1   Predator odor stress produces sex- and subpopulation-specific increases in alcohol drinking,

2   anxiety-like behavior, and lateral hypothalamic *crh* expression

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24 **Highlights**

25 • Male Avoider rats show elevated two-bottle choice alcohol drinking after predator odor

26 stress

27 • More avoidant males show more aversion-resistant alcohol drinking

28 • Female Avoider rats show heightened anxiety-like behavior 4 weeks after stress

29 • Low frequency USVs predict Non-Avoider behavior in male rats

30 • *Crh* expression in the LH is correlated with avoidance and alcohol drinking in male rats

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Abstract

Traumatic stress leads to maladaptive avoidance behaviors and alcohol misuse in some people. In rats, predator odor ("traumatic") stress produces persistent avoidance of stress-paired contexts and escalated alcohol self-administration in some animals (Avoiders), but not others (Non-Avoiders). This mirrors the individual differences in stress responsivity and alcohol misuse seen in humans. Here, we used a quinine-adulterated alcohol drinking procedure to model compulsive-like alcohol drinking in humans. Male and female Wistar rats were given 12 weeks of intermittent access to 20% (v/v) alcohol, followed by three weeks of limited access. Rats were then indexed for avoidance using predator odor stress exposure, and limited access drinking resumed for three additional weeks after stress. During this period, the alcohol solution was adulterated twice weekly with increasing concentrations of quinine. More Avoidant males were more resistant to quinine adulteration and Avoider males increased in non-quinine alcohol drinking. Using ultrasonic vocalizations (USVs) as a measure of affective state, we found that Non-Avoider males emitted more lower frequency USVs (<32 kHz) preceding, during, and following predator odor stress. Finally, quantification of *crh, crhr1, crhr2, crhbp* gene expression in the lateral hypothalamus revealed a strong positive correlation between greater *crh* transcripts and avoidance in males and a positive correlation between *crh* transcripts and less anxiety-like behaviors in females. Together, these results suggest that the intersection of stress and compulsive-like alcohol drinking is sex-specific and dependent on individual differences in stress outcomes. This work reinforces the importance of considering sex differences in stress and alcohol use disorder research.

1. Introduction

Living through a traumatic event leads to post-traumatic stress disorder (PTSD) in some individuals but not others (Goldstein et al., 2016). Key symptoms of PTSD include avoidance of trauma-related stimuli and increased negative affect after trauma (American Psychiatric Association, 2013). Individuals with PTSD are more likely to develop alcohol use disorder (AUD) (Straus et al., 2018), with about one-third of people diagnosed with PTSD at some point in their lives also meeting criteria for AUD (Blanco et al., 2013). Further, studies show that individuals with greater PTSD symptom severity consume more alcohol and are more likely to report that they drink to cope with negative symptoms (Lehavot et al., 2014; Ranney et al., 2021). While PTSD rates are higher in women after traumatic events (Goldstein et al., 2016). rates of AUD are generally greater in men (Erol & Karpyak, 2015), but this gap appears to be narrowing (Guinle & Sinha, 2020). The prevalence of comorbid AUD and PTSD across sex is nuanced, warranting further study (Gilpin & Weiner, 2017; Saraiya et al., 2023). Regardless, consensus falls on the idea that individuals with PTSD are particularly vulnerable to AUD due to stress-induced alterations in affective neural circuitry that increase negative affect and drive alcohol drinking via negative reinforcement (Koob, 2013a, 2013b, 2014; Kwako, Schwandt, et al., 2015; Straus et al., 2018). Animal models are necessary to explore the neurobiology of these disorders with consideration of potential sex differences.

In lab rats, some aspects of PTSD symptomatology and individual differences in stress responsivity are recapitulated via a predator odor stress model. In this paradigm, a single, inescapable exposure to bobcat urine produces persistent avoidance of the odor-paired context and prolonged increases in anxiety-like behavior in a subset of outbred Wistar rats, termed "Avoiders" (Edwards et al., 2013; Whitaker & Gilpin, 2015; Schreiber et al., 2017; Albrechet-Souza & Gilpin,

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93   2019; Albrechet-Souza et al., 2020; Weera et al., 2023). All predator odor-exposed animals show

94   evidence of acute stress, such as elevated serum corticosterone and ACTH in the hours after

95   predator odor exposure, blunted body weight gain, and heightened anxiety-like behavior over a

96   period of several days after stress (Whitaker & Gilpin, 2015; Weera et al., 2020). However, only

97   Avoiders show escalated alcohol self-administration (SA) (Edwards et al., 2013; Whitaker &

98   Gilpin, 2015; Schreiber et al., 2017; Weera et al., 2020, 2023) and hyperalgesia after stress (Itoga

99   et al., 2016). The heightened anxiety-like behavior after stress also appears to be more persistent

100   in Avoider rats, lasting at least up to 9 days post-stress (Weera et al., 2023). Studies also show that

101   Avoider or Non-Avoider status remains stable over weeks after stress and is not altered by repeated

102   odor exposure (Schreiber et al., 2017; Weera et al., 2020). Importantly, we highlight that most of

103   this work was performed in male rats, although more recent studies point towards similar

104   behavioral phenotypes in female rats following predator odor stress (Albrechet-Souza et al., 2020;

105   Weera et al., 2023).

106   Neurocircuit studies using this animal model implicate the lateral hypothalamus (LH) as

107   an important node downstream of the amygdala for mediating Avoider-related stress responses.

108   For instance, activity of inputs into the LH from the central amygdala (CeA) is required for

109   supporting avoidance of a stress-paired context (Weera et al., 2021), and inhibition of CeA-to-LH

110   inputs that express corticotropin-releasing factor (CRF) type-1 receptors (CRF1) is sufficient for

111   normalizing the escalated alcohol self-administration and anxiety-like behavior in male and female

112   Avoider rats. Within the LH, antagonism of CRF1 reduces binge-like alcohol drinking in male

113   mice (Bendrath et al., 2025) and rescues heightened anxiety-like behavior after acute stress

114   (Eghtesad et al., 2022). Together, these findings implicate the CRF neuropeptide system within the

115   LH in driving post-stress alcohol drinking and anxiety-related behaviors.

116   The main goal of this study was to test the hypothesis that both male and female Avoider

117   rats will show increased free-choice alcohol drinking after stress. In addition to free-choice alcohol

118   drinking, we also tested Avoider, Non-Avoider, and unstressed Control rats' consumption of

119   alcohol solutions that were adulterated with an aversive stimulus (i.e., quinine). Humans with AUD

120   typically show sustained alcohol consumption despite negative consequences (also called

121   'compulsive-like' alcohol drinking) (Sinha, 2009; Koob & Volkow, 2010; Vendruscolo et al., 2012;

122   American Psychiatric Association, 2013), and the quinine-adulterated alcohol drinking procedure

123   is a model of aversion-resistant drinking (Simms et al., 2008; De Oliveira Sergio et al., 2023).

124   Given that alcohol drinking may be driven by negative emotional states, we tested the enduring

125   nature of stress-induced anxiety-like behavior in Avoider and Non-Avoider rats, weeks after

126   predator odor exposure, as well as their emotional states before stress, during stress, and during

127   expression of avoidance or non-avoidance of stress-paired contexts using ultrasonic vocalizations

128   (USVs) as a proxy. Finally, we quantified levels of *crh, crhr1, crhr2,* and *crhbp* in the LH. The

129   findings here contribute to the growing understanding of subpopulation- and sex-specific effects

130   of stress.

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132   **2. Methods**

133   **2.1 Animals**

134   Adult (8 weeks old) male and female Wistar rats (Charles River, Kingston, NY) were

135   single-housed in a temperature- and humidity-controlled vivarium (22°C) on a 12-hour reversed

136   dark/light cycle (lights off at 9 am EST). Rats were provided with tap water and chow (Teklad

137   2919) *ad libitum.* All rats were handled and weighed at least once each week during the duration

138 of the experiments. Behavioral tests occurred during the dark period. All experiments were

139 approved by the Tufts University Institutional Animal Care and Use Committee (IACUC).

2.2 Predator Odor Conditioned Place Aversion

141 All rats underwent a 4-day predator odor conditioned place aversion (CPA) procedure as

142 previously described (Edwards et al., 2013; Weera et al., 2023). Briefly, on the first day, rats

143 explored a three-chamber arena with a center zone and two chambers with distinct tactile (mesh

144 vs. holes) and visual (stripes vs. dots) stimuli for 5 minutes in a dimly lit room (maximum light 13

145 lumens; Pretest). Duration spent in each chamber was recorded by experimenters blinded to rat

146 identities. Placement of three paws inside of striped or dotted chambers was required for an entry

147 to be counted. On the second day, individual rats were confined to either the stripes or dots chamber

148 for 15 minutes (neutral chamber). On the third day, individual rats were confined to the other

149 chamber with a bobcat urine-soaked paper towel (or nothing for unstressed controls) for 15

150 minutes (odor chamber). On the final day, rats were again allowed to explore the entire arena for

151 5 minutes (Posttest). The change in duration spent in the odor-paired chamber from Pretest to

152 Posttest was recorded for odor-exposed (stressed) rats. Stressed rats with a decrease in time spent

153 in the odor-paired chamber that was greater than 10 seconds were classified as "Avoiders." While

154 other stressed rats were designated "Non-Avoiders" (Edwards et al., 2013; Albrechet-Souza &

155 Gilpin, 2019). Non-odor exposed animals were considered unstressed Controls. The behavioral

156 apparatus was cleaned with chlorine dioxide between each animal on all days, and with soap,

157 water, and an enzyme-based odor remover (PureAyre Odor Eliminator, Clean Earth Inc., Kent,

158 WA) at the end of predator odor exposure.

2.3 Ultrasonic Vocalization Recording and Analysis

160   During all days of predator odor place conditioning, ultrasonic vocalizations (USVs) were

161   recorded from the actively behaving rat. An Avisoft Bioacoustics Condenser Microphone

162   (CM16/CMPA, Avisoft Bioacoustics UltraSoundGate Model 116Hb) was suspended 18 inches

163   above the center of the place conditioning apparatus and Avisoft RECORDER software was used

164   to monitor recording. Audio files were processed in DeepSqueak using the built-in Long Rat and

165   Rat neural networks to generate call detection files which were manually edited for vocalization

166   identification accuracy by a team of analysts blinded to rat condition (Coffey et al., 2019). Data

167   files were analyzed for quantity of calls as well as number of calls with the average principal

168   frequency of less than 32 kHz which is the maximum range for vocalizations typically emitted in

169   aversive situations (Brudzynski, 2001; Burgdorf et al., 2008; Takahashi et al., 2010). For the 15-

170   minute odor day, only the first 5 minutes of the audio file was analyzed to allow comparison to the

171   5-minute pre- and post-stress sessions.

172   **2.4 Alcohol Drinking**

173   Procedures were adapted from published studies (Simms et al., 2008; De Oliveira Sergio

174   et al., 2023). Sixty rats (30 male, 30 female) were single housed to accurately measure free-choice

175   water and alcohol consumption in each rat. Rats were given 12 weeks of Intermittent Access two-

176   bottle choice alcohol (20% v/v) drinking on a Monday, Wednesday, Friday schedule, followed by

177   3 weeks of Limited Access (20-minute) two-bottle choice drinking on a Monday to Friday

178   schedule. On the third week of Limited Access drinking, the alcohol solution was adulterated with

179   10 mg/L quinine monohydrochloride dihydrate (Sigma-Aldrich) on Tuesday and Thursday. Next,

180   rats were counterbalanced into Stress and unstressed Control groups based on average

181   consumption of quinine adulterated alcohol (i.e., Quinine Baseline) and subjected to the 4-day

182   (Monday-Thursday) predator odor CPA procedure described above (2.2). Limited Access alcohol

183 drinking resumed on the next day (Friday) and for three additional weeks on a Monday-Friday

184 schedule. Across these 3 weeks, the alcohol solution was adulterated with increasing

185 concentrations of quinine (10 mg/L, 60 mg/L, 100 mg/L) twice each week (Figure 1A).

2.5 Behavioral Assays Following Alcohol Drinking

187 Rats were subjected to a 5-minute elevated plus maze (EPM) test in a dim (maximum 40

188 lumens in open arms), novel room 5-6 days after the final drinking session. Rats were introduced

189 to the center zone of the EPM and allowed to explore undisturbed. Behavior was recorded by an

190 overhead video camera and processed using Ethovision XT (Noldus Information Technology,

191 v17.5) to score time in open arms, entries into each zone, and locomotion. Hand scoring for stretch

192 attempts was conducted by an independent experimenter blinded to rat condition. Criteria for

193 stretch attempts included entry of the front two paws into the open arms followed by retreat.

2.6 Quantitative PCR (qPCR)

195 Four weeks following predator odor stress, rats were sacrificed, and brains were snap

196 frozen in 2-methyl butane and stored at -80˚C until dissection. Bilateral punches (1 mm diameter,

197 0.5mm depth) of the lateral hypothalamus (LH) were collected at +2.2 A/P, ±2.0 M/L, -8.8 D/V

198 from Bregma according to the atlas of Paxinos and Watson. for Quantitative PCR (qPCR) across

199 all rats and stored at -80˚C. RNA was extracted using the Qiagen RNeasy RNA extraction kit

200 (Hilden, Germany) according to manufacturer's instructions.

201 RTqPCR: Complementary DNA (cDNA) was synthesized using the RETROscript kit from

202 Applied Biosystems (Carlsbad, CA, USA). PCR was performed using a Quant3 (Fisher Scientific)

203 under standard amplification conditions: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C

204 and 60 s at 60 °C. All PCR primers were TaqMan® Gene Expression Assays purchased from

205 Applied Biosystems. The amplification efficiency of each of these assays has been validated by

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206 Applied Biosystems and averages 100% (± 10). Assay ID and accession numbers were as follows:

207 *gapdh:* Rn01775763_g1*; crh:* Rn01462137_m1*; crhbp:* Rn00594854_m1*; crhr1:*

208 Rn00578611_m1; *crhr2:* Rn00575617_m1. Final quantification of mRNA was obtained using the

209 comparative cycle threshold (CT) method (User Bulletin #2, Applied Biosystems). Data are

210 reported as fold change in transcription relative to a calibrator cDNA. In brief, the housekeeping

211 gene (*gapdh*) was used as an internal control against which each target signal was normalized

212 (ΔCT). Validation studies confirmed that the raw CT values of *gapdh* did not vary by treatment

213 group (Supplemental Figure S3A). The ΔCT was then normalized against a calibrator (i.e. the

214 mean of the control male group for the target gene) to provide the ΔΔCT relative to the control

215 group. Finally, data are transformed $2^{\wedge}$ ΔΔCT and expressed as fold change.

216 **2.7 Statistical Analysis**

217 All data were analyzed using omnibus ANOVAs with Sex and Stress Group as between-

218 subjects factors, and Day as a within-subjects factor for alcohol drinking. Significant main effects

219 were followed up with lower order ANOVAs and Tukey's post hoc tests. Correlations were

220 performed using simple linear regression analyses. Alpha was set at 0.05. Outliers were determined

221 using Grubbs Outlier Test (≤1/group). Analysis was performed in Prism 10 (GraphPad) and SPSS

222 Statistics (IBM). Data are shown as Mean ± SEM.

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224 **3. Results**

225 **3.1 Male Avoider rats show elevated free-choice alcohol drinking**

226 All rats had two-bottle choice intermittent access to 20% (v/v) alcohol in the home cage

227 for 12 weeks (Intermittent Access) followed by two weeks of Limited Access and one week of

228 Quinine Baseline (Figure 1A). Alcohol intake during Intermittent Access, 24-hour drinking

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229   sessions was similar between sexes and between animals that were subsequently counterbalanced

230   into Stress or unstressed Control groups (Supplemental Figure S1A). In the Limited Access phase

231   in which rats had alcohol access for 20 min 5x/week, alcohol intake increased over two weeks

232   (Repeated Measures Day x Sex ANOVA, significant main effect of Day, $F_{(4.193, 243.2)} = 10.09$,

233   $p<0.0001$) and stabilized (Supplemental Figure S1B). A mixed-effects analysis (Sex x Day) of

234   percent preference for alcohol (volume of alcohol consumed/total liquid consumed*100) during

235   Limited Access revealed that females had greater preference for alcohol ($F_{(1, 58)} = 4.220$,

236   $p=0.0445$) and, for both sexes, alcohol preference increased across drinking sessions ($F_{(4.166,}$

237   $_{239.8)} = 4.671$, $p=0.0010$) (Supplemental Figure S1C). A mixed-effects analysis was utilized to

238   correct for five randomly missing values due to water bottle leakage.

239   Rats were assigned to Control or Stress conditions in a counterbalanced fashion based on

240   their average consumption of quinine-adulterated alcohol the week before stress (Quinine

241   Baseline; Supplemental Figure S1D). Following predator odor stress, 30% of male rats and 55%

242   of female rats showed avoidance of stress-paired contexts (Figure 1B). A subset of animals was

243   kept as unstressed, handling Controls and were tested in parallel to stressed animals.

244   One purpose of this experiment was to test the hypothesis that Avoiders would show

245   increased free-choice unadulterated alcohol drinking. Post-stress changes in unadulterated alcohol

246   drinking were quantified as a percent change relative to the pre-stress Alcohol Baseline (i.e.,

247   average alcohol consumed over the Monday, Wednesday, and Friday before the week of stress

248   procedures). Change from Alcohol Baseline was averaged for Monday, Wednesday, Friday for

249   each post-stress week. Outliers were excluded and then the post-stress unadulterated alcohol

250   drinking values for each week were averaged. We found that male Avoider rats drank more alcohol

251   (without quinine adulteration) across 3 weeks following predator odor stress compared to

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252   unstressed Controls (Stress Group x Sex ANOVA, interaction, $F_{(2,54)}$ = 4.341, p = 0.0179;

253   Tukey's post-hoc, p=0.0407 for Avoiders vs. Controls) (Figure 1C). Alcohol intake across

254   Monday-Wednesday-Friday of each post-stress week remained stable (i.e., no statistical effect of

255   day or week).

256   Another purpose of this experiment was to test the hypothesis that Avoider rats would show

257   greater compulsive-like alcohol drinking using a free-choice quinine-adulterated alcohol drinking

258   procedure. Following the week of predator odor stress CPA, rats underwent three weeks of Monday

259   to Friday limited access drinking sessions, with the alcohol solution adulterated with quinine twice

260   weekly on Tuesdays and Thursdays. The concentration of quinine increased from 10 mg/L (Week

261   1 post-stress) to 60 mg/L (Week 2 post-stress) to 100 mg/L (Week 3 post-stress) (Figure 1A).

262   Within each sex, within subjects RM two-way ANOVAs for intake of quinine-adulterated alcohol

263   across all quinine concentrations (i.e., across all three weeks post-stress) showed significant main

264   effects of quinine concentration (Stress Group x Quinine Concentration; males:

265    $F_{(1.407, 37.98)}$ = 39.32, p<0.0001; females: $F_{(1.560, 42.11)}$ = 20.48, p<0.0001; Supplemental

266   Figure S1E, F). Further analyses tested the effects of Stress Group on quinine-adulterated alcohol

267   drinking within each quinine concentration.

268   In male rats (Figure 1D), we found an inverse correlation between avoidance scores and

269   intake of quinine-adulterated alcohol, such that greater avoidance of stress-paired contexts

270   predicted greater intake of alcohol that was adulterated with 60 mg/L quinine (R^2=0.2653,

271   p=0.0201). This relationship was not detected in female rats (R^2=0.02424, p=0.5122; Figure 1E).

272   There were no stress group or sex differences in alcohol consumption when the alcohol

273   solution was adulterated with a mild dose of quinine (10 mg/L) (Figure 1F). With moderate quinine

274   challenge (60 mg/L), female rats consumed more alcohol than males (Stress Group x Sex ANOVA,

275   main effect of Sex, $F_{(1, 53)} = 8.877$, $p=0.0044$). (Figure 1G). At a higher quinine dose (100 mg/L),

276   female rats still consumed more alcohol than males, but there were no Stress Group effects (Stress

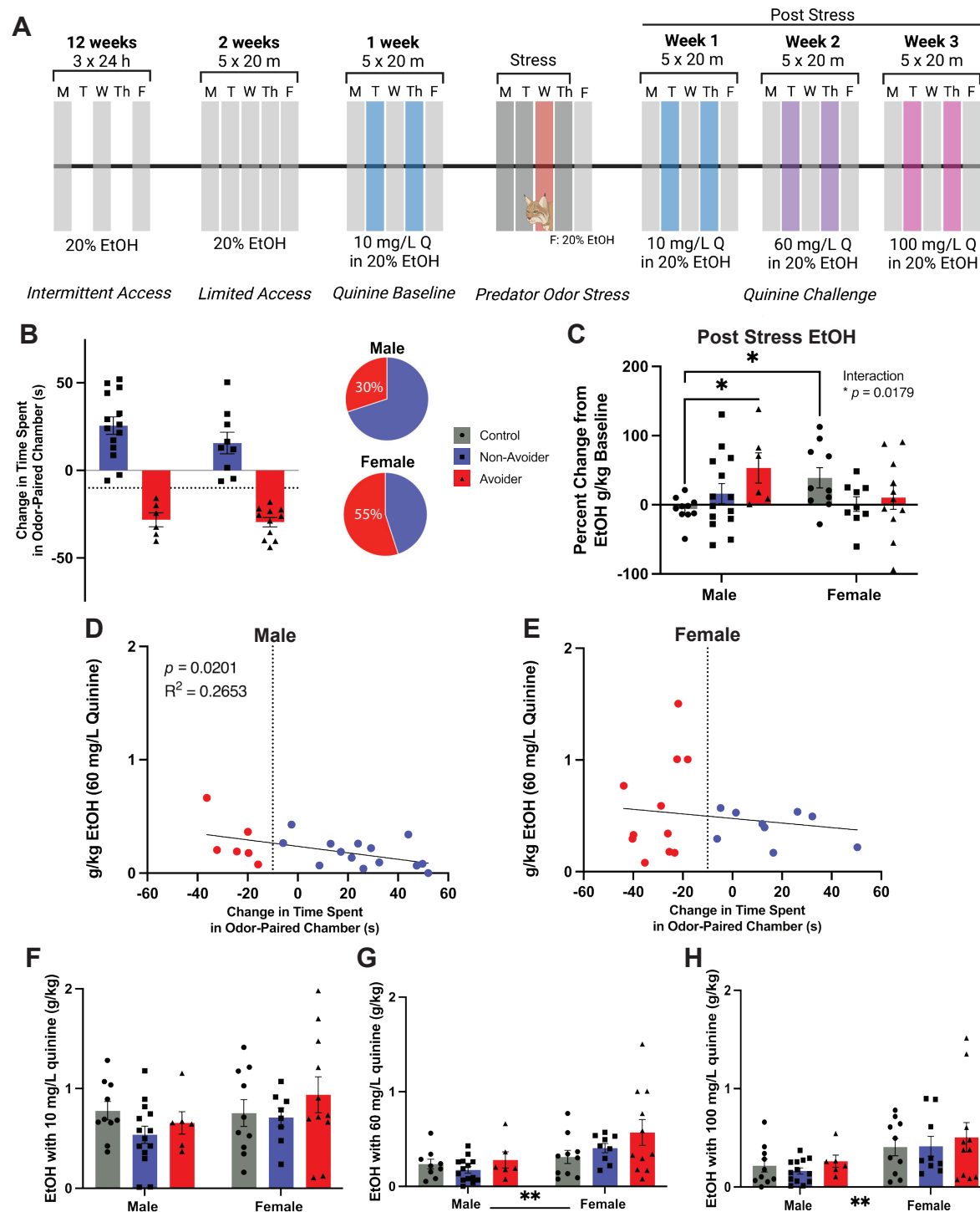277   Group x Sex ANOVA, significant main effect of Sex, $F_{(1, 53)} = 8.371$, $p=0.0055$) (Figure 1H).

Figure 1. Avoidant males show increased free-choice alcohol drinking and quinine-resistant drinking

A) Timeline schematic of the experiment. **B)** Distribution of avoidance vs. non-avoidance of predator odor stress-paired contexts (male Avoiders = 6, female Avoiders = 11). **C)** Post-stress alcohol consumption averaged across 3 weeks of post-stress non-quinine drinking sessions. Expressed as percent change from pre-stress alcohol-only drinking sessions during the Quinine

Baseline week (i.e., averaged across Monday, Wednesday, and Friday of that week). **D)** Relationship between change in time spent in odor-paired chamber (avoidance score) and consumption of alcohol adulterated with 60 mg/L in male and **E)** female rats. **F)** Post-stress consumption of alcohol adulterated with 10 mg/L, **G)** 60 mg/L, or **H)** 100 mg/L quinine**.** Error bars represent Mean ± SEM. *p <0.05, **p < 0.01. Created in BioRender. Bonauto, S. (2025) https://BioRender.com/w93w369.
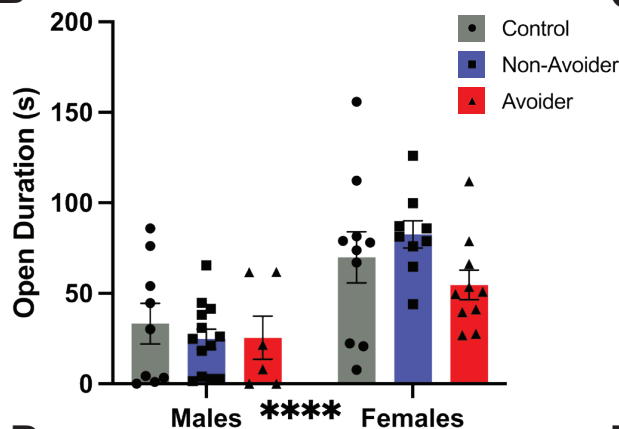
3.2 Female Avoider rats show heightened anxiety-like behavior 4 weeks after predator odor stress

The purpose of this experiment was to test the enduring nature of stress-induced anxiety-like behavior in male and female Avoider vs. Non-Avoider rats. Animals were subjected to an EPM test 28 days after stress (Figure 2A). In general, female rats spent more time in the open areas (Stress Group x Sex ANOVA, main effect of Sex, $F_{(1, 51)}$ = 25.42, p<0.0001) (Figure 2B), and had more open arm entries than male rats (Stress Group x Sex ANOVA, main effect of Sex, $F_{(1, 51)}$ = 42.43, p<0.0001) (Figure 2C). Measurements of stretch attempts showed that female Avoider rats had more aborted attempted entries into the open arms of the plus maze (Stress Group x Sex ANOVA, interaction, $F_{(2, 51)}$ = 4.066, p=0.0230; Tukey's post-hoc in female Avoiders vs. Controls, p=0.0198), indicating elevated anxiety-like behavior in these animals (Figure 2D). Additionally, both Avoider and Non-Avoider females executed more stretch attempts than males in the same stress groups (Tukey's post-hoc Avoiders male vs female: p = 0.0005; Non-Avoiders male vs female: p = 0.0406). Finally, distance moved throughout the assay was similar across stress groups, though females showed more locomotion than males (Stress Group x Sex ANOVA, main effect of Sex, $F_{(1, 50)}$ = 27.32, p<0.0001) (Figure 2E).
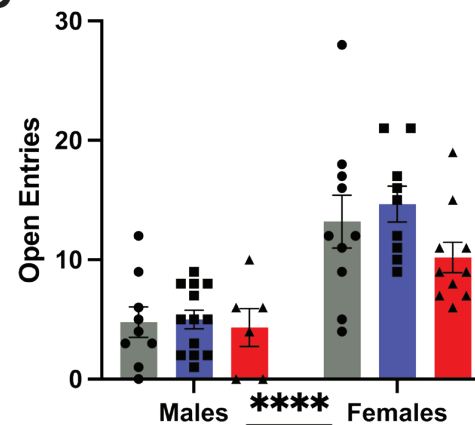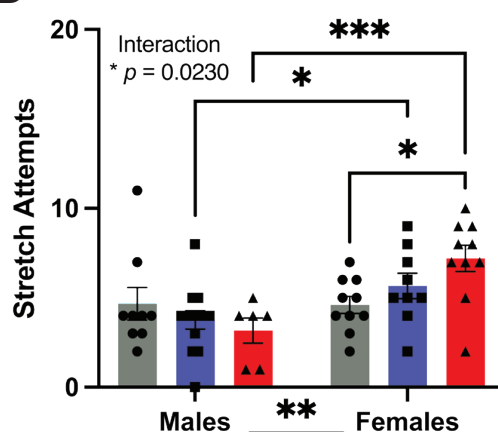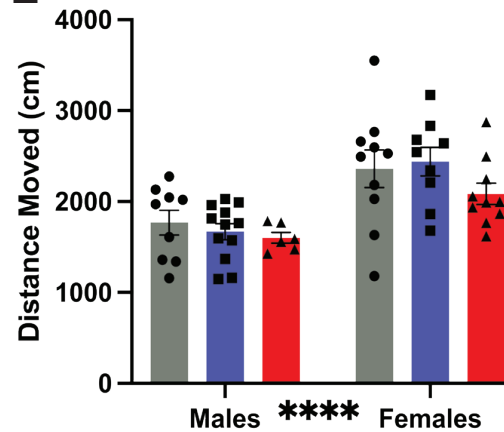
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Figure 2. Female Avoider rats show heightened anxiety-like behavior 28 days after stress
A) Timeline schematic (extension of Figure 1A). **B)** Duration in open arms of the EPM. **C)** Number of entries into open arms of the EPM. **D)** Number of stretch attempts (front two paws without entry) into the open arms of the EPM. **E)** Distance moved during EPM. Error bars represent Mean +/- SEM. *$p <0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Created in BioRender. Bonauto, S. (2025) https://BioRender.com/v69x870.

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3.3 Male Non-Avoider rats emit more <32 kHz ultrasonic vocalizations before, during, and after predator odor stress

The purpose of this experiment was to track USVs emitted by Avoider and Non-Avoider rats throughout predator odor place conditioning. During place conditioning, USVs were recorded and analyzed for principle frequency and total number of vocalizations. Throughout the place conditioning procedure, there were no group differences in the number of USVs emitted (Supplemental Figure S2A-C) and little change in the amount or type of USVs emitted by each individual rat from Pretest to Posttest (Supplemental Figure S2D, E). However, there were robust differences between Stress Groups and Sexes when we analyzed USVs within distinct frequency bands (Figure 3A). During the Pretest, males emitted significantly more <32 kHz USVs than females (Stress Group x Sex ANOVA, main effect of Sex, $F_{(1, 51)} = 9.264$, $p=0.0037$; effect of Stress Group, $F_{(2, 51)} = 2.938$, $p = 0.0620$). A direct comparison between the two subpopulations of stressed animals (i.e., Avoiders vs. Non-Avoiders) showed that males that would become Non-Avoiders with future odor stress emitted a higher percentage of <32 kHz vocalizations compared to Avoiders (unpaired t-test, t=2.383, df=16, $p=0.0299$) (Figure 3B). In males, there was also a positive correlation between eventual avoidance scores and percent of <32 kHz USVs during Pretest, such that a higher percentage of USVs in the <32 kHz range during Pretest predicted greater non-avoidance of a stress-paired context ($R^2=0.4371$, $p=0.0028$; Figure 3C).

During predator odor exposure, vocalizations from the first 5 minutes of the recording were quantified. Males again emitted a greater percentage of <32 kHz vocalizations than females (Stress Group x Sex ANOVA, main effect of Sex, $F_{(1, 51)} = 9.919$, $p=0.0027$), but vocalizations between stress groups were not significantly different (Figure 3D). Correlations between eventual

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337   avoidance scores and percent USVs <32kHz were also not significant (but in males, R^2=1615,

338   p=0.0790; Figure 3E).

339   During Posttest, 24 hours after predator odor stress, Non-Avoider males continued to emit

340   a greater percentage of <32 kHz vocalizations (Stress Group x Sex ANOVA, interaction

341     $F_{(2, 50)}$ = 3.800, p=0.0291, Tukey's post hoc in male Control vs Non-Avoiders p=0.0007 and

342   male Avoiders vs Non-Avoiders p=0.0073; Figure 3F). Additionally, there was a positive

343   correlation between avoidance scores and <32 kHz USVs for males in the Posttest, such that less

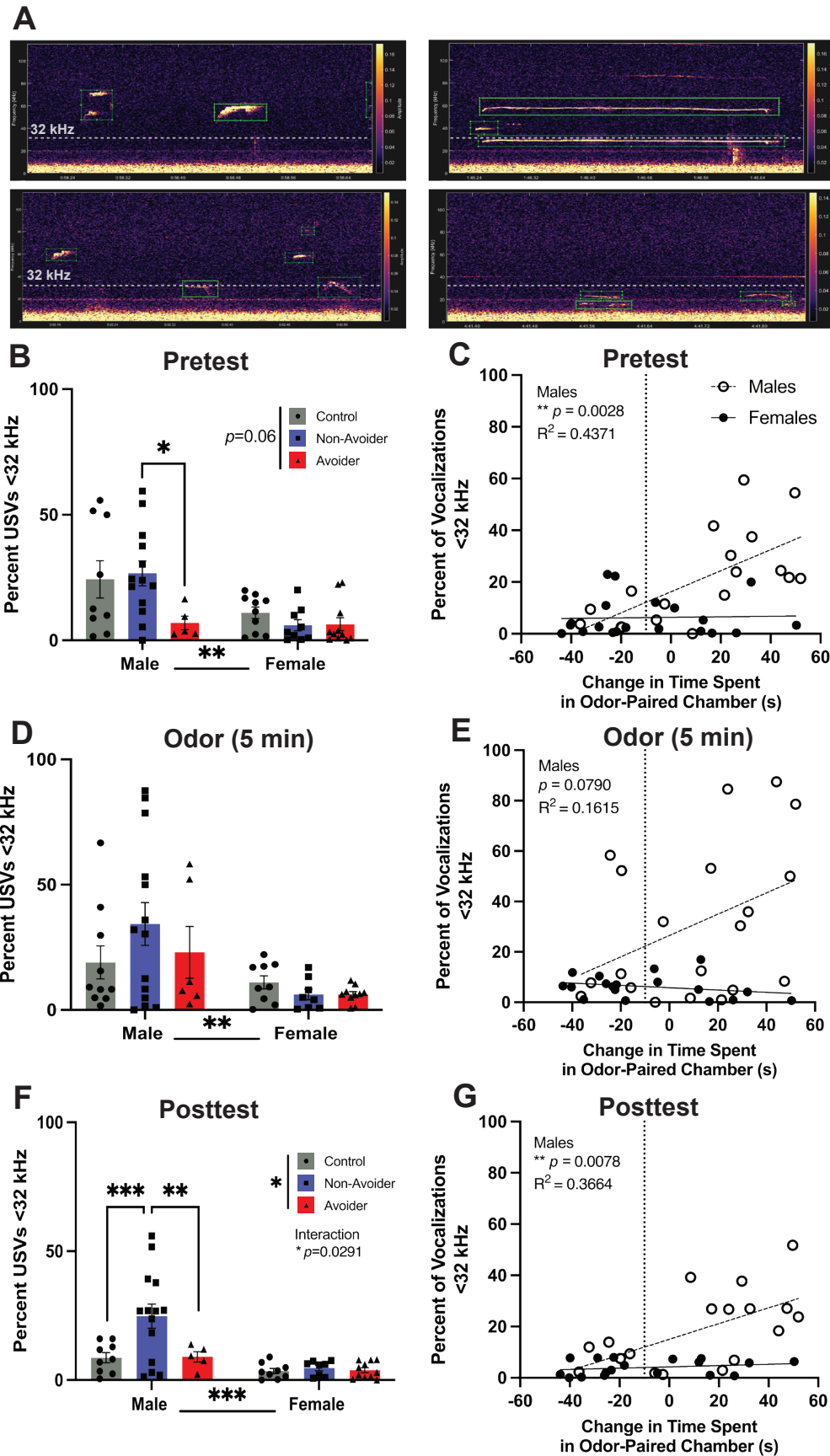344   avoidant animals emitted a greater percentage of <32 kHz USVs (p=0.0078, R^2=0.3664; Figure

345   3G).

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Figure 3. Non-Avoider males emit more <32 kHz USVs at all time points
A) Examples of <32 kHz USV spectrograms. **B)** Percentage of USVs with principle frequencies <32 kHz in the **B)** Pretest, **D)** first 5 minutes of odor exposure, **F)** Posttest. **C)** Relationship between change in time spent in odor-paired chamber (avoidance score) and percentage of vocalizations <32 kHz in the **C)** Pretest, **E)** first 5 minutes of odor exposure, **G)** Posttest. Error bars represent Mean +/- SEM. *p <0.05, **p < 0.01, ***p < 0.001. Created in BioRender. Bonauto, S. (2025) https://BioRender.com/q64q502.

3.4 Gene Expression Changes in the Lateral Hypothalamus

Genes associated with stress and the stress response were examined in the lateral hypothalamus 4 weeks after predator odor exposure. Levels of *crh* were significantly increased in male Avoiders compared with Non-Avoiders (p=0.025) and Controls (p=0.019) (Stress Group x Sex ANOVA, interaction, (F $(2,51)$ = 5.654; p=0.0061; Figure 1A). Tukey's post hoc multiple comparisons test also showed that female Control animals had higher *crh* compared to male Controls (p=0.016) and female Avoiders had less *crh* than male Avoiders (p=0.028). Expression of *crhbp* was also examined (Figure 4B). A two-way ANOVA revealed a significant main effect of Sex (F $(1,47)$ = 4.305; p=0.0435).

To test if there was a relationship between gene expression and post-stress behavioral changes, correlations were performed using all stressed rats. In male rats, Avoidance scores were negatively correlated with *crh* expression in the LH, such that greater avoidance scores predicted higher LH *crh* expression (R^2=0.3264; p=0.0106; Figure 4C). In addition, the amount of quinine (60 mg/L) adulterated alcohol consumed tended to be positively correlated with *crh* expression in males (R^2=0.1681; p=0.0812; Figure 4D). With regards to post-stress consumption of unadulterated alcohol in male rats, there was a weak relationship between percent change in alcohol drinking and *crh* expression (R^2=0.1559; p=0.0943; Figure 4E), but the relationship between percent change in drinking and *crhbp* expression was significant (R^2=0.2593, p=0.0368; Figure 5F). In females, there was a positive correlation between EPM open arm entries and *crh*

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374   expression (R^2=0.3760, p=0.0068; Figure 4G) as well as with *crhbp* expression (R^2=0.2979;

375   p=0.0191; Figure 4H).

376   The expression levels of *crhr1* and *crhr2* in the LH were similar across Stress Groups and

377   Sexes (Supplemental Figure S3B, C). However, in female stress-exposed rats, greater expression

378   of *crhr1* in the LH predicted more open arm entries in the EPM (R^2=0.2185, *p*=0.0505,
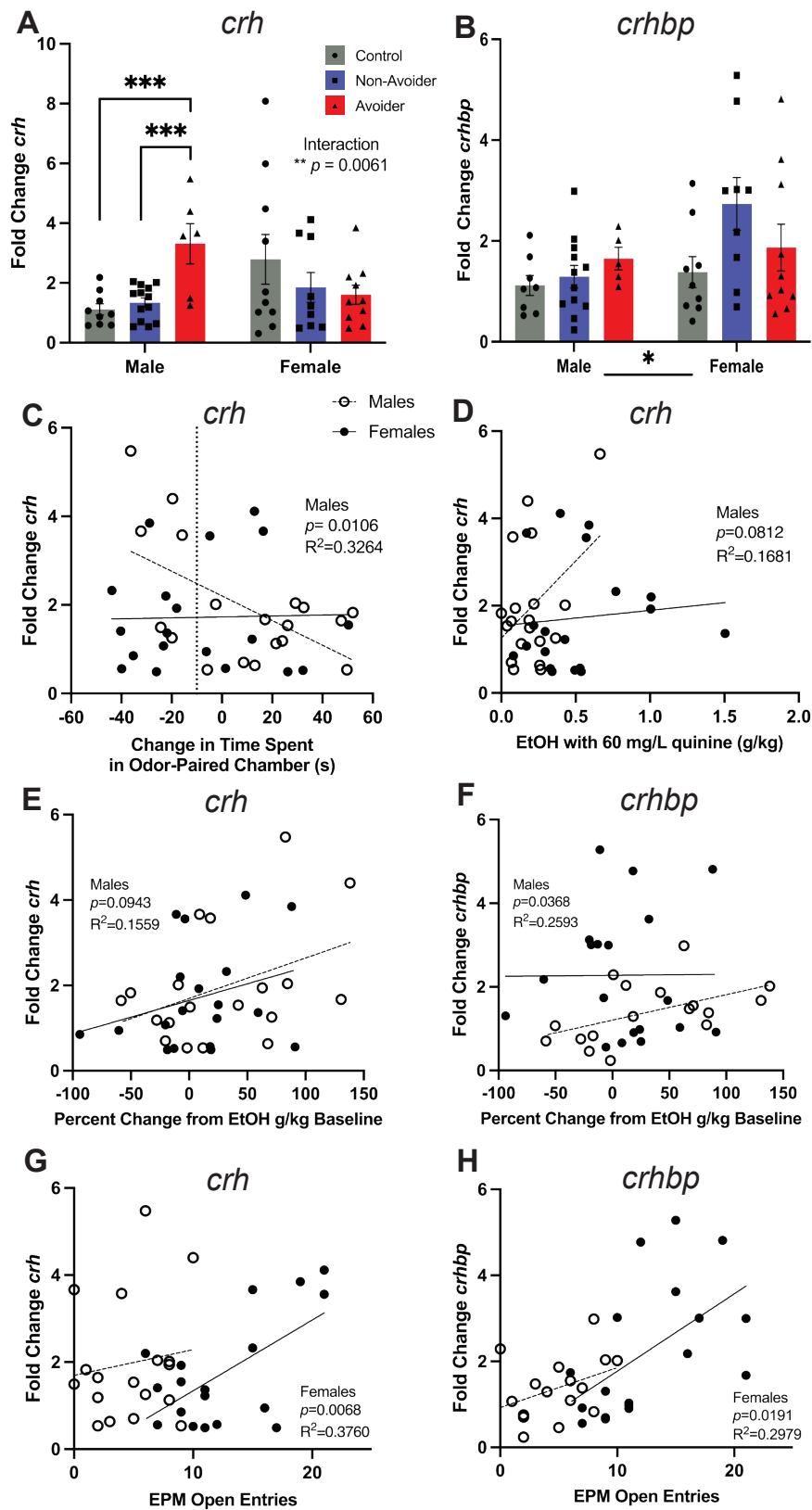
379   Supplemental Figure S3D).

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Figure 4. Gene expression changes in the lateral hypothalamus
A) Fold change in mRNA expression of *crh* and **B)** *crhbp*. **C)** Relationship between crh expression and avoidance score or **D)** amount of 60 mg/L quinine in alcohol consumed in post stress week 2. **E)** Correlation between post-stress change in unadulterated alcohol drinking relative to Alcohol Baseline and *crh* expression or **F)** *crhbp*. **G)** Relationship between open arm entries in the EPM and *crh* expression or **H)** *crhbp*. Error bars represent Mean +/- SEM. *p <0.05.

Discussion

Individuals with PTSD are more susceptible to AUD, yet the mechanisms and neurobiological factors of this phenomenon are unclear (Goldstein et al., 2016; Straus et al., 2018). In the present study, we used a predator odor stress model that recapitulates some aspects of PTSD, such as persistent avoidance of stress-paired stimuli in the absence of the stressor (i.e., conditioned avoidance; Albrechet-Souza and Gilpin, 2019) to test the effects of an acute, potentially 'traumatic,' stressor on free-choice alcohol drinking; aversion-resistant alcohol drinking; anxiety-like behavior; ultrasonic vocalizations before stress, during stress, and during expression of conditioned avoidance; and CRF system gene expression in the lateral hypothalamus in male and female rats. We found that only subsets of male (30%) and female rats (55%) developed conditioned avoidance of a stress-paired context, reflecting individual differences in stress responsivity also seen in humans, and similar to previous findings in rats (e.g., Albrechet-Souza et al., 2020). Male Avoider rats showed elevated free-choice alcohol drinking and were more likely to show aversion-resistant alcohol drinking. Female Avoider rats showed signs of heightened anxiety-like behavior 4 weeks after odor exposure. Using USV recordings, we found that increased vocalizations in the <32 kHz range before stress and during testing for conditioned avoidance predicted more non-avoidance behavior in male but not female rats. In the LH, male Avoiders had elevated *crh* gene expression, whereas all female stressed and unstressed rats had more *crhbp*, when brains were collected 4 weeks after stress (or handling control procedures). Overall, these

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408 findings expand our understanding of Avoider vs. Non-Avoider phenotypes after stress, allowing

409 us to embark on novel, mechanistic research avenues to elucidate the neurobiology of stress-

410 alcohol interactions in subpopulations of animals.

411 Previous studies reliably demonstrate that Avoider rats show heightened alcohol operant

412 self-administration after predator odor stress (Edwards et al., 2013; Schreiber et al., 2017; Weera

413 et al., 2020, 2023), but most of these data were collected in male rats. A central goal of this study

414 was to test the hypothesis that both male and female Avoider rats would show increased alcohol

415 drinking after stress in a free-choice condition. This hypothesis was supported only in male rats.

416 Greater avoidance behavior in male rats also predicted higher consumption of alcohol adulterated

417 with 60 mg/L quinine, a tastant that has been shown to be equally aversive in both male and female

418 rodents (Sneddon et al., 2019; Radke et al., 2020; Katner et al., 2022). This increase in post-stress

419 quinine-adulterated alcohol drinking is interpreted as a form of aversion-resistant or 'compulsive-

420 like' alcohol drinking, which is reminiscent of various forms of continued drinking despite

421 negative consequences seen in humans with AUD (see De Oliviera Sergio et al., 2023 for review).

422 In lab animals, the addition of a moderate dose of quinine to alcohol drinking solutions

423 typically produces a quantifiable reduction in alcohol drinking. This response can be blunted in

424 subgroups of animals by a variety of factors known to support increased alcohol drinking, such as

425 chronic intermittent alcohol access (Hopf et al., 2010), early life stress (Radke et al., 2020, 2021),

426 chronic predatory stress (Shaw et al., 2020), and a genetic predisposition for high alcohol

427 preference (Katner et al., 2022), which suggests that these factors may disrupt the motivational

428 balance between the reinforcing effects of alcohol and the aversive, anti-reinforcing effects of

429 quinine. The present results demonstrate that an acute stressor (predator odor) can also blunt the

430 effectiveness of an aversive stimulus in reducing alcohol intake in individual stress-reactive

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431 animals, in this case, in more avoidant male rats. Previous work using an operant procedure found

432 that male Avoider rats will also show operant responding for alcohol that is more resistant to

433 quinine challenge (Edwards et al., 2013).

434 Five days after the end of alcohol drinking (28 days after stress), we tested rats for their

435 anxiety-like behavior using the EPM. Female Avoider rats showed heightened anxiety-like

436 behavior, as indicated by more stretch attempts (i.e., aborted entries into open areas) compared to

437 unstressed Controls. Male Avoider rats, on the other hand, did not show any differences in behavior

438 in the EPM compared to Non-Avoiders and Controls. Previous work shows that predator odor

439 stress heightens anxiety-like behavior, as measured using the EPM and open field tests, in both

440 male Avoiders and Non-Avoiders up to 5 days post-stress (Whitaker & Gilpin, 2015). By day 9

441 post-stress, anxiety-like behavior in male and female Non-Avoiders return to Control levels,

442 whereas anxiety-like behavior in Avoiders remain elevated (Weera et al., 2023). Here, we tested

443 anxiety-like behavior at a more protracted time point, that is 28 days after stress, in both male and

444 female rats, and found that the heightened anxiety-like behavior in male but not in female Avoiders

445 had returned to Control levels at this time point. It is currently unclear how long-lasting predator

446 odor stress effects are on anxiety-like behavior in female Avoider rats, but our results show that

447 stress-heightened anxiety-like behavior persist for at least 1 month after stress. It is important to

448 note that the animals in the present study have undergone multiple months of alcohol drinking,

449 similar to those in Weera et al. (2023), but the rats in Whitaker and Gilpin (2015) were alcohol

450 naïve. Another important previous finding to consider is that, in alcohol-drinking male and female

451 rats that had undergone multiple acoustic startle tests, open arms times and entries on the EPM

452 were similar between stressed, and unstressed rats 17 days after predator odor exposure

453 (Albrechet-Souza et al., 2020).

454   Rats produce a variety of ultrasonic vocalizations (USVs) which may correspond with their

455 affective state (Knutson et al., 2002; Litvin et al., 2007; Brudzynski, 2013). For instance,

456 vocalizations in the 18-32 kHz frequency band are emitted during a variety of aversive experiences

457 and are proposed to serve as alarm calls indicating distress or threat (Brudzynski, 2001, 2021;

458 Burgdorf et al., 2008; Takahashi et al., 2010). We recorded USVs emitted by Avoider, Non-

459 Avoider, and unstressed Control rats before stress (i.e., during place conditioning Pretest), during

460 stress, and after stress during place conditioning testing (Posttest) to track the affective state of

461 these animals throughout the procedure. While the total number of USVs were fairly consistent

462 across Avoiders and Non-Avoiders and throughout the predator odor place conditioning procedure,

463 we found that, in males, Non-Avoiders exhibited a greater proportion of USVs in the <32 kHz

464 range compared to Avoiders during both Pretest (i.e., before stress) and Posttest (i.e., after stress).

465 Avoidance scores also correlated with percent of USVs in the <32 kHz range, such that male rats

466 that emitted more of their vocalizations in the <32 kHz range during Pretest were more likely to

467 not avoid a stress-paired context, and this pattern persisted into Posttest. At this time, it is unclear

468 why male Non-Avoider rats emit more vocalizations within the "aversive" range than male

469 Avoiders. Perhaps, males that respond to novel environments (i.e., the novel place conditioning

470 apparatus during the Pretest) with greater numbers of low frequency alarm calls are more likely to

471 be active coping individuals, thereby exhibiting a Non-Avoider status (or approach coping

472 strategy) following predator odor stress. USVs functioning as alarm calls are often emitted from a

473 place of safety following an adverse exposure (Litvin et al., 2007; Brudzynski, 2021). In the

474 Pretest, Non-Avoiders experienced the anxiogenic novel apparatus but may have perceived

475 relatively more safety than Avoiders and thus initiate alarm call social communication. Female rats

476 emitted far fewer USVs in the <32 kHz range compared to males, regardless of stress group.

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477   During fear conditioning, female Wistar rats emit very few low frequency USVs (Schwarting,

478   2018; Willadsen et al., 2021) which suggests that this type of social communication during

479   aversive experiences is generally specific to males.

480   To begin to elucidate the neural mechanisms behind the subpopulation-specific changes in

481   alcohol drinking and anxiety-like behaviors after predator odor stress, we quantified gene

482   expression of various CRF neuropeptide system components in the LH. In male rats, Avoiders had

483   significantly increased levels of *crh* in the LH compared to both controls and Non-Avoiders. Given

484   that brains were collected 4 weeks after exposure to predator odor, this prolonged increase in *crh*

485   may represent a potential mechanism underlying avoidance behavior as well as the increase in

486   alcohol drinking observed post-stress in male Avoider rats. Indeed, *crh* was significantly correlated

487   with the change in time spent in the odor-paired chamber (i.e., avoidance scores), such that greater

488   avoidance predicted higher levels of *crh* in the LH. Moreover, *crh* expression also tended to be

489   correlated with consumption of quinine-adulterated alcohol (60 mg/L) and post-stress change in

490   total unadulterated alcohol consumption. In addition, there was a correlation between CRH binding

491   protein gene (*crhbp*) expression in the LH and change in alcohol drinking from pre-stress to post-

492   stress, such that greater increases in drinking post-stress predicted higher levels of *crhbp* in male

493   rats. In Non-Avoider female rats, levels of LH *crh* and *crhbp* predicted lower anxiety-like behavior,

494   as indicated by more open arm entries in the EPM. Collectively, these findings highlight the

495   importance of the CRH system within the LH in mediating subpopulation (i.e., Sex- and Stress

496   Group-specific) differences in stress-alcohol interactions and inform our ongoing circuit-based

497   studies centered on the LH.

498   Extrahypothalamic CRF, especially in the extended amygdala, is well known to mediate

499   escalated forms of alcohol drinking, and preclinical studies showed that inhibition of CRF1

500   receptors rescues the escalated alcohol self-administration seen in alcohol-dependent animals

501   (Funk et al., 2006, 2007). However, clinical trials with CRF1 antagonists have not yielded success

502   for alleviating AUD symptoms (Kwako, Spagnolo, et al., 2015; Schwandt et al., 2016),

503   highlighting the need to further elucidate CRF-CRF1 circuit and cellular mechanisms in alcohol

504   drinking and related neuropsychiatric conditions (Pomrenze et al., 2017; Weera & Gilpin, 2024).

505   Indeed, circuit-based studies show that the role of CRF-CRF1 signaling in alcohol drinking may

506   have subpopulation-dependent effects. For instance, silencing CRF+ neurons projecting from the

507   central amygdala (CeA) to the LH or CRF1 antagonism within the LH blunts binge-like alcohol

508   consumption in male but not female mice (Bendrath et al., 2025), while silencing CRF1+ CeA-to-

509   LH neurons reduces alcohol self-administration in stress-exposed male and female Avoider but not

510   Non-Avoider rats (Weera et al., 2023). Our ongoing work is focused on understanding the roles of

511   LH cells and circuits, including those that express CRF/CRF1, in stress-alcohol interactions across

512   subpopulations of animals.

513   Collectively, these studies show that acute predator odor stress, which recapitulates some

514   aspects of traumatic stress, increases free-choice alcohol drinking, aversion-resistant alcohol

515   drinking, and anxiety-like behavior in a manner that is dependent sex and stress responsivity (i.e.,

516   Avoider vs. Non-Avoider phenotype). We highlight the utility of pre-stress ultrasonic vocalizations

517   for predicting Avoider vs. Non-Avoider status after stress. Finally, our gene expression analysis

518   suggests that the CRF system within the lateral hypothalamus plays an important role in post-stress

519   behavioral outcomes.

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Declaration of Interests

The authors declare they have no conflict of interest.

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