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ORIGINAL INVESTIGATION

Persistent escalation of alcohol consumption by mice exposed to brief episodes of social defeat stress: suppression by CRF-R1 antagonism

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Abstract

Rationale Episodic bouts of social stress can precede the initiation, escalation, or relapse to disordered alcohol intake. Social stress may engender neuroadaptations in the hypothalamic-pituitary-adrenal (HPA) axis and in extrahypothalamic stress circuitry to promote the escalation of alcohol intake.

Objectives We aimed to (1) confirm a pattern of escalated drinking in socially defeated mice and to (2) test drugs that target distinct aspects of the HPA axis and extrahypothalamic neural substrates for their effectiveness in reducing murine, stress-escalated drinking.

Methods Male C57BL/6J (B6) mice were socially defeated by resident Swiss-derived males for ten consecutive days receiving 30 bites/day. Ten days after the final defeat, cohorts of B6 mice received continuous or intermittent access to 20% EtOH (*w*/*v*) and water. After 4 weeks of drinking, mice were injected with weekly, systemic doses of the CRF-R1 antagonist, CP376395; the glucocorticoid receptor antagonist, mifepristone; the 11-beta-hydroxylase inhibitor, metyrapone; or the 5-alpha-reductase inhibitor, finasteride.

Results Prior to drug treatments, defeated mice reliably consumed more EtOH than non-defeated controls, and mice given alcohol intermittently consumed more EtOH than those with continuous access. CP376395 (17–30 mg/kg) reduced continuous, but not intermittent EtOH intake (g/kg) in socially defeated mice. Mifepristone (100 mg/kg), however, increased drinking by defeated mice with intermittent access to alcohol while reducing drinking during continuous access. When administered finasteride (100 mg/kg) or metyrapone (50 mg/kg), all mice reduced their EtOH intake while increasing their water consumption.

Conclusions Mice with a history of episodic social defeat stress were selectively sensitive to the effects of CRF-R1 antagonism, suggesting that CRF-R1 may be a potential target for treating alcohol use disorders in individuals who escalate their drinking after exposure to repeated bouts of psychosocial stress. Future studies will clarify how social defeat stress may alter the expression of extrahypothalamic CRF-R1 and glucocorticoid receptors.

Keywords Social defeat stress · Alcohol · Intermittent access to alcohol · CRF-R1 · CP376395 · Glucocorticoids · Metyrapone · Mifepristone · Finasteride · C57BL/6J mice

The onset of escalated alcohol consumption and relapse to disordered drinking is often preceded by episodes of social stress (Boden et al., 2014; Brown et al. 1990; Field and Powell 2007; Laws et al. 2017; Miller et al. 1974; Sinha 2001; Uhart and Wand 2009). Similarly, in some preclinical rodent models, animals subjected to daily social defeats subsequently escalate their alcohol consumption compared to non-defeated or dominant individuals (Albrechet-Souza et al. 2017; Caldwell and Riccio 2010; Croft et al. 2005; Hwa et al. 2016a; Karlsson et al. 2017; Kudryavtseva et al. 2006; Kudryavtseva et al. 1991; Nelson et al. 2017; Norman et al. 2015; Ribeiro Do et al. 2009). Converging evidence

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suggests that this pattern of drinking may arise from stress-induced neuroadaptations, culminating in the sensitization of hypothalamic, extrahypothalamic, and mesocorticolimbic stress- and reward-related circuitries (Covington et al. 2005; Han et al. 2017; Holly et al. 2016; Hwa et al. 2016a; Laine et al. 2017; Martinez et al. 2002; Matsuda et al. 1996; Miczek et al. 2008; Nikulina et al. 2004; Nikulina et al. 2008). Here, we use a mouse model of episodic social defeat stress-escalated drinking to examine pharmacological interventions that target distinct aspects of the hypothalamic-pituitary-adrenal (HPA) axis and extrahypothalamic stress circuitry. In addition, we assessed whether these effects are specific to the schedule of chronic alcohol access by administering drugs to socially defeated and non-defeated mice with either continuous or intermittent access to alcohol.

Activation of the HPA axis prompts corticotropin-releasing factor (CRF, i.e., CRH) release from median eminence terminals originating in the paraventricular nucleus of the hypothalamus. CRF subsequently stimulates adrenocorticotropic hormone (ACTH) secretion from the anterior pituitary and glucocorticoid secretion from the adrenal cortex. In addition to other physiological effects, glucocorticoids regulate HPA axis activity and stress responding through negative feedback. In contrast, distinct CRF signaling in extrahypothalamic brain regions is associated with the regulation of affective behavior (Heinrichs and Koob, 2004). Within the extended amygdala, CRF is upregulated upon exposure to stress or alcohol, and sensitization of the extrahypothalamic CRF system contributes to the anxiogenic effects of alcohol withdrawal (Gilpin and Roberto 2012). Peripherally, CRF-R1 antagonists block ACTH secretion (Rivier et al. 1984; Rivier et al. 1999; Webster et al. 1996), while central CRF-R1 antagonism has been proposed to act via the extended amygdala to normalize stress-escalated drug-taking (Gilpin and Roberto 2012).

Though consistent evidence demonstrates that CRF-R1 blockade reduces alcohol intake in animal models of chronic or binge-like drinking (Cippitelli et al. 2012; Correia et al. 2015; Gehlert et al. 2007; Gilpin et al. 2008; Hwa et al. 2013, 2016a, 2016b; Koob 2010; Lodge and Lawrence 2003; Lowery et al. 2010; Rinker et al. 2017; Sparta et al. 2008, 2009), so far, similar compounds have been ineffective in suppressing stress-induced alcohol craving in samples of anxious, alcohol-dependent patients (Kwako et al. 2015; Schwandt et al. 2016). We propose that stress-induced dysregulation of glucocorticoid secretion and of CRF and dopamine release in response to ethanol may escalate intake by individuals with a stress history, and that targeting CRF signaling may underlie the potentially therapeutic effects of CRF-R1 antagonists (Molander et al. 2012; Sinha 2008). To clarify the potential role of CRF-R1 in stress-escalated continuous or intermittent alcohol intake, we treated socially defeated and non-defeated mice with doses of the CRF-R1 antagonist, CP376395.

Previously, we and others have reported escalated levels of circulating corticosterone in mice subjected to moderate, episodic social defeat stress (Croft et al. 2008; Norman et al. 2015). In animal models, self-administered corticosterone escalates local dopamine release in the ventral striatum and promotes alcohol consumption (Fahlke and Hansen 1999; Piazza et al. 1993, 1996), while acutely administered alcohol can increase cortical and hippocampal glucocorticoid concentrations (Porcu et al. 2014). In rats, blocking glucocorticoid receptors (GR) with mifepristone suppresses alcohol drinking and stress-induced alcohol seeking (Koenig and Olive 2004; Simms et al. 2012; Vendruscolo et al. 2015). Similarly, in alcohol-dependent patients, mifepristone significantly diminishes alcohol craving (Vendruscolo et al. 2015). We hypothesized that elevated levels of circulating glucocorticoids may increase the rewarding value of alcohol to promote alcohol consumption by mice with a history of episodic social defeat stress. To assess glucocorticoid functioning in stress-escalated drinking, defeated and non-defeated male mice received either systemic doses of the 11β-hydroxylase inhibitor, metyrapone, to block synthesis of corticosterone, or doses of the GR antagonist, mifepristone, to prevent receptor activation by corticosterone.

Finally, we tested the 5α-reductase inhibitor, finasteride, in our murine model of stress-escalated chronic alcohol drinking. Inhibiting allopregnanolone and tetrahydrodeoxycorticosterone (THDOC) synthesis with acute doses of finasteride can transiently suppress alcohol intake (Ford et al. 2005a, 2008; Ramaker et al. 2011); conversely, low doses of the neurosteroid allopregnanolone, a potent positive allosteric modulator of $GABA_A$ receptors, can increase voluntary alcohol consumption in C57BL/6J mice (Ford et al. 2005b, 2007; Sinnott et al. 2002). Stress exposure, whether in the form of episodic social defeat or intermittent periods of forced abstinence from chronic alcohol, may alter how neurosteroids interact with the $GABA_A$ receptors (Sarkar et al. 2011). To address the potential role of neurosteroid synthesis in mice with a history of repeated social stress, animals maintained on continuous or intermittent schedules of alcohol access were treated systemically with acute doses of finasteride.

In the present experimental work, we assessed the role of (1) signaling via CRF-R1 receptors, (2) corticosterone synthesis and its actions on glucocorticoid receptors, and (3) endogenous neurosteroid synthesis in a murine model of social defeat stress-escalated alcohol intake. The 11β-hydroxylase inhibitor, metyrapone, and the 5α-reductase inhibitor, finasteride, reduced alcohol intake in mice, regardless of their stress history. In contrast, the glucocorticoid receptor antagonist, mifepristone, increased alcohol intake in mice with a stress history and intermittent access to alcohol while the same dose of

mifepristone decreased drinking in mice with continuous alcohol access. Upon receiving the CRF-R1 antagonist, socially defeated mice with continuous access reduced their alcohol drinking, adding to the accumulating evidence that CP376395 may diminish chronically escalated alcohol consumption. In light of these findings, we propose that CP376395 may be most effective in individuals who initiate or intensify their drinking after experiencing episodes of psychosocial stress.

Materials and methods

Animals

Eight-week-old male C57BL/6J mice (B6 mice; Jackson Laboratories, Bar Harbor, ME, USA) were housed individually. Aggressive residents were adult Swiss Webster (CFW) males (Charles River Laboratories, Wilmington, MA, USA) maintained in breeding pairs with CFW females for at least 3 weeks before aggressive encounters to facilitate territorial agonistic behavior toward male conspecifics (Miczek and O'Donnell 1978). All mice were housed in clear polycarbonate cages (28 × 17 × 14 cm) lined with pine shavings, and had unlimited access to tap water and rodent chow (Purina LabDiet 5001) through stainless steel wire mesh cage lids. Food, water, and bedding were changed weekly at least 24 h prior to systemic drug injections and behavioral testing. The vivarium was temperature-regulated and maintained on a 12-h reverse light/dark photocycle (lights off from 0700 to 1900 h). Mice were cared for according to the NIH *Guide for the Care and Use of Laboratory Animals* (National Research Council 2011), and procedures were approved by the Institutional Animal Care and Use Committee of Tufts University.

Drugs

Ninety-five percent ethyl alcohol (EtOH; Pharmco-AAPER Products) was diluted with tap water to achieve a concentration of 20% EtOH (*w*/*v*). EtOH solutions were made weekly, and any remaining solution was disposed of at the end of each week.

The CRF-R1 antagonist, CP376395 (Tocris, Minneapolis, MN, USA), was dissolved in 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) in dH_2O and administered in doses of 10, 17, and 30 mg/kg. Metyrapone (Sigma-Aldrich, St. Louis, MO, USA), an 11β-hydroxylase inhibitor, was dissolved in 0.9% saline and injected in doses of 10, 30, and 50 mg/kg. Twenty percent (2-hydroxypropyl)-β-cyclodextrin (Sigma-Aldrich, St. Louis, MO, USA) in dH_2O served as the vehicle for suspensions of the glucocorticoid receptor antagonist, mifepristone (i.e., RU38486; Sigma-Aldrich, St. Louis, MO, USA; 17, 30, 56, and 100 mg/kg). Twenty percent β-cyclodextrin vehicle was also used to prepare suspensions of the 5α-reductase inhibitor, finasteride (Sigma-Aldrich, St. Louis, MO, USA; 10, 30, and 100 mg/kg). Drug doses, selected based on previous research and on pilot studies (Hwa et al. 2016b; Lowery et al. 2010; Ramaker et al. 2011), were prepared immediately prior to their administration and given systemically in an injection volume of 10 mL per kg of body weight.

Moderate episodic social defeat stress

Male B6 mice were randomly assigned to the non-defeated, control group or to the moderate episodic social defeat stress group (Table 1). Control mice were weighed daily and returned to their home cage. Moderate episodic social defeat stress occurred as previously described (Norman et al. 2015; Yap et al. 2005). Briefly, social defeat episodes occurred every 24 h for ten consecutive days; each episode consisted of a 5-min pre-defeat threat period, followed by the social defeat, and concluding with a 5-min post-defeat threat period. First, the female and any pups were temporarily removed from a resident CFW's home cage and replaced by a B6 male in a perforated, protective cage (15 × 7 × 7 cm). Immediately after this pre-defeat threat, the B6 male was removed from the protective cage and returned to the resident's home cage. This social defeat was terminated either after the experimental mouse received 30 bites, after 5 min had elapsed, or in the rare case that the B6 mouse attacked the resident male. Following the defeat, the B6 mouse was placed back in the protective cage for a post-defeat threat period in the resident's home cage. After the post-defeat threat period, B6 mice were returned to

Table 1 Sample sizes (*n*/group)

	CAA	IAA
CP376395		
Non-defeated	12	10
Socially defeated	12	11
Metyrapone		
Non-defeated	8	8
Socially defeated	15	10
Mifepristone		
Non-defeated	12	12
Socially defeated	11	11
Finasteride		
Non-defeated	7	10
Socially defeated	14	10
BEC analysis		
Non-defeated	23	16
Socially defeated	26	20

CAA Continuous access to alcohol, *IAA* Intermittent access to alcohol, *BEC* Blood ethanol concentration

their home cages until the next social defeat episode on the following day. B6 mice that were aggressive toward a resident CFW ($n = 6$ of 100) could not be defeated and were therefore excluded from further experimental work.

Voluntary EtOH intake: intermittent or continuous access to 20% EtOH (*w*/*v*)

Ten days after the final social defeat episode, defeated and non-defeated B6 males received either intermittent or continuous access to 20% EtOH (*w*/*v*) and water (i.e., two-bottle choice), as described previously (Hwa et al. 2011). Defeated and non-defeated mice with intermittent access to alcohol (IAA) were presented with 20% EtOH and water, 3 h into the dark photocycle on Mondays, Wednesdays, and Fridays. On all other days, both bottles dispensed water. Separate groups of defeated and non-defeated mice were assigned to receive daily, continuous access to 20% EtOH and water (CAA). For both continuous and intermittent access protocols, EtOH presentation alternated between the left and right side of the cage lid to prevent the development of a side preference. Body weights were recorded every 48 h, and bottles were weighed prior to and after each 24-h EtOH access period. To control for fluid loss not due to drinking (i.e., drip values), bottle measurements were recorded from an empty cage; these values were subtracted from the recorded intake values of each mouse to account for leakage.

Stress-escalated voluntary EtOH intake and systemic pharmacology

During the third week of continuous or intermittent access to EtOH, mice were habituated every 48 h to handling and intraperitoneal (IP) vehicle injections. Weekly IP injections of CP376395, metyrapone, mifepristone, or finasteride began during the fourth week of intermittent or continuous EtOH access (Table 1). Mice continued to consume alcohol, with weekly drug treatments, for up to six additional weeks (i.e., 10 weeks total; Fig. 1). Drugs were administered 2 h into the dark phase of the photocycle. An hour later, bottles were emptied, refilled with 20% EtOH (*w*/*v*) and water, weighed, and returned, signifying the onset of the EtOH access period (i.e., 0 h, Fig. S1). EtOH and water intake and drip values were recorded after 2, 4, and 24 h of EtOH access to evaluate brief and long-lasting drug effects on fluid consumption. Animals received weekly, IP drug doses in a non-systematic order with the highest doses administered last to minimize the potential for carryover effects. Mice were maintained on two-bottle choice protocols, allowing us to determine if effective drug doses selectively reduced alcohol consumption or if they were non-selective and reduced both alcohol and water intake, possibly due to sedative effects or effects on thirst or fluid regulation.

After receiving their final drug treatment, a subset of mice (Table 1) continued to drink with either continuous or intermittent access to alcohol for at least one more week. Blood was then collected from the submandibular vein after 4-h access to EtOH and water to determine blood ethanol concentration (BEC; mg/dL). Samples were centrifuged for 10 min (3000 rpm, 4 °C), and plasma (5 μL) was extracted and analyzed with the Analox AM1 Alcohol Analyzer (Analox Instruments Inc., Lunenberg, MA, USA).

Statistics

Twenty-four-hour alcohol consumption is presented as grams of EtOH consumed per kilogram of body weight (g/kg; (mL EtOH intake × 0.2) / body weight in kg)) and as percent preference (100 × (mL EtOH intake / mL EtOH intake + mL water intake)). For the first 4 weeks of intermittent or continuous access to EtOH, average individual daily intake values were analyzed by two-way analyses of variance (two-way ANOVA) to detect interactions between the EtOH access protocol and history of social defeat stress. To detect changes in drinking over time, average individual EtOH intake (g/kg) and percent preference values were calculated for the first and fourth weeks of intermittent or continuous access; these data were analyzed by two-way repeated measures ANOVA (two-way RM ANOVA). EtOH intake (g/kg), water intake (mL), and BEC (mg/dL) data collected after IP drug administration were analyzed within drug group and drinking protocol. Main effects and interactions between drug and social defeat history were detected by two-way RM ANOVA. Statistically significant main effects were followed by Dunnett's post hoc comparisons, conducted to identify differences between vehicle and specific drug doses and between socially defeated and non-defeated groups. A square root transformation was used when assumptions of normality or equal variance were not met; figures and tables portray the back-transformed data. All statistical analyses were conducted with α set at 0.05 using SigmaPlot v13.0 (Systat Software Inc., San Jose, CA, USA).

Results

Stress-escalated voluntary EtOH intake

A history of moderate, episodic social defeat stress and intermittent alcohol access both independently escalated 20% EtOH intake by male C57BL/6J mice. Two-way ANOVA revealed significant main effects of stress history ($F[1, 169] = 71.98$, $p < 0.001$) and the schedule of EtOH access ($F[1, 169] = 215.23$, $p < 0.001$) on EtOH consumption (g/kg; Fig. 2a, b). Specifically, mice that underwent moderate episodic social defeat stress significantly increased their EtOH consumption compared to non-defeated control mice, and

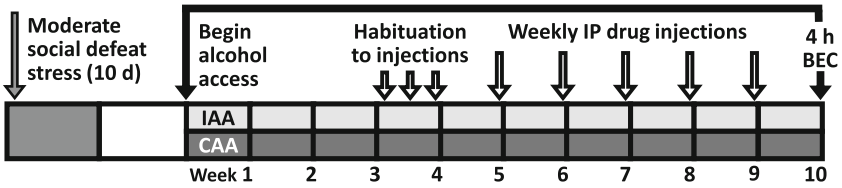

Fig. 1 Male C57BL/6J (B6) mice underwent ten consecutive days of moderate, episodic social defeat stress, receiving 30 bites/day from an aggressive resident CFW male; alternatively, non-defeated B6 control mice were weighed daily. Ten days after the final social defeat session, defeated and non-defeated B6 males received either intermittent *or* continuous access to 20% EtOH (*w/v*) and water. Mice with intermittent access to alcohol (IAA) received 20% EtOH and water on Mondays, Wednesdays, and Fridays, and two bottles of water on the remaining days. Mice with continuous access to alcohol (CAA) received 20% EtOH and water daily. During the third week of IAA or CAA, mice were habituated to intraperitoneal (IP) injections on Mondays, Wednesdays, and Fridays 1 h prior to the onset of the alcohol access period. Beginning in the fourth week of IAA or CAA, mice received weekly IP injections of CP376395 (0, 10, 17, 30 mg/kg), metyrapone (0, 10, 30, 50), mifepristone (0, 17, 30, 56, 100), *or* finasteride (0, 10, 30, 100). At least 1 week after the final IP drug treatment, blood samples were collected from the submandibular vein 4 h after the onset of the alcohol access period and analyzed for blood plasma ethanol concentration (BEC)

intermittent access to EtOH yielded greater levels of drinking compared to continuous access. Likewise, social defeat stress ($F[1, 169] = 31.94$, $p < 0.001$) and intermittent EtOH access ($F[1, 169] = 205.6$, $p < 0.001$) significantly increased preference for 20% EtOH over water (Fig. 2c, d). Only mice with intermittent access to alcohol showed an escalation in their EtOH intake ($F[1, 80] = 95.46$, $p < 0.001$) and EtOH preference ($F[1, 80] = 119.70$, $p < 0.001$) from the first to the fourth week of drinking. In accordance with these findings, blood ethanol concentrations (BECs) taken from a representative subset of mice revealed higher BECs in animals with intermittent access to alcohol ($F[1, 81] = 53.82$, $p < 0.001$) and in those with a history of moderate, episodic social defeat stress ($F[1, 81] = 4.52$, $p = 0.037$; Table 2).

Selective suppression of stress-escalated, continuous access EtOH drinking by CRF-R1 antagonism

In socially defeated mice with continuous access to alcohol, injections of the CRF-R1 antagonist, CP376395, reduced daily alcohol intake. Two-way RM ANOVA revealed a significant interaction between stress history and CP376395 ($F[3, 66] = 3.74$, $p = 0.015$) and a main effect of drug treatment ($F[3, 66] = 4.08$, $p = 0.01$), with 17 and 30 mg/kg doses selectively reducing daily alcohol intake in mice with a history of moderate, episodic social defeat stress (Fig. 3a). This interaction developed over the course of the 24-h access period following drug administration, but was not detectable early in the drinking episode. Rather, CRF-R1 antagonism reduced 2- and

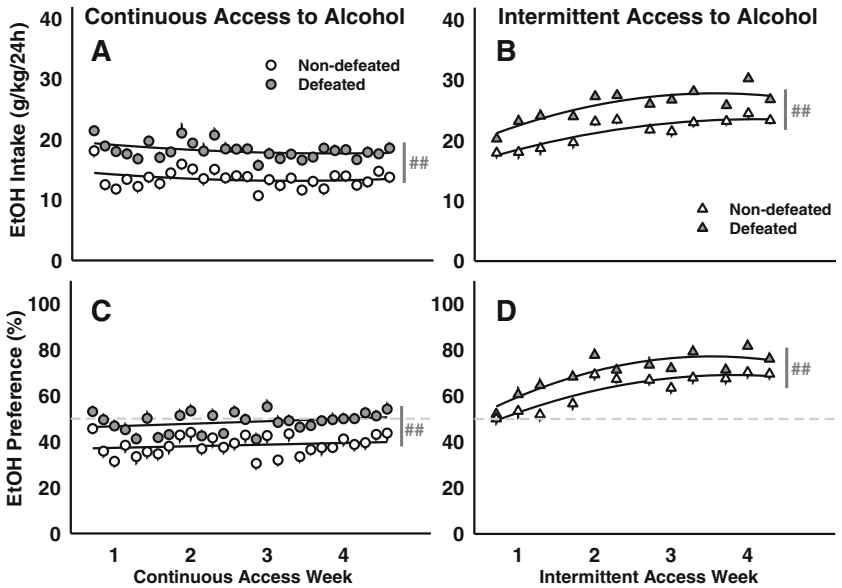

Fig. 2 Socially defeated and non-defeated C57BL/6J males received 20% EtOH and water for 4 weeks prior to receiving weekly, systemic drug injections. Daily mean ± SEM intake (g/kg/24 h) for mice with **a** continuous access to alcohol or **b** intermittent access to alcohol. The amount of alcohol consumed as a percentage of the total daily intake (EtOH Preference) is depicted as daily mean ± SEM for mice with **c** continuous or **d** intermittent access to alcohol. Best-fit curves are in black. Dark gray lines represent significant main effects: ##$p < 0.01$ non-defeated vs. defeated. Dashed lines mark the preference cutoff: values > 50% indicate a preference for alcohol over water, while values < 50% indicate a preference for water over alcohol

Table 2 BECs (mg/dl) after 4-hour access to 20% EtOH and water

	CAA	IAA*
Non-defeated	30.13 ± 4.34	90.01 ± 13.85
Socially defeated#	46.39 ± 11.18	133.32 ± 15.30

Mean ± SEM; * $p<0.05$, CAA vs. IAA; # $p<0.05$, socially defeated vs. non-defeated; blood ethanol concentration (BEC); continuous access alcohol (CAA); intermittent access alcohol (IAA)

4-h alcohol intake by all mice, regardless of stress history (2 h: $F[3, 66] = 9.37$, $p < 0.001$; 4 h: $F[3, 66] = 5.93$, $p = 0.001$; Table 3). Importantly, water drinking was not suppressed by drug administration, suggesting that CP376395 specifically reduces alcohol consumption (Table S1). By 4 h into the access period, defeated mice consumed significantly more alcohol than their non-defeated counterparts ($F[1, 22] = 7.67$, $p = 0.011$); this effect persisted for the entire, 24-h access period in defeated mice injected with vehicle or the lowest dose of CP376395 (10 mg/kg; Fig. 3a). In our analysis of water consumption (mL), we found that social defeat stress significantly interacted with CP376395 ($F[3, 66] = 3.01$, $p = 0.036$). In general, stress-escalated alcohol intake was associated with lower levels of water drinking compared to the control condition. However, upon receiving doses of CP376395 that effectively reduced their alcohol consumption, defeated mice compensated by drinking slightly more water while controls consumed slightly less water than in the vehicle condition (Table 1, Fig. S2A).

CRF-R1 antagonism decreased 2- and 4-h alcohol intake in defeated and non-defeated mice with intermittent access to alcohol

Antagonism of CRF-R1 with CP376395 reduced 2- and 4-h alcohol intake by mice maintained on the intermittent access schedule (2 h: $F[3, 48] = 21.63$, $p < 0.001$; 4 h: $F[3, 48] = 15.83$, $p < 0.001$). This drug effect was no longer evident by 24 h of two-bottle choice (Fig. 3b, Table 4). In general, defeated animals consuming less water than controls, especially during the first 2 h of access (2 h: $F[1, 16] = 8.42$, $p = 0.01$). Though the CRF-R1 antagonist selectively reduced alcohol intake without suppressing water consumption, two-way RM ANOVA detected a significant interaction between defeat stress and CP376395 (24 h: $F[3, 48] = 2.94$, $p = 0.042$). Given the 17.0 mg/kg dose, non-defeated mice drank more water compared to defeated animals (Table S2, Fig. S2A), though intake did not significantly differ from the vehicle condition for either group.

Inhibition of 11β-hydroxylase reduced alcohol intake in defeated and non-defeated mice

Treatment with the highest dose of the 11β-hydroxylase inhibitor, metyrapone (50 mg/kg), diminished alcohol drinking during 2, 4, and 24 h of continuous access (2 h: $F[3, 60] = 6.23$, $p < 0.001$; 4 h: $F[3, 60] = 5.55$, $p = 0.002$; 24 h: $F[3, 60] = 5.00$, $p = 0.004$; Fig. 3c, Table 3). Mice consumed less alcohol after receiving metyrapone, and compensated by increasing their water intake, but only for the initial 2 and 4-h access periods (2 h: $F[3, 60] = 8.86$, $p < 0.001$; 4 h: $F[3, 60] = 3.94$, $p = 0.012$; Table S1, Fig. S2B).

Animals with intermittent access also reduced their alcohol intake upon receiving the highest dose of metyrapone (50 mg/kg; Fig. 3d). Both stressed and non-stressed mice were sensitive to this effect during 2, 4, and 24 h of alcohol access (2 h: $F[3, 48] = 7.31$, $p < 0.001$; 4 h: $F[3, 48] = 9.16$, $p < 0.001$; 24 h: $F[3, 48] = 16.95$, $p < 0.001$; Table 4). Stress-escalated alcohol intake was observed at every time point in socially defeated mice compared to their non-defeated counterparts (2 h: $F[1, 16] = 9.80$, $p = 0.006$; 4 h: $F[1, 16] = 10.33$, $p = 0.005$; 24 h: $F[1, 16] = 29.11$, $p < 0.001$). An analysis of water intake data revealed a significant interaction between stress history and metyrapone (2 h: $F[3, 48] = 2.93$, $p = 0.043$; 4 h: $F[3, 48] = 3.09$, $p = 0.036$; Table S2). While metyrapone (50 mg/kg) reduced alcohol intake by all mice, only non-defeated animals compensated by drinking more water during the initial 2- and 4-h access periods. Though alcohol intake remained suppressed, water intake was no longer elevated by the 24-h time point (Fig. S2B).

Glucocorticoid receptor antagonism increased EtOH intake by defeated mice and decreased EtOH intake by non-defeated mice with intermittent access to alcohol

For animals with intermittent access to alcohol, stress history interacted with mifepristone to increase alcohol consumption (24 h: $F[4, 84] = 2.65$, $p = 0.039$). Specifically, mice subjected to episodic social defeat stress consumed significantly more alcohol than controls when given the highest drug dose (100 mg/kg). In contrast, given a moderate dose of mifepristone (30 mg/kg), non-defeated animals reduced their alcohol intake (Fig. 3f, Table 4). Stress-escalated drinking was evident after 2, 4, and 24 h of alcohol access (2 h: $F[1, 21] = 8.81$, $p = 0.007$; 4 h: $F[1, 21] = 4.63$, $p = 0.043$; 24 h: $F[1, 21] = 17.18$, $p < 0.001$; Fig. 3f), while a main effect of mifepristone was only detected for the full, 24-h access period ($F[4, 84] = 4.03$, $p = 0.005$).

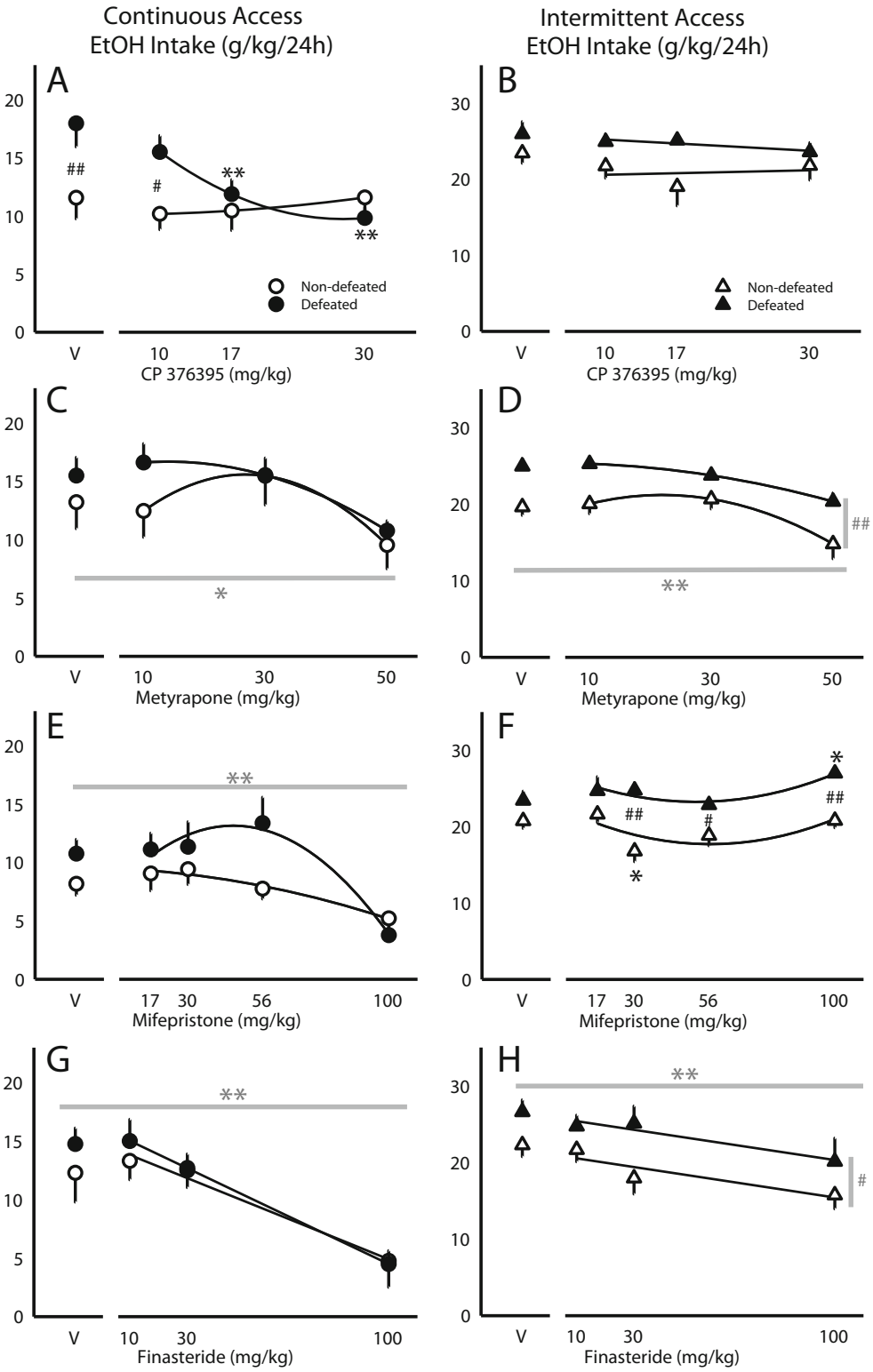

Fig. 3 Daily alcohol intake (g/kg/24 h) shown as mean ± SEM for socially defeated and non-defeated C57BL/6J males with either (**a**, **c**, **e**, **g**) continuous or (**b**, **d**, **f**, **h**) intermittent access to alcohol. Mice received systemic doses of **a**, **b** CP376395, **c**, **d** metyrapone, **e**, **f** mifepristone, or **g**, **h** finasteride prior to the onset of the alcohol access period. For interactions between social defeat stress and drug treatment, significant post-hoc comparisons are depicted as $*p < 0.05$, $**p < 0.01$ vehicle vs. drug; $\#p < 0.05$, $\#\#p < 0.01$ non-defeated vs. defeated. Best-fit curves are in black. Dark gray lines represent significant post hoc comparisons in the presence of main effects: $*p < 0.05$, $**p < 0.01$ vehicle vs. drug; $\#p < 0.05$, $\#\#p < 0.01$ non-defeated vs. defeated

In analyzing water intake during the initial 2 h of intermittent alcohol access, two-way RM ANOVA identified a significant interaction between defeat stress history and mifepristone ($F[4, 84] = 2.52$, $p = 0.047$), a main effect of reduced water intake by defeated mice ($F[1, 21] = 8.85$, $p = 0.007$), and a main effect of drug treatment ($F[4, 84] = 5.73$, $p < 0.001$). Compared to vehicle, non-defeated mice diminished their water intake after receiving moderate doses of mifepristone (17, 56 mg/kg), whereas defeated animals were only sensitive to this effect at higher doses (56, 100 mg/kg;

Table 3 Two- and four-hour continuous access alcohol intake (g/kg) by socially defeated or non-defeated mice after systemic injections of CP376395, metyrapone, or mifepristone

		Non-defeated		Socially defeated	
Drug	mg/kg	2hr	4hr	2hr	4hr
CP 376395[d]	veh	2.60 ± 0.43	3.93 ± 0.77	3.46 ± 0.38	6.75 ± 0.95
	10[a]	1.56 ± 0.32	2.90 ± 0.50	2.19 ± 0.25	4.29 ± 0.42
	17[a b]	1.48 ± 0.27	2.98 ± 0.45	1.60 ± 0.25	3.55 ± 0.46
	30[a b]	1.81 ± 0.35	2.72 ± 0.56	1.25 ± 0.28	2.59 ± 0.51
Metyrapone	veh	1.43 ± 0.34	4.69 ± 1.13	2.04 ± 0.26	4.03 ± 0.41
	10	2.12 ± 0.32	3.74 ± 0.61	2.61 ± 0.47	5.26 ± 0.74
	30	1.99 ± 0.51	4.31 ± 0.93	1.80 ± 0.43	4.16 ± 0.61
	50[a b]	1.25 ± 0.35	2.65 ± 0.55	0.99 ± 0.12	2.64 ± 0.29
Mifepristone	veh	1.32± 0.21	3.50 ± 0.55	1.31 ± 0.34	3.20 ± 0.92
	17	1.23 ± 0.32	3.40 ± 0.92	1.16 ± 0.31	3.12 ± 0.63
	30	1.50 ± 0.39	3.28 ± 0.51	1.11 ± 0.41	2.57 ± 0.63
	56	0.62 ± 0.16	2.08 ± 0.56	1.01 ± 0.30	3.05 ± 0.62
	100[a b]	0.16 ± 0.06	0.35 ± 0.13	0.40 ± 0.19	0.86± 0.52

Mean ± SEM; main effects of drug followed by post-hoc comparisons of vehicle vs. drug dose: [a] $p<0.05$ at 2hr, [b] $p<0.05$ at 4hr; main effects of social stress followed by post-hoc comparisons of socially defeated vs. non-defeated: [c] $p<0.05$ at 2hr, [d] $p<0.05$ at 4hr

Table S2). By 4 h of alcohol access, all doses of mifepristone significantly reduced water intake regardless of stress history ($F[4, 84] = 5.89$, $p < 0.001$). This effect was no longer detectable by the 24-h time point, though the main effect of defeat stress persisted ($F[1, 21] = 4.34$, $p = 0.049$; Fig. S2C).

Mifepristone diminished alcohol consumption in mice with continuous access to alcohol

Regardless of their stress history, mice with continuous access to alcohol reduced their 2-, 4-, and 24-h EtOH intake after

Table 4 Two- and four-hour intermittent access alcohol intake (g/kg) by socially defeated or non-defeated mice after systemic injections of CP376395, metyrapone, mifepristone or finasteride

		Non-defeated		Socially Defeated	
Drug	mg/kg	2hr	4hr	2hr	4hr
CP 376395	veh	3.67 ± 0.26	6.20 ± 0.28	4.72 ± 0.42	7.09 ± 0.60
	10[a]	3.65 ± 0.72	6.44 ± 0.75	3.03 ± 0.55	5.19 ± 0.58
	17[a b]	1.65 ± 0.38	4.42 ± 1.02	2.37 ± 0.33	4.21 ± 0.50
	30[a b]	1.38 ± 0.31	2.27 ± 0.55	1.19 ± 0.26	3.22 ± 0.59
Metyrapone[c d]	veh	2.41 ± 0.35	3.95 ± 0.47	3.65 ± 0.23	5.24 ± 0.45
	10	2.27 ± 0.32	3.44 ± 0.53	3.73 ± 0.27	5.36 ± 0.44
	30	2.42 ± 0.36	3.63 ± 0.70	3.10 ± 0.43	4.58 ± 0.47
	50[a b]	1.58 ± 0.37	1.80 ± 0.42	2.21 ± 0.28	3.66 ± 0.27
Mifepristonec d	veh	1.78 ± 0.29	4.17 ± 0.68	2.61 ± 0.28	5.20 ± 0.71
	17	1.71 ± 0.34	5.19 ± 0.81	2.43 ± 0.36	4.73 ± 0.93
	30	1.55 ± 0.28	2.92 ± 0.53	2.18 ± 0.29	4.99 ± 0.59
	56	1.40 ± 0.33	3.36 ± 0.66	1.68 ± 0.42	4.10 ± 0.77
	100	1.40 ± 0.20	3.02 ± 0.37	2.46 ± 0.23	4.56 ± 0.49
Finasteride[c d]	veh	1.66 ± 0.38	3.46 ± 0.85	2.75 ± 0.39	6.06 ± 0.68
	10	1.88 ± 0.41	4.11 ± 0.66	2.19 ± 0.45	5.17 ± 0.84
	30[a b]	0.50 ± 0.16	1.94 ± 0.46	1.84 ± 0.48	4.58 ± 0.99
	100[a b]	0.29 ± 0.20	0.97 ± 0.32	0.58 ± 0.38	2.21 ± 0.58

Mean ± SEM; main effects of drug followed by post-hoc comparisons of vehicle vs. drug dose: a$p<0.05$ at 2hr, b$p<0.05$ at 4hr; main effects of social stress followed by post-hoc comparisons of socially defeated vs. non-defeated: c$p<0.05$ at 2hr, d$p<0.05$ at 4hr

receiving the highest dose of mifepristone (100 mg/kg; 2 h: $F[4, 80] = 8.42$, $p < 0.001$; 4 h: $F[4, 80] = 13.86$, $p < 0.001$; 24 h: $F[4, 80] = 9.34$, $p < 0.001$; Fig. 3e, Table 3). For water intake, two-way RM ANOVA identified a significant interaction between social stress and mifepristone treatment (2 h: $F[4, 80] = 2.88$, $p = 0.028$; 4 h: $F[4, 80] = 3.46$, $p = 0.012$; 24 h: $F[4, 80] = 3.86$, $p = 0.006$; Table S1). While non-defeated mice generally consumed more water than their socially defeated counterparts (2 h: $F[1, 20] = 7.19$, $p = 0.014$; 4 h: $F[1, 20] = 4.38$, $p = 0.049$; Table S1), mifepristone (100 mg/kg) selectively increased water drinking by defeated animals, yielding values comparable to controls (Fig. S2C). Mifepristone (56, 100 mg/kg) also briefly diminished water drinking by controls, though this effect was no longer evident by the 24-h time point (Fig. S2C, Table S1).

Inhibition of 5α-reductase suppressed daily alcohol consumption by defeated and non-defeated mice

Given intermittent access to alcohol, defeated and non-defeated mice treated with finasteride (100 mg/kg) reduced their 2-, 4-, and 24-h alcohol intake (2 h: $F[3, 54] = 12.59$, $p < 0.001$; 4 h: $F[3, 54] = 14.54$, $p < 0.001$; 24 h: $F[3, 54] = 7.42$, $p < 0.001$; Fig. 3h, Table 4). While the moderate dose (30 mg/kg) also suppressed 2- and 4-h alcohol drinking, only the highest dose (100 mg/kg) suppressed 24-h intake (Table 4). During the first 2 h of access, finasteride diminished both alcohol (g/kg) and water (mL) consumption (EtOH: $F[3, 54] = 12.59$, $p < 0.001$; H_2O: $F[3, 54] = 8.36$, $p < 0.001$; Tables 4 and S2); however, by 24 h, finasteride-treated animals selectively increased their water drinking ($F[3, 54] = 5.56$, $p = 0.002$; Fig. S2D), while maintaining low alcohol intake ($F[3, 54] = 7.42$, $p < 0.001$; Fig. 3h; Table 4). Two-way RM ANOVA also revealed significant main effects of escalated 2-, 4-, and 24-h alcohol intake by mice with a history of defeat stress (2 h: $F[1, 18] = 4.92$, $p = 0.04$; 4 h: $F[1, 18] = 6.62$, $p = 0.019$; 24 h: $F[1, 18] = 4.82$, $p = 0.042$). During the first 2 h of intermittent access, stress-escalated alcohol intake was concurrent with reduced water consumption ($F[1, 18] = 7.5$, $p = 0.013$).

As in the intermittent access group, mice with continuous access reduced their daily alcohol consumption upon receiving finasteride (100 mg/kg; $F[3, 63] = 40.78$, $p < 0.001$; Fig. 3g, Table 3). This effect was specific for alcohol, as finasteride did not suppress 24-h water intake (Fig. S2D, Table S1). For this experiment, only 24-h drinking data were collected, and thus, we cannot exclude the possibility of a non-specific effect of finasteride early in the access period, as observed in the intermittent access experiment.

Discussion

In some individuals, social stress experiences precede an escalation in alcohol drinking (Sinha 2001; Sinha 2008). Here, we first confirm our earlier findings, showing that episodic social defeat stress can promote persistently escalated alcohol consumption in male C57BL/6J mice (Hwa et al. 2016a); likewise, we demonstrate that intermittent access to alcohol can produce significantly higher levels of voluntary alcohol intake and BECs compared to continuous access, as illustrated in our previous work (Hwa et al. 2011; Hwa et al. 2013; Hwa et al. 2016a). After establishing a pattern of stress-escalated drinking, mice were administered drugs targeting specific aspects of the HPA axis, including CRF-R1 receptors, glucocorticoid synthesis and receptor activation, and neurosteroid synthesis. We found that (1) the CRF-R1 antagonist, CP376395, selectively reduced voluntary alcohol intake by mice with continuous alcohol access and a history of episodic social defeat stress, (2) blocking glucocorticoid receptors increased alcohol intake by socially defeated mice with intermittent access to alcohol, and (3) inhibiting corticosterone or neurosteroid synthesis diminished alcohol intake, regardless of stress history or the schedule of alcohol access.

Interactions between stress neuropeptides and mesocorticolimbic dopamine signaling may mediate stress-escalated alcohol consumption (Melis et al. 2009; Spanagel et al. 2014). Acute alcohol intake promotes HPA axis activation, and CRF and corticosterone contribute to dopamine release in the nucleus accumbens (Fahlke et al. 1996; Lemos et al. 2012; Piazza et al. 1996; Richardson et al. 2008). Importantly, a history of repeated social defeat stress also increases mesolimbic CRF and plasma corticosterone and results in cross-sensitization to alcohol, a phenomenon characterized by increased locomotor behavior and amplified accumbal dopamine release in response to acute drug challenge (Han et al. 2017; Holly et al. 2016; Norman et al. 2015; Phillips et al. 1997; Roberts et al. 1995; Yavich and Tiihonen 2000). An increase in alcohol-induced dopamine release after exposure to episodic social defeat stress may promote the rapid escalation of drinking by stressed mice (Phillips et al. 1997; Quadir et al. 2016). By targeting BNST-VTA-NAcc-mPFC circuitry, CRF-R1 antagonism may selectively reduce the escalation of alcohol consumption in stress-experienced animals (Albrechet-Souza et al. 2017; Hwa et al. 2016a; Silberman et al. 2013).

In previous work, we and others have shown that chronic intermittent access to alcohol can promote significantly escalated voluntary alcohol drinking by C57BL/6J mice relative to consumption during continuous access (Hwa et al. 2011, 2013, 2016a; Melendez 2011; Newman et al. 2016; Osterndorff-Kahanek et al. 2013; Rosenwasser et al. 2013; but see Crabbe et al. 2012). In dependence-inducing protocols, alternating bouts of exposure to ethanol vapor and

withdrawal can upregulate extrahypothalamic *Crh* and *Crhr1* gene expression and suppress hypothalamic *Crh* mRNA while increasing subsequent voluntary intake. These intermittent exposure effects contrast with those seen after continuous ethanol vapor exposure (in rats: O'Dell et al. 2004; Richardson et al. 2008; Sommer et al. 2008; in mice: Eisenhardt et al. 2015). Intermittency may sustain escalated, persistent alcohol consumption by reducing CRF-mediated glucocorticoid secretion and negative feedback in the HPA axis (Breese et al. 2011; Edwards et al. 2015; Rivier et al. 1984) and by increasing CRF signaling and sensitivity in extrahypothalamic brain areas associated with negative affect in withdrawal (Koob and Kreek 2007).

Blocking CRF-R1 receptors with CP376395 reduced alcohol intake in stressed mice with continuous access without suppressing their water consumption. Yet, CP376395 only transiently diminished alcohol drinking by animals given alcohol intermittently, a schedule of access shown to escalate alcohol consumption compared to continuous access. Previous reports suggest that CRF-R1 antagonists may selectively diminish intake by animals classified as high drinkers; we hypothesize that the alcohol- and stress-induced neuroplastic changes in extrahypothalamic versus hypothalamic CRF systems may be more indicative of later responding to CRF-R1 antagonists than alcohol intake alone (Correia et al. 2015; Heilig and Koob 2007; Hwa et al. 2013; Sparta et al. 2008). Because hypothalamic and extrahypothalamic CRF sites undergo divergent modifications during chronic alcohol drinking, the effectiveness of systemically administered CRF-R1 antagonists may depend on the extent to which receptors have been upregulated in the extended amygdala and downregulated along the HPA axis (Edwards et al. 2015; Eisenhardt et al. 2015; O'Dell et al. 2004; Richardson et al. 2008; Sommer et al. 2008). This possibility is supported through studies in *Crhr1* knockout mice; while a global gene knockout promotes social stress-escalated alcohol consumption (Molander et al. 2012; Sillaber et al. 2002), a conditional, forebrain-specific *Crhr1* knockout blocks stress-escalated drinking. These observations suggest that CRF-R1 in the HPA axis may counteract stress effects on drinking whereas extrahypothalamic CRF-R1 activation may stimulate drinking by mice subjected to episodic social defeat stress.

Considerable preclinical work shows that CRF-R1 antagonists can effectively suppress measures of alcohol intake (Koob 2010). However, two recent clinical trials conducted in patients with alcohol use disorders (AUD) report no effect of CRF-R1 antagonism on alcohol cue-induced craving after inpatient detoxification (Kwako et al. 2015; Schwandt et al. 2016; but see Shaham and de Wit 2016, Pomrenze et al. 2017). CRF-R1 antagonists may be specifically effective in reducing alcohol intake by individuals who are currently drinking, and possibly, in those who escalate their drinking after experiencing episodic stress. Elevated circulating cortisol is characteristic of individuals who consume considerable amounts of alcohol, whereas patients in early abstinence tend to have low levels of cortisol; therefore, different drugs may be necessary to normalize glucocorticoid production depending on current drinking status. Reducing glucocorticoid levels with a CRF-R1 antagonist may suppress drinking and the likelihood of relapse during acute withdrawal; conversely, compounds that can increase cortisol levels may diminish cravings during early abstinence (Stephens and Wand 2012). As such, antagonists targeting CRF-R1 should be tested in future clinical trials conducted in non-treatment seeking individuals diagnosed with AUDs (Pomrenze et al. 2017), and in those who escalate their drinking after exposure to repeated bouts of psychosocial stress.

Studies using the glucocorticoid receptor antagonist, mifepristone, demonstrate suppressed alcohol intake and alcohol-cued craving after drug administration in patients suffering from AUDs and reduced alcohol consumption by dependent rats (Vendruscolo et al. 2012, 2015) whereas mifepristone appears to have no effect on drinking in non-dependent rats (Fahlke et al. 1995; Fahlke et al. 1996; Vendruscolo et al. 2012; Vendruscolo et al. 2015). In the present work, we show that a high dose of mifepristone (100 mg/kg) can actually increase intermittent alcohol consumption by defeated mice while a modest dose (30 mg/kg), administered to non-defeated animals, can reduce intermittent alcohol intake. Interestingly, we found that the same dose of mifepristone that escalated drinking in stressed mice with intermittent access effectively reduced alcohol intake in defeated and non-defeated mice with continuous access to alcohol. These findings suggest that moderate antagonism of glucocorticoid receptors may be effective in reducing drinking while higher doses may interfere with HPA axis negative feedback mechanisms; however, the effectiveness of mifepristone appears to depend on individual differences in stress and the pattern of alcohol intake.

Mifepristone may escalate drinking in stressed mice by blunting corticosterone-mediated negative feedback of the HPA axis. Animals with a history of social defeat stress exhibit a potentiated dopamine response to acute alcohol (Yavich and Tiihonen 2000) and may experience greater subjective reinforcing effects of the drug. In repeated bouts of alcohol intake, the eventual downregulation of HPA axis glucocorticoid signaling may accelerate stress-induced drinking, which could be sustained through sensitization of extrahypothalamic brain areas associated with negative affect in withdrawal (Breese et al. 2011; Edwards et al. 2015; Koob and Kreek 2007; Rivier et al. 1984). This profile of neuroadaptations may cause mifepristone to further impair negative feedback, particularly in animals that escalate their drinking on an intermittent schedule of access. As such, increased levels of circulating corticosterone would

subsequently stimulate CRF transcription in the extended amygdala, thus worsening negative affect and increasing alcohol consumption (Shepard et al. 2006; Tran and Greenwood-Van Meerveld 2012). While antagonism of glucocorticoid receptors with mifepristone may have therapeutic effects in some alcohol-dependent patients, its use may be limited by the patient's history of psychosocial stress exposure and their pattern of ongoing alcohol consumption.

Consistent with previous findings (Fahlke et al. 1994; Koenig and Olive 2004; Simms et al. 2012; Vendruscolo et al. 2015), we found a suppression of alcohol intake when glucocorticoid synthesis was inhibited with systemically administered doses of metyrapone. Likewise, inhibiting peripheral glucocorticoid secretion through adrenalectomy suppresses alcohol consumption by rats, and drinking is recovered through systemic or intracerebral injections of corticosterone but not aldosterone (Fahlke et al. 1994, 1995; Lamblin and De Witte 1996), indicating that metyrapone likely diminishes intake by blocking corticosterone synthesis, and not through its inhibition of mineralocorticoid synthesis. Importantly, when alcohol drinking was suppressed in our experiments, animals compensated by consuming greater volumes of water, suggesting that metyrapone selectively diminishes voluntary alcohol intake by non-dependent animals.

Similarly, finasteride treatment suppressed alcohol intake by defeated and non-defeated mice that received continuous or intermittent access to alcohol. This effect may result from the inhibition of allopregnanolone and THDOC synthesis; however, finasteride treatment may also cause an accumulation of precursor and recruitment of an alternative neurosteroid synthetic pathway. Thus, a reduction in allopregnanolone and THDOC concentrations may precede an increase in concentrations of neurosteroids that act as negative allosteric modulators of the $GABA_A$ receptor (Morrow et al. 1990). While the inhibition of 5α-reductase effectively suppressed chronic, voluntary drinking in C57BL/6J mice, the mechanism by which finasteride affects alcohol drinking requires further investigation.

In mice with continuous alcohol access, CP376395 selectively suppressed social stress-escalated drinking. Neuroadaptations in glucocorticoid receptor expression during intermittent access may preclude CRF-R1 antagonists from reducing stress-escalated drinking. Because activation of glucocorticoid receptors can modulate *Crh* transcription in the extended amygdala (Shepard et al. 2006; Tran and Greenwood-Van Meerveld 2012), intermittency-induced neuroadaptations in GR expression may also promote mifepristone-escalated drinking in defeated mice. Interestingly, intra-VTA infusions of CP376395 can selectively reduce alcohol intake in mice with a history of episodic stress that receive alcohol intermittently (Hwa et al. 2016a). These findings along with our present results suggest that local inhibition of CRF-R1 receptors within the VTA may target circuitry that is distinct from regions that undergo neuroadaptive changes in response to intermittency-escalated drinking, possibly within the extended amygdala. Using a combination of pharmacological tools that target CRF/urocortin 1 and optogenetics in CRF-Cre mice, we plan to identify stress-associated neuroadaptations occurring in CRF-expressing neurons projecting from the CeA and BNST to the VTA (Silberman et al. 2013).

The current studies explore the potential roles of several molecular targets, highlighting CRF-R1 antagonism as a promising candidate for the treatment of stress-escalated alcohol drinking. We continue to characterize the effectiveness of CRF-R1 antagonists by using an ethologically relevant defeat protocol to investigate CRF mechanisms of stress-escalated alcohol drinking in females that have been repeatedly defeated by aggressive, female conspecifics. Using this protocol, our ongoing work explores the role of sexually dimorphic brain regions such as the BNST that may be involved in stress-escalated drinking (Allen and Gorski 1990). Here, we show that CP376395 can reduce stress-escalated alcohol consumption in a dose range that does not influence concurrent water intake. Further studies are necessary to examine the effects of social defeat stress and CRF-R1 antagonism in mice with a prior history of alcohol consumption (e.g., Karlsson et al. 2017) and to determine if CRF-R1 antagonism generally suppresses the motivation to obtain and consume natural rewards or if such compounds can selectively reduce chronic, stress-escalated alcohol drinking.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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