS exposure ($n=26-35$ animals per group). All mice showed a main effect of CIE exposure on drinking but only males showed a main effect of FSS alone on test drinking. #Significantly different from baseline average (mixed model ANOVA, *post hoc* Tukey's multiple comparisons test, # $p<0.05$, ## $p<0.01$, ### $p<0.001$); *Significant difference between exposure history groups (mixed model ANOVA, *post hoc* Sidak's multiple comparisons test, ** $p<0.01$, *** $p<0.001$). All data shown as mean $\pm$ SEM. (c, d) CIE and FSS results in a greater proportion of high drinkers as compared to low and medium. In both male (c) and female (d) mice, CIE and CIE/FSS groups exhibit a higher proportion of high vs. low and medium drinkers. Also of note, in males, but not females, FSS alone results in a

greater proportion of medium drinkers relative to AIR/NS. (e, f) CIE and FSS drives strong individual changes in drinking in most exposure groups. Individual differences in drinking at baseline (last 2 weeks; y axis) was strongly correlated with differences in test drinking in male (e) and female (f) mice (x axis). In males, FSS produced escalations in drinking irrespective of baseline consumption in both air and CIE vapor groups. CIE alone escalated individual drinking, and drinking levels were related to baseline consumption. Combined CIE and FSS strongly right shifted consumption in animals from both sexes regardless of baseline consumption. *Significant correlation between baseline and test intake (Pearson correlation, ** $p < 0.01$, *** $P < 0.001$)

Fig. 3.

Ethanol and stress exposure escalates marble burying, and this can be reduced by systemic prazosin. (a, b) Marble burying increases in both sexes after CIE and FSS exposure. Total number of marbles buried in male (a) and female (b) mice (5 min bins). (c) Prazosin (0.75 mg/kg) decreases the number of marbles buried in both sexes across stress and exposure history. Marbles buried at 10 mins. Main effect of prazosin treatment (3-way ANOVA, ### $p=0.0002$); Main effect of exposure history group (** $p=0.0038$). (d) Prazosin increases latency to bury first marble regardless of exposure history. Main effect of prazosin treatment (3-way ANOVA, ### $p=0.0006$); Main effect of exposure history groups (* $p=0.0106$). $n=6-11$ animals per group. \$=significant posthoc effect against vehicle within history group. All data shown as mean $\pm$ SEM

Fig. 4.

Intra-LC CRF antagonism did not impact marble burying in mice with a history of ethanol and stress exposure. (a, b) Marble burying increases after CIE and FSS exposure with and without intra-LC CP infusions. Number of marbles buried in male (a) and female (b) mice (5 min bins). (c) Number of marbles buried at 10 mins. Main effect of exposure history group (**** $p < 0.0001$) (d) CIE and FSS exposure decrease latency to bury first marble and this is not impacted by CRF antagonism in LC. Main effect of exposure history group (*** $p = 0.0005$). N= 5–9 animals per group. All data shown as mean $\pm$ SEM