Systems/Circuits

Impact and Role of Hypothalamic Corticotropin Releasing Hormone Neurons in Withdrawal from Chronic Alcohol Consumption in Female and Male Mice

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Worldwide, alcohol use and abuse are a leading risk of mortality, causing 5.3% of all deaths (World Health Organization, 2022). The endocrine stress system, initiated by the peripheral release of corticotropin releasing hormone (CRH) from primarily glutamatergic neurons in the paraventricular nucleus of the hypothalamus (PVN), is profoundly linked with alcohol use, abuse, and relapse (Blaine and Sinha, 2017). These PVN CRH-releasing (PVN^{CRH}) neurons are essential for peripheral and central stress responses (Rasiah et al., 2023), but little is known about how alcohol affects these neurons. Here, we show that two-bottle choice alcohol consumption blunts the endocrine-mediated corticosterone response to stress during acute withdrawal in female mice. Conversely, using slice electrophysiology, we demonstrate that acute withdrawal engenders a hyperexcitable phenotype of PVN^{CRH} neurons in females that is accompanied by increased glutamatergic transmission in both male and female mice. GABAergic synaptic transmission was unaffected by alcohol history. We then tested whether chemogenetic inhibition of PVN^{CRH} neurons would restore stress response in female mice with a history of alcohol drinking in the looming disk test, which mimics an approaching predator threat. Accordingly, inhibition of PVN^{CRH} neurons reduced active escape in hM4Di alcohol history mice only. This study indicates that stress-responsive PVN^{CRH} neurons in females are particularly affected by a history of alcohol consumption. Interestingly, women have indicated an increase in heavy alcohol use to cope with stress (Rodriguez et al., 2020), perhaps pointing to a potential underlying mechanism in alcohol-mediated changes to PVN^{CRH} neurons that alter stress response.

Key words: alcohol; behavior; electrophysiology; hypothalamus; mice; withdrawal

Significance Statement

Paraventricular nucleus of the hypothalamus neurons that release corticotropin releasing hormone (PVN^{CRH}) are vital for stress response. These neurons have been understudied in relation to alcohol and withdrawal despite profound relations between stress, alcohol use disorders (AUD), and relapse. In this study, we use a variety of techniques to show that acute withdrawal from a history of alcohol impacts peripheral stress response, PVN^{CRH} neurons, and behavior. Specifically, PVN^{CRH} are in a hyperactive state during withdrawal, which drives an increase in active stress coping behaviors in female mice only. Understanding how alcohol use and withdrawal affects stress responding PVN^{CRH} neurons may contribute to finding new potential targets for the treatment of alcohol use disorder.

Received June 22, 2023; revised Aug. 24, 2023; accepted Sep. 29, 2023.

Author contributions: S.N., J.S.B., and T.L.K. designed research; S.N., S.L., L.A.H., T.S., S.L.D., and K.M.B. performed research; S.N., S.L., L.A.H., T.S., and S.L.D. analyzed data; S.N. wrote the first draft of the paper; S.N., J.S.B., and T.L.K. edited the paper; S.N. wrote the paper.

This work was supported by the National Institute of Alcohol Abuse and Alcoholism grants: U01AA020911 and P60AA011605 to T.L.K. The Howard Hughes Medical Institute fellowship: GT13514 and National Institute of Alcohol Abuse and Alcoholism grant: T32AA007573 to S.N., and The Canadian Institutes of Health Research to J.S.B.

The authors declare no competing financial interests.

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https://doi.org/10.1523/JNEUROSCI.1153-23.2023

Introduction

Alcohol use disorders (AUDs) account for the majority of substance use disorders worldwide, with ~3 million deaths per year attributed to harmful alcohol use (World Health Organization, 2022). Alcohol consumption is also increasing, with recent reports from the United States indicating that women especially are engaging in heavy binge drinking behaviors that are linked to relief from COVID-19-associated stressors (Pollard et al., 2020; Rodriguez et al., 2020). Despite the established relationship between alcohol, withdrawal, and

stress (Sinha et al., 2009; Becker, 2012; Blaine and Sinha, 2017; Peltier et al., 2019), few studies have investigated alcohol-induced alterations to paraventricular nucleus of the hypothalamus corticotropin releasing hormone (PVN^{CRH}) neurons, which initiate the endocrine stress response via the hypothalamic-pituitary-adrenal (HPA) axis (Kudielka and Kirschbaum, 2005; Stephens and Wand, 2012).

There is a blunting of the HPA-axis stress hormone, cortisol (corticosterone in rodents), to acute stress in both male and female AUD or binge drinking withdrawal subjects (Wand and Dobs, 1991; Errico et al., 1993; Bernardy et al., 1996; Junghanns et al., 2003; Adinoff et al., 2005; Blaine et al., 2019). The only study we are aware of that has investigated this in rodents had similar findings; Lee et al. (2000) subjected 24 h withdrawal male rats to acute stressors and found reduced *Fos* expression in the PVN as well as reduced ACTH levels compared with controls. Despite rodent investigations offering a direct ability to study alterations to PVN^{CRH} neurons themselves, few studies have explored how chronic alcohol alters these neurons, and fewer still have studied how females are affected.

Female rodents show a greater activation of the HPA-axis stress system to different stressors, including acute alcohol challenges, and display more active stress responses in behavioral tasks that activate this system (Rivier, 1999; Palanza, 2001; Kudielka and Kirschbaum, 2005; Palanza and Parmigiani, 2017; Bangasser et al., 2018), perhaps suggesting interactions between chronic alcohol and stress response. Indeed, females consume high levels of alcohol in a two-bottle choice (2-BC) voluntary alcohol paradigm, and compared with water controls, display greater active escape behaviors during acute withdrawal in the looming disk test that mimics an approaching predator (Neira et al., 2022). PVN^{CRH} neurons are activated before escape initiation in this task, and optogenetic inhibition of these neurons reduces escape behavior, indicating that PVN^{CRH} neurons are necessary for active escape responses to the looming disk (Daviu et al., 2020). Additionally, a history of alcohol has been shown to increase glutamatergic synaptic transmission in PVN^{CRH} neurons in male monkeys (Jimenez et al., 2019) and cause biophysical and synaptic alterations to these neurons in protracted abstinence in rats (Marty et al., 2020; Munier et al., 2022). This evidence led to our hypothesis that PVN^{CRH} neurons are more excitable in acute withdrawal after a history of alcohol consumption, resulting in more active stress coping behaviors. Although some studies suggest blunted endocrine stress system function in withdrawal, hormonal output and alterations to PVN^{CRH} neurons might be detangled. Indeed, PVN^{CRH} neurons have recently been implicated in various behaviors independent of HPA-axis mediated hormone release (Füzesi et al., 2016; Kim et al., 2019; Yuan et al., 2019; Daviu et al., 2020).

Despite the prevalence and harm stemming from AUD, few treatments exist to mitigate this chronic relapsing disorder. Relapse rates have been associated with stress response systems, and most often, relapse occurs early in abstinence (Junghanns et al., 2003; Karim et al., 2010; Blaine and Sinha, 2017; Wemm et al., 2019). It is therefore imperative to understand physiological alterations in early withdrawal, particularly those associated with stress response. Thus, we used fluorescent in situ hybridization (FISH), *ex vivo* slice electrophysiology, and chemogenetic *in vivo* inhibition to examine how a history of intermittent 2-BC alcohol consumption alters PVN^{CRH} neurons during acute withdrawal in female and male mice. We find drastic effects in PVN^{CRH} neuronal modulation by chronic alcohol intake as well as PVN^{CRH}-driven changes in stress coping behavior in alcohol history female mice only.

Materials and Methods

2-BC alcohol paradigm

At least 4 d before the start of 2-BC, mice were transferred to a temperature-controlled 12 h reverse dark/light cycle vivarium, single-housed, and switched to Isopro RMH 3000 chow (LabDiet), which supports high alcohol intake levels (Marshall et al., 2015). Mice were given *ad libitum* access to water and food. During the 2-BC procedure, alcohol access female and male mice over 8 weeks of age were given access to water and intermittent 24 h alcohol (20% w/v) sessions with forced 24-48 h withdrawals between each session (i.e., M/W/F, 24 h Alcohol Sessions; Tu/Th/Sat/Sun, No Alcohol). Alcohol and water bottles were weighed at the start and end of each alcohol session, which began 2-3 h into the dark cycle. Averaged drip values from an empty cage were subtracted from all water and alcohol raw values. Water access control mice were matched for age, weight, and litter and received only water throughout the 2-BC procedure. The Institutional Animal Care and Use Committee at UNC Chapel Hill approved all experimental procedures, which were performed in accordance with the National Institutes of Health's *Guide for the care and use of laboratory animals*.

Home-cage test

At least 24 h after the final 2-BC alcohol session, mice were removed from the animal facility and transferred to a behavioral testing room, where they were allowed to habituate to the room for a minimum of 40 min. After habituation, the home-cage with only the bedding and mouse was placed in a ~60 lux low-light sound attenuated chamber that contained an overhead camera for filming behavior. Rearing, digging, grooming, and locomotion were analyzed for a 15 min period using DeepLabCut and SimBA-mediated automatic analysis as previously discussed (Neira et al., 2022).

Plasma corticosterone and FISH

C57BL/6J (The Jackson Laboratory) mice were exposed to 18 alcohol sessions in a 2-BC procedure. To measure basal corticosterone and *Fos* mRNA levels in PVN^{CRH} neurons during acute withdrawal, 24-36 h after the final alcohol drinking session, mice (females: 10 water controls for corticosterone and 9 for *in situ* because of technical errors, 10 alcohol access; males: 10 water controls, 10 alcohol access) were placed in an isoflurane chamber with minimal handling before being transferred out of their animal facility housing room. Once fully anesthetized, mice underwent rapid decapitation, trunk blood was collected in a 2 ml tube for plasma corticosterone analysis, and brains were rapidly frozen over aluminum and dry ice and stored in a −80°C freezer for FISH experiments.

A separate set of mice were also exposed to 18 sessions in a 2-BC procedure (females: 11 water controls, 12 alcohol access for corticosterone and 13 for *in situ* because of technical errors; males: 12 water controls, 13 alcohol access). Twenty-four to 36 h after the final alcohol session, mice were subjected to the home-cage test, a mild acute stressor that involves transfer to a behavioral testing room and extra handling. Mice were immediately placed in an isoflurane chamber, and blood and brains were collected to measure corticosterone and *Fos* mRNA levels in PVN^{CRH} neurons during acute withdrawal following the home-cage test.

Plasma corticosterone. Trunk blood was centrifuged at 4°C for 10 min at 3000 RCF, and 30 µl plasma was extracted and stored at −4°C. To measure corticosterone, the DetectX Corticosterone Enzyme Immunoassay Kit (Arbor Assays) was used and followed according to the manufacturer's instructions. Data were analyzed using a Spectra Max Plate Calorimetric 96 well microplate reader and the MyAssays Arbor Assays analyzer.

FISH. Brains were sliced into 12 µl coronal sections containing the PVN using a Leica CM3050 S cryostat (Leica Microsystems) and mounted directly on slides. FISH was performed using the Affymetrix ViewRNA 2-plex assay according to the manufacturer's instructions using probes for *Crh* (category #316091) and *Fos* (category #316921-C2). A blinded individual visually determined the slice containing the highest density of CRH neurons concentrated in the medial PVN and chose one hemisphere counterbalanced per mouse for analysis. FISH data were analyzed using QuPath version 0.4.3 (Bankhead et al., 2017).

Total number of *Crh*- and/or *Fos*-positive neurons were counted, and PVN^{CRH} activation was determined by the percent of *Crh*-expressing neurons that also expressed *Fos*.

Ex vivo *slice electrophysiology*

C-fos is an indirect measure of neuronal activation. Therefore, to more directly test how PVN^{CRH} neurons are affected in acute withdrawal by chronic alcohol consumption, we performed slice electrophysiology using a CRH-reporter mouse strain to visually identify fluorescent, CRH-containing neurons in the PVN. These mice were bred in-house by crossing *CRH-ires-Cre (B6(Cg)-Crh$^{tm1(cre)Zjh}$/J)* mice to Ai9 cre-dependent tdTomato fluorescent reporter (*B6.Cg-Gt (ROSA)26Sor$^{tm9(CAG-tdTomato)Hze}$/J*) mice. Mice were exposed to 18-26 sessions of intermittent alcohol using the 2-BC choice procedure and underwent the home-cage test 24-30 h after the final session. Immediately after, mice were placed in an isoflurane chamber until fully anesthetized and brains were rapidly removed and submerged in an ice-cold carbogen (95% O_2/5% CO_2) saturated sucrose aCSF cutting solution (in mM as follows: 194 sucrose, 20 NaCl, 4.4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1.2 NaH_2PO_4, 10 D-glucose, and 26 $NaHCO_3$). Brains were then blocked for the PVN, and 250 µm coronal slices were prepared on a Leica VT 1000S vibratome (Leica Biosystems) and transferred to a holding chamber with 34°C heated and carbogen saturated aCSF (in mM as follows: 124 NaCl, 4.4 KCl, 1 NaH_2PO_4, 1.2 $MgSO_4$, 10 D-glucose, 2 $CaCl_2$, and 26 $NaHCO_3$). Slices were allowed to equilibrate for at least 1 h, then transferred to a recording chamber (Warner Instruments) with oxygenated and 30 ± 2°C heated flowing aCSF (2 ml/min). Neurons were visualized using a 40× immersion objective on a Scientifica Slicescope II with differential interference contrast, and a 550 LED was used to visualize fluorescent, CRH-containing PVN^{CRH} neurons. Whole-cell patch-clamp experimental recording signals were acquired using an Axon MultiClamp 700B (Molecular Devices), the data were sampled at 10 kHz, low-pass filtered at 3 kHz, and analyzed in pClamp 10.7 (Molecular Devices) or Easy Electrophysiology. One to three neurons were recorded per mouse for each experiment using borosilicate glass capillary micropipettes pulled with a 2-5 MΩ electrode tip resistance using a Flaming/Brown P97 electrode puller (Sutter Instruments). Any changes >20% from the initial access resistance throughout the experiment or a membrane capacitance >30 MΩ led to the exclusion of that neuron from analysis. Cells were also excluded from analysis if the current to hold the cell membrane voltage at −80 mV in current-clamp mode exceeded −60 pA.

Intrinsic excitability experiments. Experiments were recorded in current-clamp mode with a potassium gluconate-based intracellular solution (in mM as follows: 135 K-gluconate, 5 NaCl, 2 $MgCl_2$, 10 HEPES, 0.6 EGTA, 4 Na_2ATP, 0.4 Na_2GTP, pH 7.3, 289-292 mOsm). Input resistance was calculated using steps from −70 to −80 mV in the current–voltage relationship in voltage clamp immediately after breaking into the cell. Resting membrane potential (RMP) was measured after stabilization in current clamp. Current was injected to hold cells at a common membrane potential of −80 mV, and changes in excitability were measured by the frequency of action potentials fired at increasing 10 pA current steps (−40 to 80) lasting 250 ms. In total, 11 female water, 9 female alcohol, 10 male water, and 9 male alcohol mice were used.

Synaptic transmission experiments. Excitatory post synaptic currents (EPSCs) were recorded in voltage-clamp mode with a potassium gluconate-based intracellular solution. Spontaneous transmission (sEPSCs, total mice: 8 male and female water mice and 7 male and female alcohol-exposed mice) was recorded at −80 mV in the presence of 100 µM GABA-A receptor antagonist picrotoxin and miniature events (mEPSCs, total mice: 4 water and alcohol females, 4 water males and 6 alcohol males) were recorded in the presence of 100 µM picrotoxin and 500 nM sodium channel blocker TTX.

Inhibitory post synaptic currents (IPSCs) were recorded in voltage-clamp mode with a potassium-chloride gluconate-based intracellular solution (in mM as follows: 80 KCl, 70 K-gluconate, 10 HEPES, 1 EGTA, 4 Na_2ATP, 0.4 Na_2GTP, pH 7.2, 285-290 mosmol). Spontaneous transmission (sIPSCs, total mice: 8 male, 6 female water mice and 8 male and 7 female alcohol-exposed mice) was recorded at −80 mV in the presence of 10 µM AMPA and kainate receptor antagonist DNQX and miniature events (mIPSCs, total mice: 4 water and alcohol females, 4 water males and 6 alcohol males) were recorded in the presence of 10 µM DNQX and 500 nM TTX.

hM4Di chemogenetic inhibition

CRH^{cre} in-house bred mice aged 6 weeks or older underwent stereotaxic surgery for infusion of mCherry control or hM4Di virus into the PVN. After at least 5 d of recovery, mice began a 6 week 2-BC procedure. In the final 3 weeks of 2-BC, including the day before the first CNO experiment, mice were subjected to at least four saline habituation injections on alcohol off days; 0.5 mg/kg CNO (i.p.) was administered to mCherry control and hM4Di mice for all behavioral tests 30-50 min before the test. One day after the final, 18th, 2-BC alcohol session ended, mice were subjected to the home-cage test immediately followed by a free social interaction test. Three and 4 d after the final 2-BC session, mice were once again brought to the behavior room and allowed to habituate for at least 40 min. A saline injection was administered intraperitoneally 20-30 min before a 20-min-long exposure to the looming disk arena (41 × 19 × 20.5 cm mirrored floor plastic arena with a 13 × 12 × 10 cm protective shelter in one corner) to habituate them to the injection, arena, and the location of the protective shelter. On the fifth day following 2-BC, after at least 40 min habituation to the room, mice were administered CNO and underwent the looming disk test. Mice were perfused and unilateral or bilateral PVN injections were verified, as we saw no differences in behavior between unilateral and bilateral hits. After verification, total *N* values include the following: females, 13 water mCherry, 13 alcohol mCherry, 9 water hM4Di, 11 alcohol hM4Di; males, 10 water mCherry, 10 alcohol mCherry, 10 water hM4Di, 12 alcohol hM4Di.

Stereotaxic surgery. After 4% isoflurane anesthesia induction, mice were transferred to a stereotaxic frame and kept at 1%-2% maintenance in oxygen (1-2 L/min). The scalp was sterilized (70% ethanol and betadine) and the skull exposed. Two small holes were made in the skull above the injection sites and 200 nl (100 nl/min) of AAV8-hSyn-DIO-mCherry or AAV8-hSyn-DIO-hm4Di-mCherry virus (Addgene) was infused in both hemispheres of the PVN using a 1 µl Hamilton syringe (0° angle, mm relative to bregma: AP: –0.55, ML: ±0.20, DV: –5.15). The virus was allowed to diffuse for 5 min following infusion.

Free social interaction. Immediately following the home-cage test as described above, mice were transferred to a fresh cage with bedding and allowed to habituate for 2 min. Following habituation, a juvenile, same-sex mouse was placed in the cage and their interactions were filmed for 10 min.

Looming disk. Mice had a minimum 3 min habituation to the looming disk arena. Following habituation, five episodes of the looming, advancing disk paradigm, separated by at least 1 min between each episode, were triggered when the mouse was not in the shelter or heading toward it. Each episode requires 8 s, starting with a 3 s, 2 cm black disk on a light gray background, which then expands to its full 20 cm size over 2 s and remains on the screen for an additional 3 s. An individual blinded to the experimental manipulation determined whether the mouse escaped to the hut within the 8 s loom presentation. If escape did not occur, the mouse was scored as freezing, or neither escape/freezing for each of the five looms. Freezing was determined as no movement other than breathing for at least 1 s throughout the loom presentation and escape was counted if the mouse entered the shelter at any moment during loom presentation.

Statistics

Female and male data were analyzed separately because of existing differences in total alcohol intake during 2-BC as shown in Table 1. FISH and corticosterone data were analyzed using unpaired *t* tests and Mann–Whitney two-tailed test for water versus alcohol conditions. Slice electrophysiology data were analyzed using two-way repeated-measures ANOVA for current, and condition (water or alcohol); and when only comparing water versus alcohol groups, nested *t* tests were used to account for multiple cells from the same mice being included in the analysis. Behavioral data for the home-

Table 1. 2-BC paradigm first, last, and total alcohol intake per experiment[a]

Experiment	Sex	First session intake (g/kg)	Last session intake (g/kg)	Total intake (g/kg)
Baseline FISH and corticosterone	Females	15.04 ± 10.28	24.53 ± 6.78	420.06 ± 107.24*
	Males	20.13 ± 5.58	16.04 ± 2.53	300.00 ± 40.60*
Acute stress FISH and corticosterone	Females	25.00 ± 9.42	29.57 ± 5.82	502.19 ± 60.58**
	Males	20.64 ± 5.23	17.95 ± 4.84	343.66 ± 72.38**
Slice electrophysiology	Females	20.57 ± 6.85	29.19 ± 6.96	543.62 ± 130.25**
	Males	16.12 ± 4.25	15.64 ± 4.28	311.11 ± 75.16**
Chemogenetics	Females mCherry	21.41 ± 8.12	31.30 ± 5.43	500.56 ± 94.11**
	Males mCherry	13.60 ± 4.44	16.78 ± 7.49	289.89 ± 87.30**
	Females hM4Di	20.67 ± 5.94	30.55 ± 6.75	467.89 ± 69.89**
	Males hM4Di	13.29 ± 6.20	16.56 ± 5.88	272.53 ± 62.82**

[a]Data are mean ± SD.
*$p < 0.01$; **$p < 0.0001$; unpaired Student's *t* test.

cage and social interaction tests in the hM4Di chemogenetic experiment were analyzed using two-way ANOVAs for condition (water vs alcohol) and virus (mCherry vs hM4Di). Looming disk data were analyzed using χ^2 tests, as the data were not numerical (escape, freeze, or neither escape nor freeze responses). A p value <0.05 was determined to be significant, and Sidak-corrected multiple comparisons were conducted when an ANOVA indicated an interaction effect. Statistical tests were conducted in GraphPad Prism 9.2.0 (GraphPad Software). ROUT Outlier tests were conducted on all data. One female alcohol mouse was removed from the plasma corticosterone analysis and the home-cage grooming data analysis per the ROUT outlier analysis after they were confirmed as outliers.

Results

To determine whether a history of alcohol in rodents alters PVN^{CRH} *Fos* expression and corticosterone in early withdrawal, we subjected female and male WT mice to 6 weeks of 2-BC and brains and trunk blood was collected for FISH (*Crh* and *Fos* mRNA) and plasma corticosterone measurements (Fig. 1*A*,*M*). Female water and alcohol-exposed mice showed similar basal corticosterone levels (Fig. 1*B*, unpaired two-tailed *t* test: $t_{(18)} = 0.224$, $p = 0.825$). Interestingly, while the total number of *Fos*-positive cells in the PVN was lower in alcohol-exposed mice (Fig. 1*C*, unpaired two-tailed *t* test: $t_{(17)} = 2.226$, $p = 0.033$), the total number of *Crh* neurons (Fig. 1*D*, unpaired two-tailed *t* test: $t_{(17)} = 0.897$, $p = 0.382$) and PVN^{CRH} activation (Fig. 1*E*, Mann–Whitney test: [$U = 28$, $p = 0.182$]), measured as the percent of *Crh*-expressing neurons that were also positive for *Fos*, was unchanged in acute withdrawal in females compared with water controls. Previous literature suggests that acute stressors can blunt PVN and hormone responses during withdrawal from vapor exposure in male rats (Lee et al., 2000). Here, we tested response to a mild acute stressor in a voluntary drinking paradigm by subjecting mice to 6 weeks of 2-BC followed by a home-cage test (which will be referred to as a mild stress) in acute withdrawal 1 d after the final alcohol session. Female mice exposed to mild stress during withdrawal had significantly lower levels of corticosterone (Fig. 1*H*, unpaired two-tailed *t* test: $t_{(20)} = 2.509$, $p = 0.021$), fewer *Fos*-positive neurons in the PVN (Fig. 1*H*, unpaired two-tailed *t* test: $t_{(22)} = 2.634$, $p = 0.015$), and less PVN^{CRH} activation (Fig. 1*K*, Mann–Whitney two-tailed test: [$U = 25$, $p = 0.006$]) compared with water controls. This was not because of differences in *Crh* total number of neurons, as these were not different between water and alcohol-exposed mice (Fig. 1*J*, unpaired two-tailed *t* test: $t_{(22)} = 0.199$, $p = 0.844$). Male mice exposed to alcohol had similar basal corticosterone levels as water treated mice (Fig. 1*N*, unpaired two-tailed *t* test: $t_{(18)} = 1.801$, $p = 0.088$). These two groups also had similar number of *Fos*-positive PVN neurons (Fig. 1*O*, unpaired two-tailed *t* test: $t_{(18)} = 1.077$, $p = 0.296$). While the total number of PVN *Crh*-positive neurons was lower in alcohol-exposed mice (Fig. 1*P*, unpaired two-tailed *t* test: $t_{(18)} = 2.108$, $p = 0.049$), the activation of PVN^{CRH} neurons did not differ between water and alcohol-exposed male mice (Fig. 1*E*, Mann–Whitney two-tailed test: [$U = 43$, $p = 0.631$]). Acute mild stress during withdrawal did not affect corticosterone (Fig. 1*T*, unpaired two-tailed *t* test: $t_{(23)} = 1.312$, $p = 0.203$), *Fos* (Fig. 1*U*, unpaired two-tailed *t* test: $t_{(22)} = 0.703$, $p = 0.490$), total number of PVN *Crh* (Fig. 1*V*, unpaired two-tailed *t* test: $t_{(22)} = 1.427$, $p = 0.168$), or PVN^{CRH} activation (Fig. 1*W*, Mann–Whitney two-tailed test: [$U = 49$, $p = 0.198$]) in alcohol-exposed male mice compared with water controls.

Thus far, the only electrophysiological investigations of PVN^{CRH} neurons have been conducted in protracted abstinence, long after alcohol cessation. In these experiments, we show that alcohol history also directly affects PVN^{CRH} neurons in acute withdrawal using *ex vivo* slice electrophysiology after a home-cage test in the PVN of CRH^{cre} x Ai9 male and female mice. First, we performed voltage- and current-clamp recordings to examine the intrinsic characteristics of PVN^{CRH} neurons. Female alcohol history mice had no differences in the average input resistance compared with water controls (Fig. 2*A*, nested *t* test: $t_{(19)} = 1.174$, $p = 0.255$) but did show a significantly depolarized RMP (Fig. 2*B*, nested *t* test: $t_{(20)} = 3.739$, $p = 0.001$). We then measured the AP frequency in response to current steps delivered in 10 pA increments from a membrane potential of −80 mV. There were significant differences in intrinsic excitability between female alcohol and water mice (Fig. 2*C*,*D*); a two-way repeated-measures ANOVA revealed a main effect of current ($F_{(2.273,75.00)} = 75.59$, $p < 0.001$), and a alcohol × current interaction ($F_{(8264)} = 2.854$, $p = 0.005$), with no effect of alcohol ($F_{(1,33)} = 1.466$, $p = 0.235$). *Post hoc* Sidak-corrected multiple comparisons test did not reveal any significant differences (30 pA: $p = 0.065$ and 40 pA: $p = 0.108$). We found no differences in intrinsic excitability in male mice, with no significant differences between water and alcohol-exposed males in the input resistance (Fig. 2*E*, nested *t* test: $t_{(17)} = 2.003$, $p = 0.061$), the RMP (Fig. 2*F*, nested *t* test: $t_{(17)} = 0.091$, $p = 0.929$), or the current step protocol (Fig. 2*G*,*H*, two-way repeated-measures ANOVA; main effect of current: $F_{(2.150,64.49)} = 104.2$, $p < 0.001$; main effect of alcohol: $F_{(1,30)} = 0.524$, $p = 0.475$. Interaction: $F_{(8240)} = 0.622$, $p = 0.759$).

Having established differences in intrinsic excitability in females, but not males, we next measured synaptic transmission in PVN^{CRH} neurons (Fig. 3). As hypothesized, female alcohol history mice had stronger excitatory synaptic drive compared with water mice, as mEPSC frequency (Fig. 3*A*, nested *t* test: $t_{(19)} = 2.426$, $p = 0.025$) was higher in alcohol mice, while there were no differences in sEPSC frequency (Fig. 3*D*, nested *t* test: $t_{(13)} = 1.596$, $p = 0.134$). There was no difference in the amplitude of mEPSCs (Fig. 3*B*) or sEPSCs (Fig. 3*E*) between female mice. Female alcohol and water mice also had no differences in mIPSC frequency (Fig. 3*G*) and amplitude (Fig. 3*H*) or sIPSC frequency (Fig. 3*J*) and amplitude (Fig. 3*K*). Interestingly, male alcohol history mice also displayed a high frequency of mEPSCs compared with water controls (Fig. 3*M*, nested *t* test: $t_{(22)} = 2.970$, $p = 0.007$) with no difference in mEPSC amplitude (Fig. 3*N*) or sEPSCs frequency (Fig. 3*P*) or amplitude (Fig. 3*Q*). Of note, there were some trends hinting at increases in inhibitory synaptic transmission in alcohol history male mice compared with water controls in mIPSC amplitude (Fig. 3*T*, nested *t* test:

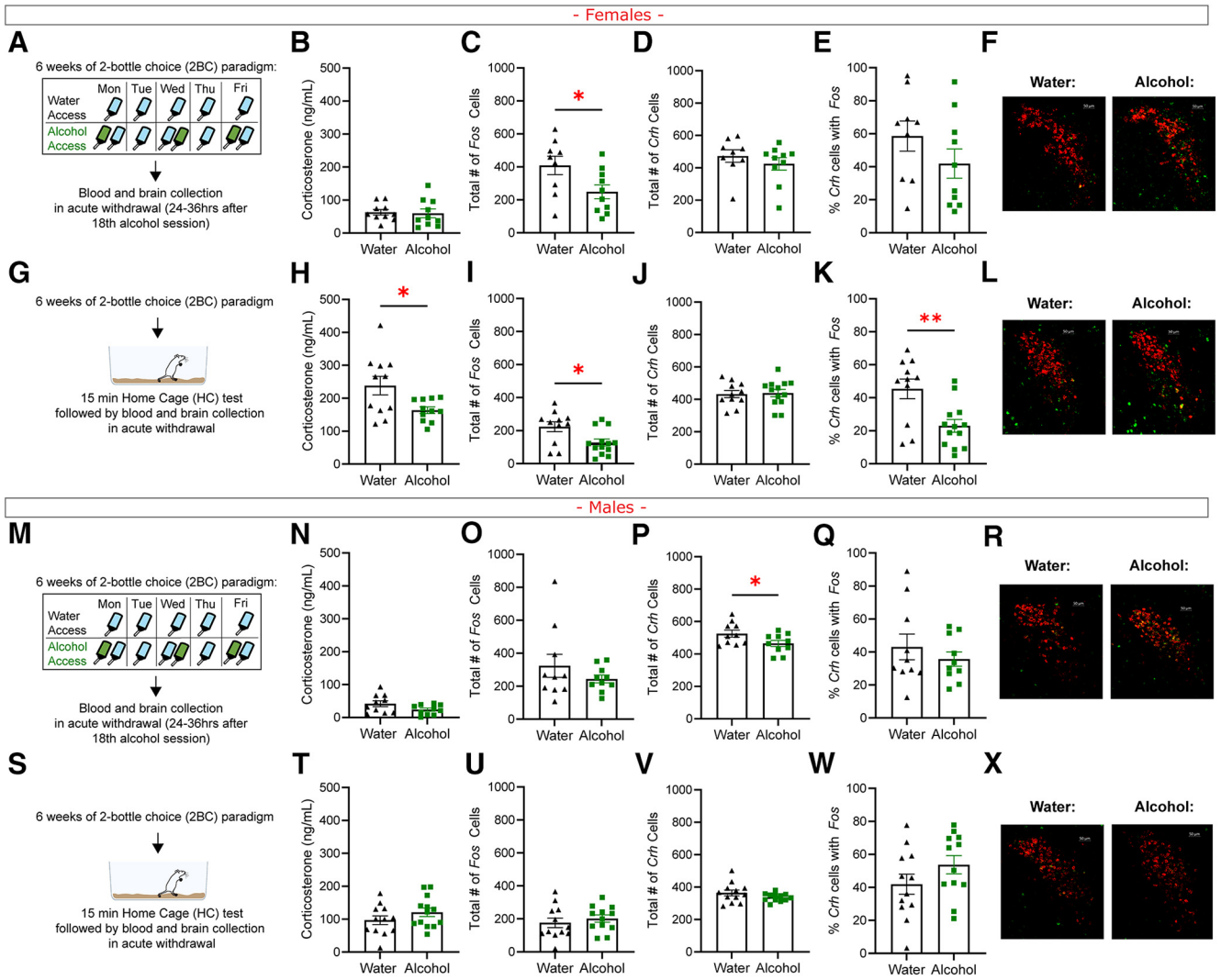

Figure 1. Acute stress in alcohol withdrawal blunts plasma corticosterone and PVN^{CRH} *Fos* mRNA expression in female alcohol history mice. In females: ***A***, Experimental design for basal measurements after 2-BC alcohol. ***B***, Corticosterone. ***C***, Total number of *Fos*-positive cells. ***D***, Total number of *Crh*-positive cells. ***E***, Percent of *Crh*-positive cells that were also positive for *Fos*. ***F***, Representative images of water (left) and alcohol (right) mice. ***G***, Experimental design for acute stress measurements after 2-BC alcohol. ***H***, Corticosterone. ***I***, Total number of *Fos*-positive cells. ***J***, Total number of *Crh*-positive cells. ***K***, Percent of *Crh*-positive cells that were also positive for *Fos*. ***L***, Representative images of water (left) and alcohol (right) mice. In males: ***M***, Experimental design for basal measurements after 2-BC alcohol. ***N***, Corticosterone. ***O***, Total number of *Fos*-positive cells. ***P***, Total number of *Crh*-positive cells. ***Q***, Percent of *Crh*-positive cells that were also positive for *Fos*. ***R***, Representative images of water (left) and alcohol (right) mice. ***S***, Experimental design for acute stress measurements after 2-BC alcohol. ***T***, Corticosterone. ***U***, Total number of *Fos*-positive cells. ***V***, Total number of *Crh*-positive cells. ***W***, Percent of *Crh*-positive cells that were also positive for *Fos*. ***X***, Representative images of water (left) and alcohol (right) mice. Data are mean ± SE. ***B,C,D,H,I,J,N,O,P,T,U,V*** student's unpaired t-test. ***E,K,Q,W*** Mann-Whitney two-tailed test. * $p < 0.05$, ** $p < 0.01$.

$t_{(8)} = 1.802$, $p = 0.109$) and sIPSC frequency (Fig. 3*V*, nested *t* test: $t_{(14)} = 2.026$, $p = 0.061$), but not in mIPSC frequency (Fig. 3*S*) or sIPSC amplitude (Fig. 3*W*).

Finally, we tested whether *in vivo* chemogenetic inhibition of PVN^{CRH} neurons during withdrawal can alter behavior. Virus placements are represented in Figure 4*B*. First, 24 h after the final alcohol session, mice were subjected to the home-cage test immediately followed by a free social interaction test (Fig. 4*A*). Female mice, regardless of alcohol history or hM4Di-mediated inhibition of PVN^{CRH} neurons, displayed similar behavioral phenotypes in the home-cage test (Fig. 4*C*). Using a two-way ANOVA, we did not find any significant main effects or interaction effect of alcohol or hM4Di in the distance moved (Fig. 4*D*), time spent rearing (Fig. 4*E*), time spent digging (Fig. 4*F*), or time spent grooming (Fig. 4*G*). Female alcohol and water exposed mice also displayed no differences in the social interaction test (Fig. 4*H*), as determined by two-way ANOVAs, in face contact (Fig. 4*I*), anogenital contact (Fig. 4*J*, main effect of hM4Di, $F_{(1,42)} = 3.439$, $p = 0.071$), allogrooming (Fig. 4*K*, main effect of hM4Di, $F_{(1,42)} = 3.267$, $p = 0.078$), or fighting (Fig. 4*L*, main effect of hM4Di, $F_{(1,42)} = 2.928$, $p = 0.094$). Male hM4di mice in the home-cage test had significantly reduced distance moved (Fig. 4*N*, two-way ANOVA main effect of hM4Di, $F_{(1,38)} = 4.546$, $p = 0.040$) regardless of alcohol history. Time spent rearing (Fig. 4*O*), digging (Fig. 4*P*), and grooming (Fig. 4*Q*) were not affected by alcohol history or virus. Interestingly, alcohol history had a significant effect on the social interaction test in males (Fig. 4*R*). Alcohol history mice, regardless of virus, spent more time in face (Fig. 4*S*, two-way ANOVA main effect of alcohol, $F_{(1,37)} = 6.150$, $p = 0.018$) and anogenital contact (Fig. 4*T*, two-way ANOVA main effect of alcohol, $F_{(1,37)} = 14.04$, $p < 0.001$) with a juvenile same sex mice than water controls. There was also a trend toward an effect of hM4Di on reduced anogenital contact (two-way ANOVA main effect of

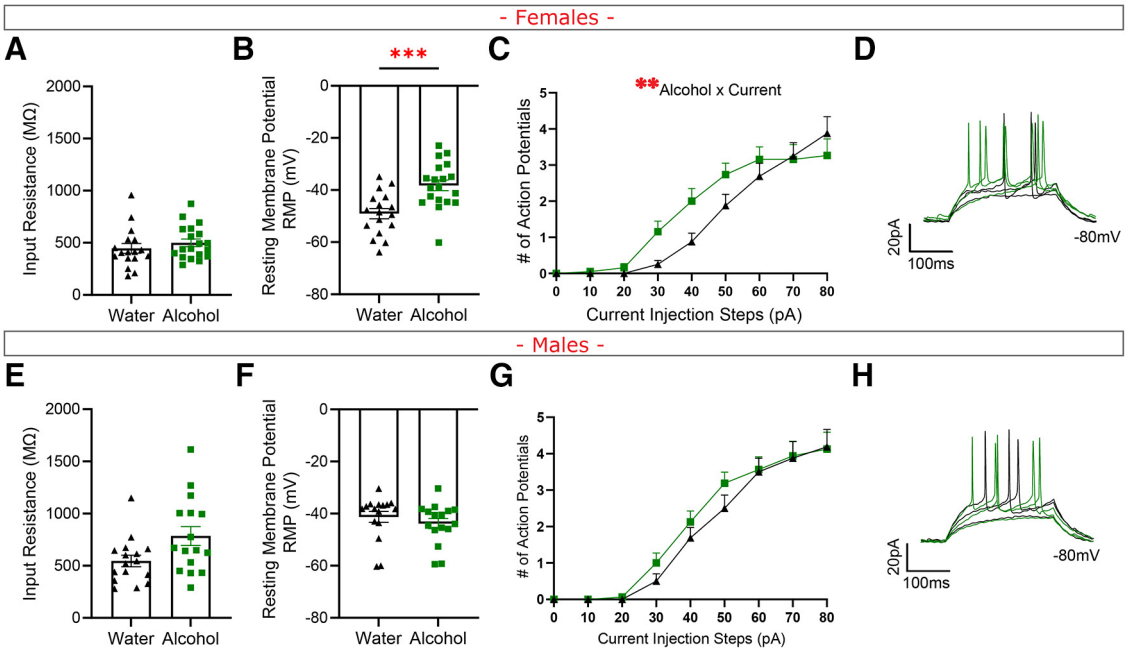

Figure 2. Chronic alcohol consumption engenders a hyperexcitable phenotype in PVN^{CRH} neurons in female alcohol withdrawal mice. In females: ***A***, Input resistance. ***B***, RMP in mV. ***C***, Current step injection plot. ***D***, Representative 30-50 pA current steps in current step injection plot. In males: ***E***, Input resistance. ***F***, RMP in mV. ***G***, Current step injection plot. ***H***, Representative 30-50 pA current steps in current step injection plot. Data are mean ± SE. ***A***, ***B***, ***E***, ***F***, nested *t*-test. ***C***, ***G***, Two-way repeated-measures ANOVA. $^{**}p < 0.01$. $^{***}p < 0.001$.

hM4Di, $F_{(1,37)} = 3.899$, $p = 0.056$). Allogrooming (Fig. 4*U*) and time spent fighting were not affected by alcohol or virus in male mice (Fig. 4*V*).

We had previously shown (Neira et al., 2022) that female mice in 6-8 h acute withdrawal had increased escape behaviors in the looming disk test; therefore, we hypothesized that inhibiting PVN^{CRH} neurons will decrease active escape behaviors, matching what was seen in water history female mice. First, we established that increased escape responses compared with mCherry water controls in 5 d alcohol withdrawal mCherry mice were also seen in the looming disk test (Fig. 5*A*). Indeed, χ^2 analysis between mCherry water and alcohol mice during Escape, Freeze, or neither Escape/Freeze behavioral responses to loom revealed significant differences in behavior (Fig 5*B*, χ^2 (1, N =130) = 11.3502, $p = 0.003$), with alcohol mCherry mice displaying greater escape responses. Importantly, hM4Di water and alcohol history female mice had near identical reactions to the looming disk test, as indicated by the nonsignificant χ^2 test (Fig 5*B*, χ^2 (1, N =100) = 0.080, $p = 0.961$). Unlike females, male mCherry alcohol history mice and water controls did not differ in their response to loom presentations (Fig. 5*C*), similar to what we had previously shown (Neira et al., 2022). Importantly, hM4Di water and alcohol history male mice also showed similar responses to the looming disk task (Fig. 5*C*).

Discussion

In this study, we find that alcohol-induced hyperactivity of PVN^{CRH} neurons drives heightened active stress coping in female mice in acute withdrawal, and inhibition of these neurons is sufficient to restore these stress coping behaviors to control levels. Specifically, using FISH, we find that *Fos* mRNA in PVN^{CRH} neurons is unaltered in basal conditions (Fig. 1*E*) but blunted in response to a mild acute stressor in female mice (Fig. 1*K*). In accordance, plasma corticosterone levels were only blunted in female alcohol history mice exposed to an acute stressor (Fig. 1*H*). Despite *Fos* mRNA reductions in PVN^{CRH} neurons during acute withdrawal, slice electrophysiology experiments demonstrated these cells are in a hyperactive state, as measured through intrinsic excitability and synaptic transmission experiments (Figs. 2 and 3). Male mice had no alterations to *Fos* mRNA and only showed increased PVN^{CRH} glutamatergic transmission. Female, not male, alcohol history mice in acute 6-8 h withdrawal from chronic alcohol consumption show greater active escape behaviors to the looming disk predator-like test compared with water controls (Neira et al., 2022), and investigations into the role of PVN^{CRH} neurons in this test indicate that active escape behavior requires an increase in PVN^{CRH} activity (Daviu et al., 2020). Thus, in line with previous research and our findings indicating hyperactivity of PVN^{CRH} in female mice, we show that *in vivo* chemogenetic inhibition of these neurons decreases active escape behavior to the looming disk test in female alcohol history mice to the levels observed in water controls (Fig. 5).

Together, our plasma corticosterone and *Fos* activation studies, and our behavioral manipulations and electrophysiological studies, align with each other but not across these measurements. Perhaps this indicates an uncoupling of the mechanisms for neuroendocrine HPA-axis activation and central PVN^{CRH} neuronal activity, such that PVN^{CRH} neurons regulate CRH release into the periphery differently than glutamate release centrally. Indeed, many recent studies have found that these neurons are involved in various stress-related behaviors independent of their neuroendocrine function (Füzesi et al., 2016; Kim et al., 2019; Yuan et al., 2019; Daviu et al., 2020). Additionally, slice electrophysiology is a direct tool that can be used to measure the activity of a neuron, while *Fos* induction can also be a response to intracellular signaling mechanisms and as such does not always correlate with neuronal depolarization, making it a more blunt tool for interpreting neuronal activation (Hoffman et al., 1993; Hoffman and Lyo, 2002; Joo et al., 2016). Alternatively, this finding could be indicative of distinct populations of PVN^{CRH} neurons with neuroendocrine and central functions (Bains et al., 2015; Lameu et al., 2023).

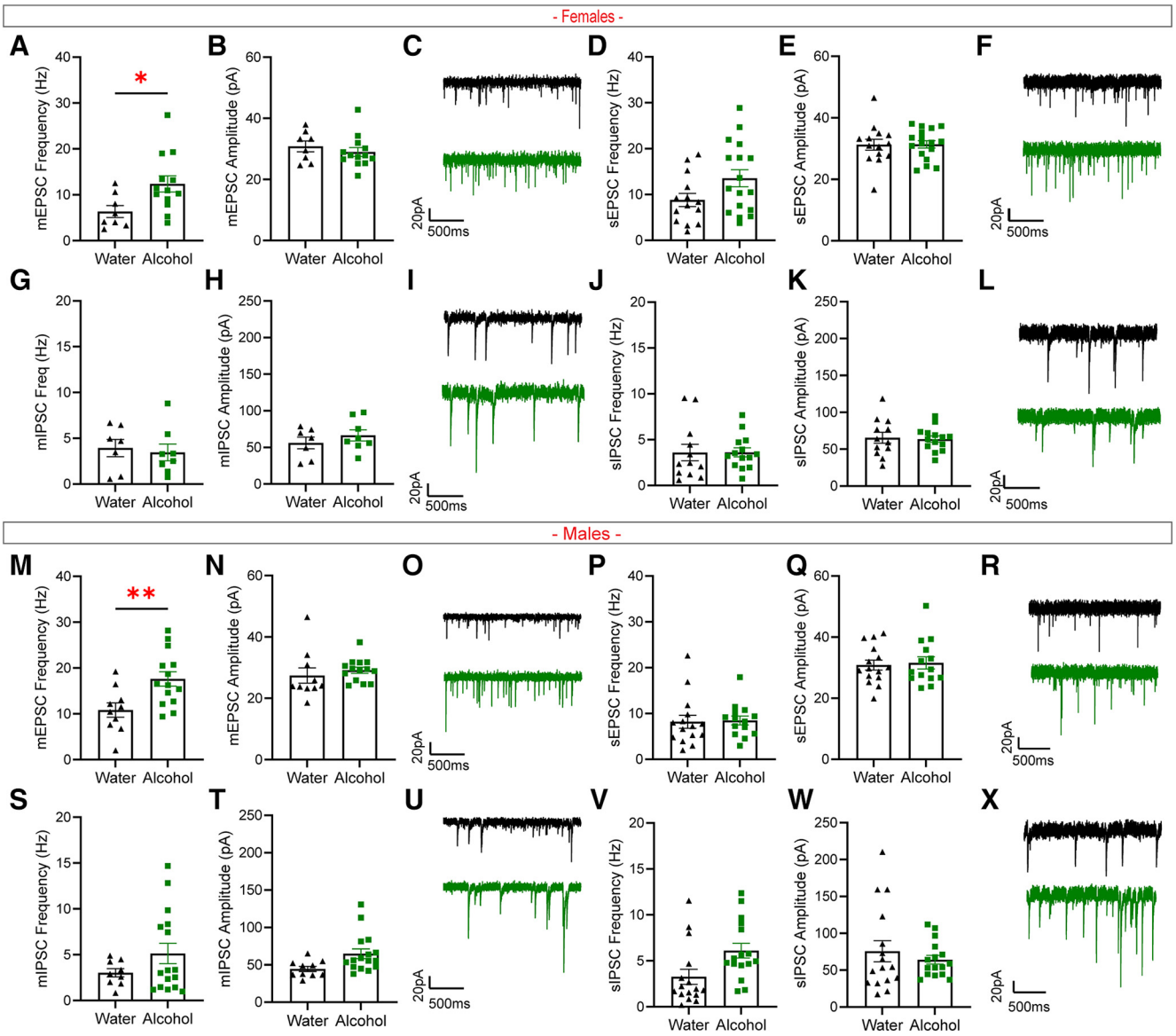

Figure 3. A history of alcohol increases glutamatergic synaptic transmission in female and male alcohol withdrawal mice. In females: ***A***, mEPSC frequency. ***B***, mEPSC amplitude. ***C***, Representative traces for water (black) and alcohol (green) mEPSCs. ***D***, sEPSC frequency. ***E***, sEPSC amplitude. ***F***, sEPSC amplitude. Representative traces for water (black) and alcohol (green) sEPSCs. ***G***, mIPSC frequency. ***H***, mIPSC amplitude. ***I***, Representative traces for water (black) and alcohol (green) mIPSCs. ***J***, sIPSC frequency. ***K***, sIPSC amplitude. ***L***, Representative traces for water (black) and alcohol (green) sIPSCs. In males: ***M***, mEPSC frequency. ***N***, mEPSC amplitude. ***O***, Representative traces for water (black) and alcohol (green) mEPSCs. ***P***, sEPSC frequency. ***Q***, sEPSC amplitude. ***R***, Representative traces for water (black) and alcohol (green) sEPSCs. ***S***, mIPSC frequency. Data are mean ± SE. ***T***, mIPSC amplitude. ***U***, Representative traces for water (black) and alcohol (green) mIPSCs. ***V***, sIPSC frequency. ***W***, sIPSC amplitude. ***X***, Representative traces for water (black) and alcohol (green) sIPSCs. *$p < 0.05$; **$p < 0.01$; nested *t*-test.

Plasma corticosterone and FISH in the PVN

To our knowledge, this is the first study to report *Fos* induction in PVN^{CRH} neurons after voluntary drinking in male and female rodents. Of interest, basal levels of *Fos* activation in PVN^{CRH} neurons were highly varied in both females and males. It was intriguing to note that, despite measures to minimize stress and handling, mice had a wide range of *Fos* activation in *Crh* neurons (Fig. 1*E*,*Q*). This variability was also present in our mild acute stress conditions for males (Fig. 1*W*) and water female mice (Fig. 1*K*), while alcohol female mice did not appear to mount a significant stress response, as *Fos* (Fig. 1*K*) and plasma corticosterone levels were blunted (Fig. 1*H*). While our mild stressor only involved transfer out of the animal facility and filming of natural behavior patterns in their home-cage, similar findings with blunting of c-fos response are seen in rodents after chronic stress conditions (Armario et al., 2004; Borrow et al., 2019; Walker et al., 2019). These mice were single housed for extended periods, which is itself a chronic stressor that can affect PVN^{CRH} neurons, particularly in females. It is possible that chronic alcohol, withdrawal episodes, and social isolation all contributed to a chronic stress phenotype in alcohol history mice. Experiments to delineate the contributions of each of these will be important to further understand alcohol-induced changes in stress function.

Slice *ex vivo* electrophysiology in PVN^{CRH} neurons

Many studies have shown that chronic stress induces a hyperexcitable state in PVN^{CRH} neurons (Flak et al., 2009; Stephens and Wand, 2012; Franco et al., 2016). Acute alcohol acts as a stressor and induces the HPA-axis system, and withdrawal itself is also a stressor on the body. Thus, chronic alcohol consumption with repeated withdrawals is likely acting as a chronic stressor and inducing a hyperactive condition in PVN^{CRH} neurons. Our

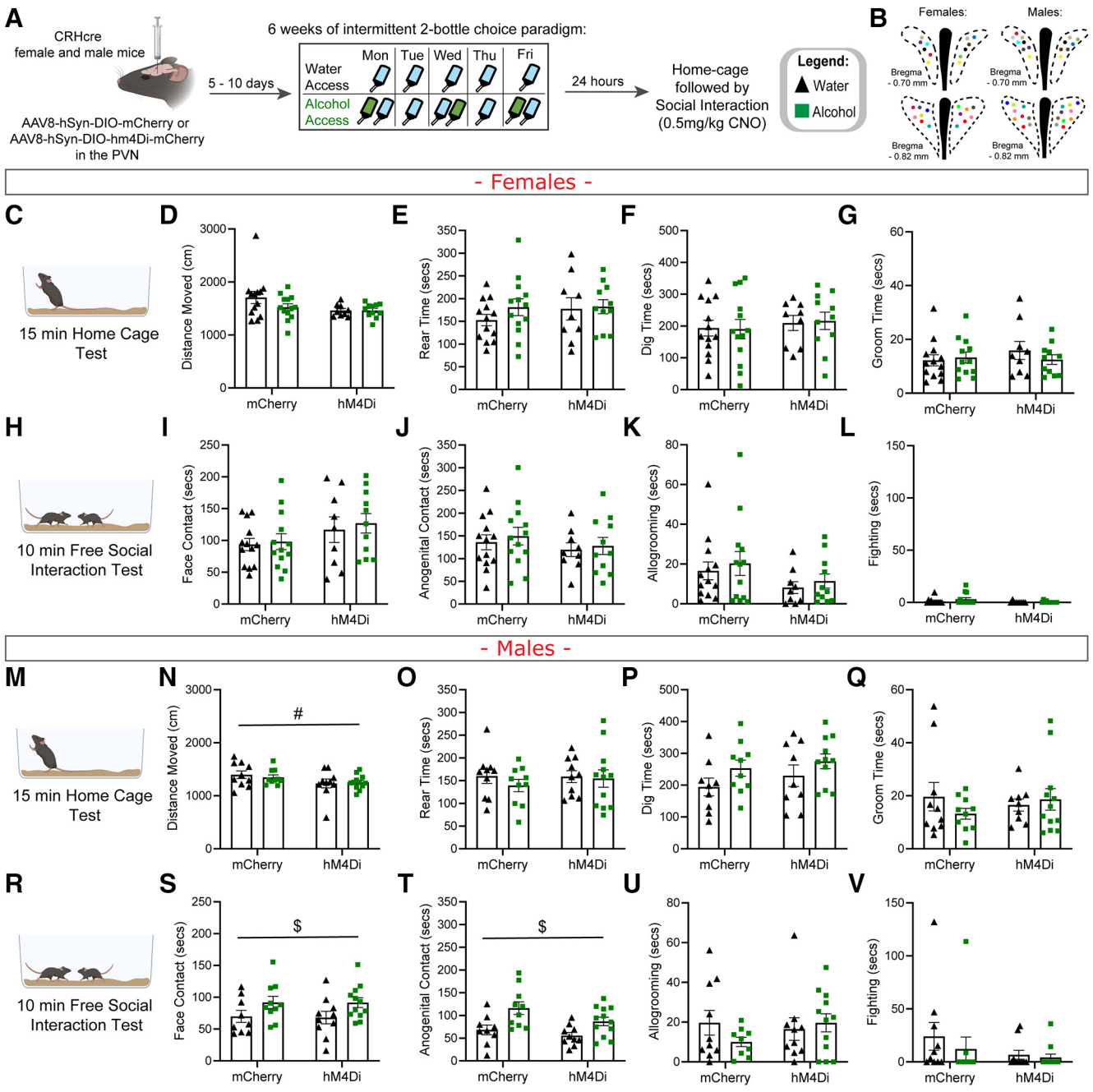

Figure 4. hM4Di-mediated chemogenetic inhibition of PVN^{CRH} neurons did not affect mouse responses to the home-cage or free social interaction test in acute withdrawal from chronic alcohol. ***A***, Experimental design for female and male CRH-cre mice. ***B***, Virus placements. Each color represents 1 mouse. In females: ***C***, Fifteen minute home-cage test. ***D***, Distance moved. ***E***, Rear time. ***F***, Dig time. ***G***, Groom time. ***H***, Ten minute free social interaction test. ***I***, Face contact. ***J***, Anogenital contact. ***K***, Allogrooming. ***L***, Fighting. In males: ***M***, Fifteen minute home-cage test. ***N***, Distance moved. ***O***, Rear time. ***P***, Dig time. ***Q***, Groom time. ***R***, Ten minute free social interaction test. ***S***, Face contact. ***T***, Anogenital contact. ***U***, Allogrooming. ***V***, Fighting. Data are mean ± SE. Two-way repeated measures ANOVA. $^{\#}$Main effect of hM4Di virus. $^{\$}$Main effect of alcohol history.

electrophysiological data support this hypothesis, particularly in female mice, as they display both increased intrinsic excitability (Fig. 2) and increased glutamatergic synaptic transmission (Fig. 3). Interestingly, male alcohol history mice also show increased glutamatergic transmission but do not display increased intrinsic excitability. Males drink less alcohol in 2-BC procedures than females (Neira et al., 2022), which might account for the lack of some effects and justifies an investigation with alcohol vapor to normalize total alcohol levels. Notably, we did observe some differences between measures of spontaneous and miniature transmission in our experiments. These could be because of multiple reasons. For example, the difference in spontaneous and mEPSC frequency could be because of alterations in action potential-dependent release of other signaling molecules, such as peptides.

Overall, these findings affirm that PVN^{CRH} neurons are impacted by acute withdrawal following chronic alcohol consumption, but the mechanisms behind these changes are still to be determined. Studies that probe AMPA/NMDA ratio as well as plasticity in these neurons will be highly informative, as both stress and alcohol alter this in PVN^{CRH} neurons (Kuzmiski et al., 2010; Bains et al., 2015; Marty et al., 2020).

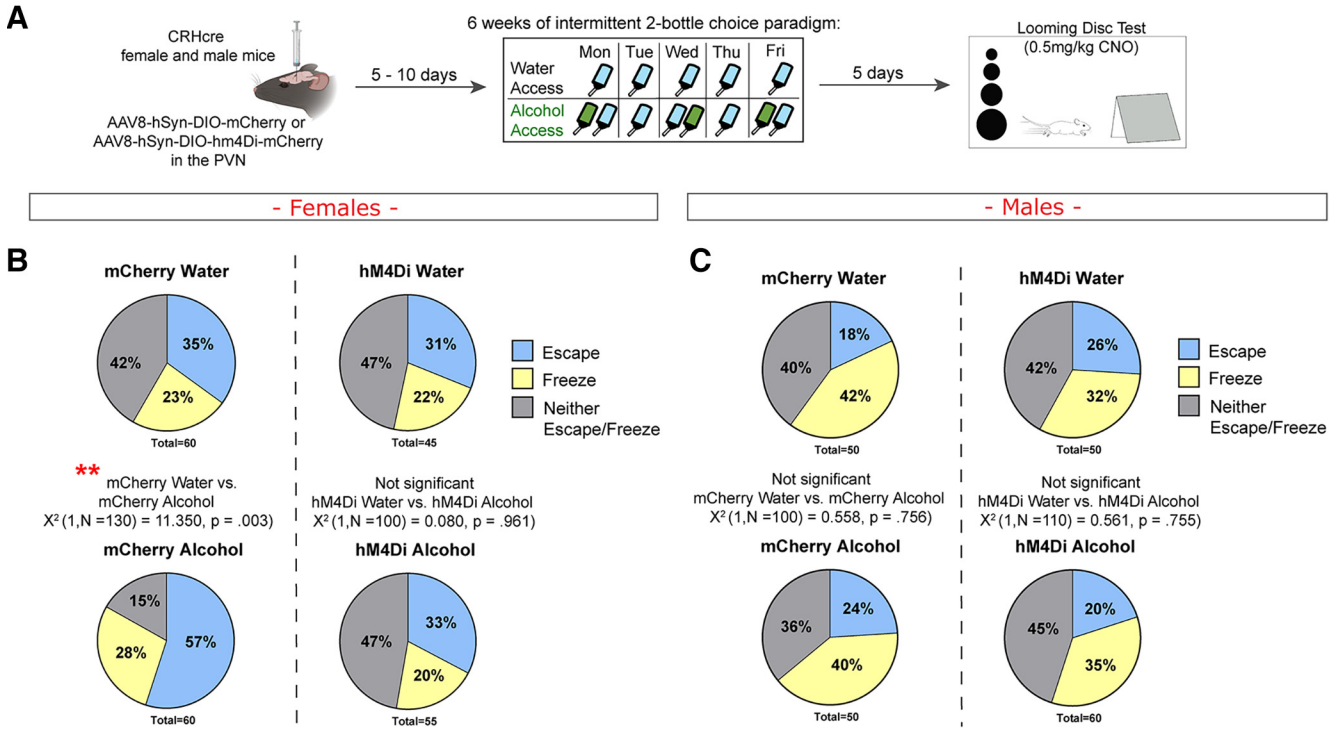

Figure 5. hM4Di-mediated chemogenetic inhibition of PVN^{CRH} neurons reduces looming disk active escape responses in female alcohol history mice during withdrawal. ***A***, Experimental design. ***B***, Female escape (blue), freeze (yellow), or neither escape nor freeze (gray) response to loom presentation. ***C***, Male escape (blue), freeze (yellow), or neither escape nor freeze (gray) response to loom presentation. Data are percent escape, freeze, or neither escape/freeze of total looming disc trials. $^{**}p < 0.01$ (χ^2).

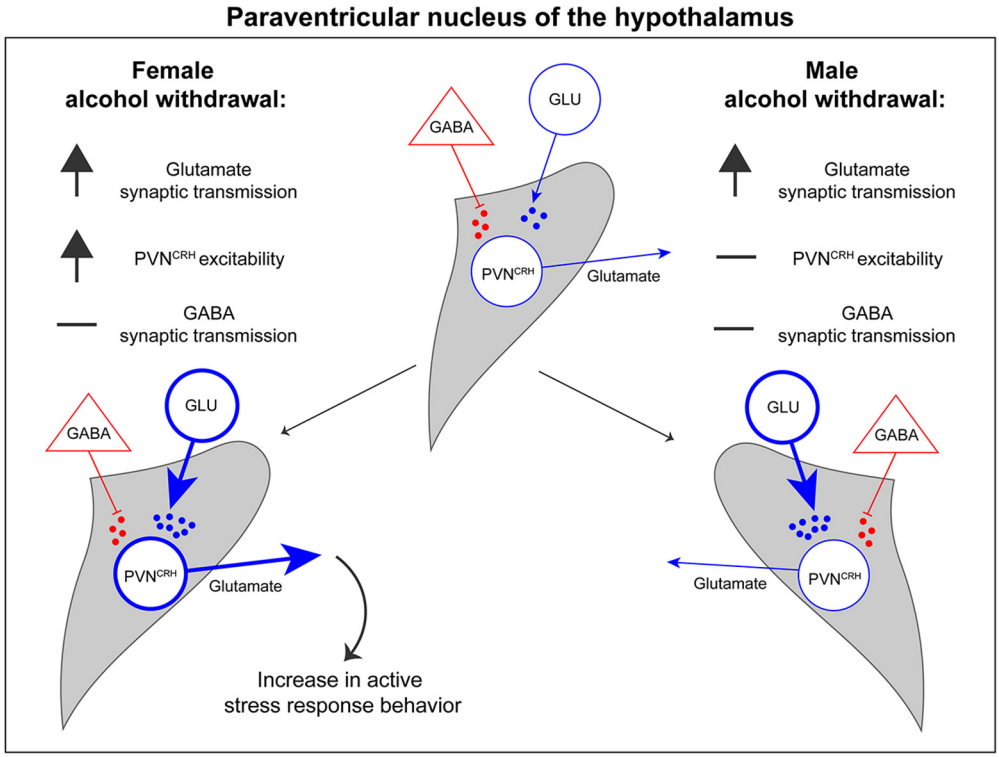

Figure 6. Working model of PVN^{CRH} neuron alterations in female and male mice. Summary of findings in females (left) and males (right) in withdrawal from a chronic history of alcohol consumption.

hM4Di chemogenetic inhibition of PVN^{CRH} neurons

In support of the hypothesis that females are more sensitive to PVN^{CRH} alterations from alcohol, we show that female alcohol history mice display greater active response in the looming disk test, and that chemogenetic inhibition decreases active escape in hM4Di female mice, returning them to levels similar to those observed in water controls (Fig. 5). Optogenetic manipulations of PVN^{CRH} neurons and acute alcohol withdrawal also alter

spontaneous behaviors, such as rearing and grooming (Füzesi et al., 2016; Yuan et al., 2019; Neira et al., 2022). Thus, we expected to find alterations to spontaneous behaviors in the home-cage test during chemogenetic inhibition of PVN^{CRH} neurons. In contrast, we did not see any changes to these behaviors in a home-cage (Fig. 4*C–G*,*M–Q*). This might be because of the relatively low dose of CNO that was used in these studies that perhaps did not override natural behavior patterns like optogenetics. Lack of alcohol history driven effects in the home-cage test might be because of the stress of the CNO injection given before the test. Indeed, the behaviors displayed by the mice were similar to those displayed by acute restraint stress mice in our previously published manuscript (Neira et al., 2022).

Immediately after the home-cage test, we conducted a free social interaction test (Fig. 4*H–L*,*R–V*). Interestingly, while we saw no effects of PVN^{CRH} inhibition on female or male mice, male alcohol history mice spent significantly more time interacting with a juvenile mouse than water controls (Fig. 4*S*,*T*). Increased social seeking behaviors in male alcohol history mice are unlikely to indicate increased aversive states in sociability. PVN^{CRH} neurons have been studied in relation to the social communication of aversive states in rodents (Sterley et al., 2018), which may explain why chemogenetic inhibition in this particular task did not affect behavior. Perhaps an investigation into other facets of social behavior may be more informative regarding the role of these neurons in social behavior after alcohol history.

Overall, because manipulations of PVN^{CRH} neurons might alter behavior in context and dose-dependent manners, follow-up experiments using higher doses of CNO, chemogenetically activating these neurons, and using optogenetics will be highly informative to understand the role PVN^{CRH} neurons play in alcohol-induced stress behaviors.

In conclusion, as summarized in our working model in Figure 6, alcohol history mice demonstrate significant alterations to PVN^{CRH} neurons in withdrawal, with females indicating more expansive alterations to these neurons and stress-related behaviors that involve the activity of these neurons. Perhaps this is because of the nature of voluntary intake in this study which leads to lower alcohol consumption in males than females, or to the social isolation that is required for 2-BC experiments that affects females greatly (Cacioppo et al., 2011; Senst et al., 2016). Alternatively, females, who already have a more active stress system in response to multiple stressors (Silva et al., 2009; Palanza and Parmigiani, 2017; Bangasser et al., 2018; Peltier et al., 2019), could be more sensitive to alcohol-induced changes in PVN^{CRH} neurons and thus suffer from the consequences of heighted activity of these neurons. This could impact their ability to cope with stressors, which might play a role in reports of women drinking for stress relief rather than the rewarding effects of alcohol consumption (Pollard et al., 2020; Rodriguez et al., 2020).

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