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Importance of sex and trauma context on circulating cytokines and amygdalar GABAergic signaling in a comorbid model of posttraumatic stress and alcohol use disorders

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Abstract

Alcohol use disorder (AUD) and anxiety disorders are frequently comorbid and share mechanisms that could be therapeutic targets. To facilitate mechanistic studies, we adapted an inhibitory avoidance-based “2-hit” rat model of posttraumatic stress disorder (PTSD) and identified predictors and biomarkers of comorbid alcohol (ethanol)/PTSD-like symptoms in these animals. Stressed Wistar rats received a single footshock on two occasions. The first footshock occurred when rats crossed into the dark chamber of a shuttle box. Forty-eight hours later, rats received the second footshock in a familiar (FAM) or novel context (NOV). Rats then received 4 weeks of two-bottle choice (2BC) ethanol access. During subsequent abstinence, PTSD-like behavior responses, GABAergic synaptic transmission in the central amygdala (CeA), and circulating cytokine levels were measured. FAM and NOV stress more effectively increased 2BC drinking in males and females, respectively. Stressed male rats, especially drinking-Vulnerable individuals (0.8g/kg average 2-hr ethanol intake with >50% ethanol preference), showed higher fear overgeneralization in novel contexts, increased GABAergic transmission in the CeA, and a profile of increased G-CSF, GM-CSF, IL-13, IL-6, IL-17a, leptin and IL-4 that discriminated between stress context (NOV>FAM>Control). However, drinking-resilient males showed the highest G-CSF, IL-13, and leptin levels. Stressed females showed increased acoustic startle and decreased sleep maintenance, indicative of hyperarousal, with increased CeA GABAergic transmission in NOV females. This paradigm promotes key features of PTSD, including hyperarousal, fear generalization, avoidance,

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and sleep disturbance, with comorbid ethanol intake, in a sex-specific fashion that approximates clinical comorbidities better than existing models, and identifies increased CeA GABAergic signaling and a distinct pro-hematopoietic, proinflammatory, and pro-atopic cytokine profile that may aid in treatment.

Keywords

PTSD; AUD; stress; sex differences; GABA; central amygdala; cytokine

Introduction

Alcohol use disorder (AUD) is a stress surfeit disorder; amplified stress signaling during alcohol (ethanol) use or withdrawal promotes greater drinking and relapse, respectively¹. AUD is comorbid with stress- and anxiety-related disorders^{2, 3}, including posttraumatic stress disorder (PTSD) and this comorbidity can differ between the sexes^{4, 5}. Individuals with stress/anxiety disorders have increased alcohol withdrawal symptoms and relapse risk⁶ and use alcohol to cope⁷. Common precipitants, such as psychogenic stress^{8, 9}, and molecular mechanisms, such as extended amygdala GABAergic neurotransmission^{10, 11} and proinflammatory cytokines¹²⁻²¹, may be implicated in both syndromes²² but remain understudied.

To identify novel molecular mechanisms, more robust animal models of AUD-stress/anxiety comorbidities are needed. Many existing models involve Pavlovian fear conditioning protocols, in which animals form associations between footshocks and otherwise neutral stimuli^{7, 23}. In reality, stress/anxiety disorders also involve fear overgeneralization, non-associative hyperarousal and fear²⁴, and operant components²⁵. Whereas Pavlovian conditioning putatively drives the “re-experiencing” symptoms of PTSD, operant conditioning drives avoidance²⁶. Models that incorporate non-Pavlovian attributes may hold advantages over pure classical conditioning models²³. Inhibitory avoidance (IA), footshock-based procedures have been used to generate PTSD-like behaviors in rodents^{27, 28} and involve both operant and Pavlovian learning under conflict²⁹. When a rodent crosses from an illuminated chamber to a dark one²⁹ they escape from aversive light but then receive an aversive footshock in the darkened chamber. Thus, the IA response evokes negative reinforcement and punishment, respectively, a conflicted outcome that may differ from classical conditioning.

Here, we characterize comorbid AUD- and PTSD-like symptoms that result from variants of a “2-hit” IA procedure that generates lasting hyperarousal, fear generalization, and extinction-resistant avoidance behavior²⁸. In the original “2-hit” procedure, a rat receives a single shock on two separate occasions with the first shock in an IA context (i.e., shuttle box) and the second in a different, novel context²⁸. The literature is inconsistent whether ethanol intake is increased more by an anticipated vs. unanticipated stressor⁷. Therefore, we compared effects of receiving the second footshock in the original IA chamber, reflecting stress re-exposure in a familiar (FAM) context, versus in an unfamiliar apparatus, or stress re-exposure in a novel (NOV) context. We then sought to apply

this novel translationally-relevant behavioral model to identify whether sex differences in physiology and biomarkers of PTSD/AUD comorbidity exist. We evaluated impacts of the 2-hit paradigms on: 1) voluntary ethanol intake via chronic intermittent 2-bottle choice (2BC) access; 2) translationally-relevant PTSD-related behavioral responses; 3) inhibitory GABAergic transmission in the central nucleus of the amygdala (CeA), due to its role in anxiety and since its elevation is considered a hallmark of alcohol dependence across species^{10, 11}; and 4) circulating cytokine profiles, due to their hypothesized biomarker and mechanistic roles in AUD and PTSD^{12–18, 20}. We hypothesized that model conditions that effectively promote PTSD/AUD phenotypes would lead to elevated CeA GABA signaling^{10, 11} and peripheral inflammation during abstinence from ethanol^{12–21} and that re-experiencing stress in a novel environment would lead to greater effects²⁸. Finally, given sex-specific responses to stress and prevalence of stress disorders, we predicted nuanced sex differences, whereby females would show greater vulnerability to develop hyperarousal symptoms and PTSD/AUD comorbidity^{5, 30, 31}.

Materials and Methods

More detailed methods are in Supplemental.

Animals

Male and female Wistar rats ($n=96$, Charles River Laboratories) weighed 425 ± 3.7 g and 247 ± 2.1 g, respectively, when experiments began. Rats (12:12 L/D cycle, food and water *ad libitum*) were pair-housed, separated by a perforated clear plexiglass divider to permit individual 2-bottle choice (2BC) drinking while reducing isolation stress³². The procedures followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals (8th edition) and were approved by The Scripps Research Institute Institutional Animal Care and Use Committee.

Footshock Paradigm

Rats were randomly assigned to treatment groups by cage, within ethanol condition. The first shock was given after the rat crossed from the illuminated to dark chamber of an IA shuttle box (see Fig. 1a&b for timeline). The second footshock occurred 48hr later, whereby half of the rats received the second footstock in the same familiar apparatus as the first (FAM). The other half of the rats received the second footshock in a novel, environmentally-distinct, single-chambered apparatus (NOV) (Fig. 1b). Each 3-mA footshock was delivered over 2 sec. Controls (CTL) were handled by experimenters and naive to stress.

2BC Testing

Beginning 2 weeks after the first footshock, half of the rats received 48-hr acclimation to ethanol (20% *v/v*) followed by chronic, intermittent (MWF: Mondays, Wednesdays, Fridays), limited 2BC access (2-hr) to ethanol at scotophase onset (Fig. 1a). These parameters were chosen because limited access sessions can promote greater binge-like drinking than continuous access³³ while ethanol exposure prior to footshock may impair 2BC escalation²³. The second cohort received dual water bottle access, in lieu of 2BC, to determine which post-stress behavioral phenotypes required concurrent ethanol access.

Abstinence Behavior Testing

After 18 2BC sessions, rats received 7 days of abstinence before behavioral testing began. In counter-balanced order, rats completed elevated plus-maze, novelty-induced hypophagia, and bottle-brush tests during the next 1.5 weeks of abstinence, followed by sleep-phase analysis, social investigation, acoustic startle, and finally fear overgeneralization testing across the subsequent 16 days of abstinence (see Fig. 1a for timeline). All non-automated procedures and data collection were carried out by treatment-blind experimenters, and the order of animals was randomized for each behavioral test.

Elevated Plus-Maze (EPM)

Time and entries in the open arm, closed arm and center of a 5-min EPM test were recorded³⁴.

Novelty-Induced Hypophagia

Latency to eat and intake of sucrose-rich pellets³⁵ in an unfamiliar double-size cage were measured. Rats received home cage pre-acclimation to the food 24-hr earlier.

Bottle-Brush Irritability

An experimenter rotated a bottle-brush (10 trials, 5 phases, 3 sec each) before the rat in an unfamiliar test cage. Two raters scored aggressive- and defensive-like behaviors³⁶.

Comprehensive Lab Animal Monitoring System (CLAMS)

Since sleep disturbances are a hallmark of PTSD³⁷, we assessed diurnal sleep maintenance in a Comprehensive Lab Animal Monitoring System (CLAMS; Columbus Instruments, Columbus, OH, USA). Such noninvasive, activity-based measurements correlate well with EEG-defined sleep³⁸. OXYmax-CLAMS units^{39, 40} were used to infer sleep parameters of habituated rats from photocell-defined motor activity across (11-hr) their inactive phase (lights on).

Acoustic Startle

Exaggerated acoustic startle responses are present in human PTSD patients and indicate hyperarousal²⁴. During a 30-min session (75 pseudorandomized trials), an SR-LAB Startle Response system⁴¹ measured startle responses to acoustic stimuli (80- to 120-dB) and no-stimulus control trials.

Social Investigation

We used a social investigation paradigm⁴² to measure stress-induced avoidance. Time near a dish containing clean bedding was measured for 3 min; then, time near a new dish containing soiled bedding from same-sex, unfamiliar conspecifics was measured over the next 3 min⁴².

Context Memory

Latency to cross (maximum 360 sec) in the original IA shuttle box was measured 61 days post-shock.

Fear Overgeneralization

Latency to cross (maximum 10 min) to the dark compartment of a novel shuttle box (different dimensions, material and color from the IA apparatus) was measured 96 days post-shock (Fig.2b).

Vulnerability

Only a subset of people with PTSD show comorbid AUD⁴³; thus, we established criteria for identifying drinking-Vulnerable vs. Resilient subjects. Vulnerable rats were designated as those exceeding previous NIAAA criteria for moderate ethanol intake (0.8 g/kg/2-hr averaged over 4-wk)^{44, 45} in conjunction with an ethanol-preferring phenotype (>50% preference ratios). This intake level also is relevant to current NIAAA criteria of 4 or 5 drinks in a 2-hr window for a woman or man respectively, assuming each drink contains ~14 g ethanol⁴⁶ and an average human weight of 70 kg⁴⁷. We used weeks 3 thru 6 of the postshock drinking phase for vulnerability analysis.

Amygdala slice preparation and electrophysiological recordings

Preparation of acute brain slices and electrophysiological recordings were performed as previously described⁴⁸ from a subset of 24-hr abstinent rats from each group that were under deep isoflurane anesthesia. Elevated CeA GABA transmission represents a hallmark of alcohol dependence across species^{10, 11}, and acute ethanol application increase CeA GABA signaling⁴⁸. Thus, we recorded pharmacologically-isolated GABA_A receptor mediated miniature inhibitory postsynaptic currents (mIPSCs) from 51 neurons in the medial subdivision of the CeA using whole-cell voltage clamp mode. Data were analyzed using Mini Analysis (Synaptosoft Inc., Fort Lee, NJ) with 3-min bins of gap-free recording⁴⁸ and only currents >5 pA accepted for analysis.

Cytokine and stress hormone assays

At the time of euthanasia, 24-hr following the final post-abstinence 2BC test, heparin-treated nocturnal trunk blood was collected from isoflurane-anesthetized rats and centrifuged. Plasma was stored (-80°C) until being assayed in duplicate using 27-plex cytokine (RECYMAG65K27PMX) and 2-plex corticosterone/adrenocorticotrophic hormone (ACTH) (RSHMAG-69K) Luminex magnetic bead panels. The comprehensive cytokine panel includes candidates from prior human or animal model PTSD- and AUD-related studies^{12-18, 21}.

Estrous Cycle

Estrous stage was determined by vaginal lavage at euthanasia and not earlier to limit stressful or reproductive effects of vaginocervical stimulation⁴⁹.

Statistical Analyses

Behavior, electrophysiology, and 2BC were analyzed using 2-way ANOVA with between-subjects repeated measures followed by within-sex planned comparisons (simple effects of Sex*Stress interactions)^{50, 51} comparing NOV and FAM rats to their unstressed control. Significant omnibus tests were followed with post-hoc Dunnett's *t*-test relative to controls.

Outliers were determined using Grubbs' test. Since many patients with PTSD engage in sporadic bouts of heavy drinking rather than increased daily drinking⁵², we quantified heavy drinking days as those 1.96 SD greater than the control mean. IA latencies were assessed using 3-way ANOVA with repeated measures examining change in latency to cross to the dark compartment during the first shock, second shock, and context memory testing. "Stress" and "Sex" are capitalized throughout when referring to main effects. Linear discriminant function analyses were performed on standardized (% control), log-transformed scores (to normalize and homogenize variance) within each sex to identify cytokine profiles that significantly discriminated subjects per Stress history or drinking-Vulnerability/Resilience. Cytokines that loaded significantly on discriminating functions per Wilks' lambdas within the LDA were further interpreted using intercorrelations (Pearson r) and pairwise difference analysis (LSD within univariate ANOVAs). Interferon gamma (interferon- γ) and human growth-regulated oncogene/keratinocyte chemoattractant (GRO/KC) were undetectable and excluded from analyses. All tests were two-sided. P 0.05 was considered statistically significant. All data are presented as mean \pm standard error of the mean (SEM). Minimum sample size was determined using a power analysis, and n is reported in the figure legends. See Supplemental for details.

Results

"2-hit" stress increases voluntary ethanol intake

A Stress main effect indicated increased ethanol intake (Fig.1). The efficacy of NOV vs. FAM stress was sex-dependent; within-sex planned comparisons showed that FAM stress increased intake by males (Fig.1c&g), whereas NOV stress did so in females (Fig.1d&h). However, if stress conditions were combined, increased intake remained significant in males and approached significance in females, reflecting the lack of a Stress*Sex interaction. Each stressor also strengthened ethanol preference in males (Fig.1e&i) and females (Fig.1f&j). Stress did not increase average total fluid or water intake, so stress-mediated increases in ethanol intake were specific to ethanol (Fig.S1a-d).

We also examined the frequency of extreme intake days (>1.96 SD vs. control mean). NOV and FAM males had greater rates of high intake days (>1.21 g/kg/2-hr) than CTL males (Fig.1k). NOV females also had more frequent high intake days (>2.57 g/kg/2-hr, Fig.1l).

The IA-based "2-hit" stress model generated anxiety-like phenotypes

Elevated Plus-Maze (EPM)—FAM and NOV males spent significantly less time in the closed arms than controls (Fig.2d), with trends for more open arm time (Fig.S2d) and entries (Fig.S2e). Conventionally, this would be interpreted as less unconditioned avoidance of the anxiogenic-like open arms. We hypothesized instead that males might be generalizing their conditioned fear of the IA box's dark compartment to the dark, closed arms of the EPM⁵³⁻⁵⁵. Accordingly, FAM males took significantly longer than controls to enter the dark compartment of a novel shuttle box (Fig.2c). Thus, FAM males may overgeneralize fear to different, but reminiscent, contexts.

Novelty-induced hypophagia—NOV and FAM males had increased latencies to eat (Fig.2f), and Stressed subjects from both sexes (main effect) ate less than controls in the novel environment. No such Stress effects were seen during home cage acclimation to the food.

Bottle-brush test—FAM males and females showed more startle responses to the brush (Fig.2h, Supplemental Table 1) and did not differ on other measures (Supplemental Table 1).

CLAMS sleep analysis—NOV stress significantly reduced the duration of the longest sleep bout in females, with a similar trend in FAM females (Fig.2i). Stress did not alter average bout duration (Fig.S2g), number of bouts (Fig.S2h), or total sleep time (Fig.S2i).

Social investigation—NOV stress significantly reduced time in the conspecific-soiled bedding zone (Fig.2j) with a similar trend on the interaction ratio (Fig.2k). Stress history did not affect time near clean bedding, a non-social, control measure (Fig.S2j).

Acoustic startle—Consistent with the bottle brush startle phenotype, FAM and NOV stress females had increased acoustic startle responses during the first block of 120-dB stimuli (Fig.2m), but not the first trial (Fig.2l). NOV (Fig.2q), but not FAM (Fig.2q), females also exhibited elevated startle responses at lower intensity stimuli, including 85- and 100-dB. Stress did not affect acoustic startle of males (Fig.2l–n,p) or pre-pulse inhibition (Fig.2o).

Ethanol history—As we were studying PTSD/AUD comorbidity, all above groups were studied during abstinence from 2BC ethanol access. To determine the requirement for 2BC access in PTSD-like phenotypes, a cohort of “2-hit” stress rats that instead received 2BC access to water were studied in the same tests (Fig.S3). No-ethanol stress groups again showed significantly decreased plus-maze closed arm time (Fig.S3a; FAM and NOV) and reduced maximum sleep bout duration (Fig.S3f; FAM) with trends for novelty-induced hypophagia (Fig.S3d, NOV female) and increased fear overgeneralization in the novel context (Fig.S3o; FAM males). Unlike 2BC groups, decreased social interaction and increased startle to bottle brush or isolated acoustic stimuli were not seen in stressed, no-ethanol animals. However, no-ethanol NOV females uniquely showed increased total defensive behaviors in the bottle brush test, decreased total sleep time and impaired startle prepulse inhibition (Fig.S3l). Thus, 2BC ethanol access influenced some, but not all, anxiety-like phenotypes, exacerbating some, while reducing others.

Vulnerable males show comorbid alcohol use- and anxiety-disorder like comorbidity

Drinking-Vulnerability was more prevalent in stressed rats (21/32; 66%) than CTLs (4/16; 25%; $P=0.0135$, Fisher’s exact test). Of stressed males, 4/8 FAM and 3/7 NOV were Vulnerable vs. 0/8 CTLs. All but 2 stressed females (2/16) met drinking-Vulnerable criteria versus half of CTL females (4/8). Because of the greater baseline drinking (and vulnerability) in control females, further vulnerability subgroup analyses were male-focused.

Vulnerable males exhibited higher ethanol intake (Fig.3a) and preference (Fig.3b) than both Resilient and unstressed CTL males during the post-shock phase. Vulnerable males also showed more extreme high intake days (>1.14 g/kg/2-hr) than CTL and Resilient

males, which did not differ (Fig.3c). Only Vulnerable males significantly overgeneralized fear compared with CTL males as indicated by increased latency in a novel shuttle box (Fig.3d) and decreased closed arm time in the EPM (Fig.3e). Vulnerable males showed higher average drinking during post-context memory testing (Fig.S4e&f). Vulnerable males did not differ from Control or Resilient counterparts on any other behavioral parameters (Supplemental Table 2).

Stress increased CeA GABA activity

To assess whether our 2-hit model increased GABA_A-receptor mediated synaptic transmission in the CeA, we recorded action-potential independent mIPSCs from 51 CeA neurons from a random subset of rats from each group. FAM and NOV males showed significantly increased mIPSC amplitudes compared with CTL (Fig.4d), and mIPSC frequency (but not amplitude) was significantly elevated in FAM females (Fig.4h), Stress did not alter mIPSC kinetics (Fig.4e,f,j,k). In neuronal recordings ($n=122$) from animals that received Stress but had no history of ethanol, FAM males had significantly elevated mIPSC frequency and faster kinetics (rise time) compared to CTL males, while CeA GABA transmission did not differ between the other groups (Supplemental Table 3).

Cytokine profile of past stress

Linear discriminant analysis (LDA) revealed sex differences in stress-induced cytokine profiles. In males, a function significantly loaded on by higher G-CSF, IL-13, GM-CSF, IL-6, IL-17A, leptin and IL-4 levels ($0.45 < r_s < 0.62$, $P_s < 0.05$; Table 1) discriminated Stress history groups, with NOV>FAM>CTL (z -scores of $7.1+0.4$ vs. $3.2+0.7$ vs. $0.0+0.4$, respectively; $F_{2,18}=48.16$, $p<0.001$; 24% of variance; all pairwise $P_s<0.001$). These 7 cytokines also intercorrelated ($M+SEM r=0.61+0.08$). Follow-up univariate analyses confirmed (Fig.4) higher G-CSF (214+37 vs. 100+16%), GM-CSF (153+21 vs. 100+8%), IL-13 (202+32 vs. 100+8%), and IL-17A (176+42 vs. 100+12%) in NOV males vs. controls ($P_s<0.05$), with similar trends for IL-6 (111+7 vs. 100+0%, $P=0.06$), leptin (106.5+3.6 vs. 100+1.7%, $P=0.078$), and IL-4 (176+42 vs. 100+12, $P=0.079$). FAM males also had elevated IL-17A (123+8 vs. 100+5%, $P<0.05$) and a trend for higher IL-13 (139+31 vs. 100+8%, $P=0.06$). Other cytokine markers were non-significantly intermediate. These cytokines were not elevated in Stressed females, yielding Stress*Sex interactions for GM-CSF, IL-13, IL-4 and IL-17A (e.g., for NOV stress, $F_{1,25}=4.16$, 6.75, 3.50 and 3.20, respectively, $0.01 < P < 0.08$). LDA did not identify a cytokine profile that discriminated females by Stress history (raw data in Supplemental Table 4).

Cytokine profile of drinking resilience

LDA revealed a unique cytokine profile for drinking-Resilient males, significantly loaded on by higher GM-CSF, leptin, and IL-13 levels ($0.46 < r_s < 0.66$, $P_s < 0.05$; Table 1), with Resilient>>Vulnerable>CTL (z -scores of $4.1+0.4$ vs. $1.7+0.4$ vs. $0.0+0.4$, respectively; $F_{2,18}=27.90$, $p<0.001$; all pairwise $P_s<0.009$). Follow-up univariate analyses confirmed that drinking-Resilient subjects had higher G-CSF ($P<0.04$; $10+3.5$ vs. $2.9+1.0$ pg/ml), IL-13 ($p<0.005$; $10.0+2.6$ vs. $2.9+0.8$ pg/ml), and leptin ($P=0.05$; $73.1+9.3$ vs. $58.2+7.6$ ng/ml), levels than controls, whereas drinking-Vulnerable subjects did not ($4.1+1.4$ pg/ml, $4.1+1.1$ pg/ml, and $57.4+7.3$ ng/ml, respectively). This yielded significantly higher IL-13 levels

between drinking-Resilient vs. Vulnerable males ($P<0.03$). However, cytokine levels did not individually correlate with the amount of ethanol consumed for male animals.

Body weight and HPA-stress hormones

There were no effects of stress on weight gain, body weight (Fig.S5e&f), or point-sampled ACTH or corticosterone levels at sacrifice (Fig.S5a–c). Plasma corticosterone levels correlated with ethanol intake during the last 2BC session, 2 days before euthanasia (Fig.S5d; Spearman $r_s=0.423$; Pearson $r=0.494$, P_s 0.003).

At the time of euthanasia, most females were in proestrus or estrus (21/24). Comparisons between proestrus vs. estrus were limited by sample size, as there were only 3 CTL, 2 NOV and 1 FAM females in proestrus, but descriptively large differences were not seen for terminal endpoints.

Discussion

We adapted a novel rat model of lasting comorbid AUD/PTSD symptoms from a “2-hit” IA-based traumatic memory consolidation model²⁸. Stressed rats developed increased ethanol intake, preference and heavy drinking days with PTSD-like symptoms, increased CeA GABAergic synaptic transmission and novel proinflammatory cytokine profiles during abstinence. Our translationally-relevant findings also demonstrate the importance of stressor context, sex, and individual factors, in vulnerability to specific comorbid AUD/PTSD symptomatology.

In females, NOV stress more robustly escalated ethanol intake severity and promoted behavioral responses translationally-relevant to PTSD. NOV females had briefer longest sleep bouts and increased startle responses, paralleling common clinical PTSD symptoms that may share common mechanisms^{24, 37}. In contrast, in males, FAM stress more robustly promoted ethanol intake and preference with PTSD-like fear overgeneralization²⁴, interpreted from increased avoidance of dark compartments in an unfamiliar shuttle box and EPM. These sex differences resonate with previous findings that females show different responses and strategies in fear learning, EPM, and other common anxiety-related paradigms³¹. Perhaps stressed males in this protocol more often overgeneralized than females due to the long (>6 wk) post-shock interval⁵⁶, multiple IA trials⁵⁶, lack of olfactory cues⁵⁷, or ethanol history⁵⁸. Male rats also overgeneralize more than females in contexts more dissimilar to the original stress context⁵⁹. Finsterwald et al. (2015) showed a second traumatic shock was necessary for male rats to develop PTSD-like avoidance responses in the IA model we adapted. To our knowledge, we are the first to try this approach in females, and a different protocol may result in more overgeneralization in females. To further study the distinct outcomes of elevated voluntary drinking and fear overgeneralization in males vs. elevated drinking and hyperarousal (sleep disturbances with increased startle) in females, we examined these sex-specific behavioral phenotypes in FAM, NOV, and CTL rats that never had access to ethanol. Stress alone elicited signs of fear overgeneralization and impaired sleep maintenance, but concurrent post-stress ethanol access influenced the expression of other behaviors, exacerbating social avoidance and modifying startle and defensive behavioral responding.

Cognitive and physical avoidance are key symptoms⁶⁰ that predict clinical PTSD and “coping” use of alcohol⁶⁰. Accordingly, drinking-Vulnerable male rats most strongly showed avoidance of trauma-reminiscent dark places. Their avoidance of unfamiliar, but trauma-reminiscent dark places resembles a prior finding that situational reminders induced avoidance of the dark side of novel shuttle boxes and EPM-closed arms in IA-trained male rats⁵³. EPM closed arms thus may be a “low similarity” reminder of IA training, (conditioned) avoidance of which indicates a greater degree of fear overgeneralization than does dark compartment avoidance in a novel shuttle box⁵³. NOV males appeared to show generalization in the “low similarity” environment, but not the subsequent higher similarity one, whereas FAM males generalized in both. This finding may mean that fear generalization was less extinction-resistant in NOV males than in FAM males, especially in the drinking-Resilient subgroup. In this view, extinction during EPM testing may have reduced later fear overgeneralization in the novel inhibitory avoidance context in NOV males. Because the overgeneralization effect was maintained in Vulnerable males from both groups, extinction-resistance may be a useful marker of persistent overgeneralization^{28, 61} and vulnerability to drink. Vulnerable males did not separate from the other groups on other behaviors suggesting their Vulnerable phenotype associates differentially with fear overgeneralization-like responses. We cannot rule out that acoustic startle testing may have differentially affected behavior of groups in subsequent fear overgeneralization testing, but we note that males did not show behavioral differences in acute response to acoustic startle.

Additional avoidance behavior also resulted from “2-hit” stress. Hyponeophagia increased after “2-hit” stress, suggesting an anxiogenic-like⁶², avoidant state⁶³. Stressed rats also avoided odors of same sex-conspecifics. Similarly, female rats previously displayed reduced social interaction after exposure to footshock and “re-experiencing” context memory testing⁶⁴. Disrupted social function is a significant clinical feature of PTSD⁶⁰, and social phobias are commonly comorbid with alcohol use disorder⁴³.

Only some people with PTSD show AUD comorbidity⁴³. Therefore, we distinguished AUD-Vulnerable from AUD-Resilient subgroups using NIAAA criteria for intake exceeding moderate levels^{44, 45} and ethanol preference. About half of stressed males (47%) developed elevated ethanol drinking, especially with comorbid fear overgeneralization. Even higher vulnerability rates in stressed females, on the background of greater basal drinking in control females, confirm the “2-hit” model’s efficacy. The model also elevated rates of extreme high drinking days, translationally relevant because individuals with PTSD have elevated rates of heavy drinking days⁵², rather than necessarily daily drinking. Future work will examine interactions between our PTSD/AUD comorbidity model and ethanol dependence models.

The previously reported Pavlovian stress-enhanced fear learning (SEFL) model also elevates ethanol intake²³. SEFL generates non-associative fear learning with freezing as its primary fear output. In contrast, our “2-hit” IA model engages different behaviors, learning mechanisms, and underlying neural circuitry²⁹. The IA model involves a complex avoidance-avoidance conflict²⁹ wherein the animal instrumentally controls the outcome. This contingency may enhance later ethanol action tendency or otherwise prepare the animal to learn associated outcomes of its actions. This conceptualization resonates with our finding that drinking-Vulnerability corresponded with over-avoidance. Other notable features of the

present model include the long-term (>6 week) nature of outcomes; the lack of pre-stress and presence of post-stress intermittent, limited ethanol access; and the use of multi-modal contextual cues to assess overgeneralization.

Exposure to “2-hit” stress without history of ethanol increased CeA inhibitory GABAergic transmission only in FAM males. Notably, Stress and ethanol increased CeA GABAergic transmission; FAM males showed increased GABA_A receptor function, while FAM females showed increased GABA release. Although voluntary intake without dependence is insufficient to alter GABAergic transmission in the CeA^{65, 66}, the heightened ethanol intake of stressed subjects may contribute to the altered CeA GABA signaling observed here. We propose that the Stress and ethanol history synergistically led to increased CeA GABA synaptic transmission, which is anxiogenic¹⁰ and seen in other conditions with heightened anxiety and ethanol intake, such as alcohol dependence^{18, 67}.

Stressed male subjects exhibited a distinct cytokine profile of increased circulating levels of specific pro-hematopoietic (G-CSF, GM-CSF), pro-inflammatory (IL-6, leptin), and pro-atopic, Th2/Th17-like cytokines (IL-13, IL-17A, IL-4 levels). Some prior reports have observed peripheral elevations of these cytokines in isolation in patients with PTSD^{68, 69} or other severe anxiety conditions^{70, 71}. Elevated levels of G-CSF and GM-CSF could signal alterations in hematopoiesis, potentially favoring myelopoiesis versus lymphopoiesis, a phenomenon that has been described with chronic social stress in mice⁷². IL-6 levels have also been reported to be positively correlated with early-life adversity and anxiety⁷³. Higher IL-6 levels could be linked to increased myelopoiesis, given that it is a major cytokine of granulocytes. Plasma levels of pro-atopic cytokines IL-4 and IL-13 may be associated with stress vulnerability⁷⁴. Similarly, IL-17 levels also correspond with stress. Our findings show that these cytokines potentially serve as a strongly intercorrelated, lasting biomarker “cluster” caused by traumatic stress. The cytokine profile was unique to males and more pronounced in subjects with a history of 2-hit stress in the novel (putatively, unexpected) vs. familiar (putatively, expected) context. Most of these cytokines were not measured in a recent study that did not find a neuroimmune profile in comorbid PTSD-AUD⁷⁵. Interestingly, several of these cytokines increase during ethanol withdrawal^{76–78} and central G-CSF reportedly increases motivation for drug reinforcers⁷⁹. A subset of these cytokines (G-CSF, leptin, IL-13) were most elevated in drinking-Resilient subjects, which also indicates that their elevations were not artifacts secondary to heightened ethanol intake. This subset potentially might have anti-ethanol intake actions or reflect a lack of ethanol suppression of cytokine levels. Consistent with either interpretation, reduced G-CSF^{80, 81} levels are seen in actively-drinking alcohol-dependent individuals. The sexual dimorphisms in cytokine predictiveness or behavioral outcomes could be explained by many factors, including estrous cycle variability; lack of females in diestrus or metestrus; sexual dimorphism in potentially relevant third variables (e.g., greater baseline ethanol drinking and vulnerability by females, body weight, body composition, exploratory activity), and/or sexually dimorphic regulation of the implicated cytokines^{82–85}.

In summary, IA-based “2-hit” stress coupled with intermittent, limited ethanol access modeled comorbid AUD and PTSD in a sex-dependent, context-sensitive manner. Importantly, these behavioral and physiological phenotypes are distinct from those generated

by either model alone, a key finding missing from many other models of comorbidity. This synergism increases the translational utility of this model, as the key behavioral phenotypes of elevated ethanol drinking with sex-specific stress and anxiety behaviors map well onto clinical PTSD experiences. The data from this initial approach will allow targeted refinement of this model to more closely match the PTSD/AUD comorbidity seen in patients. Physiological findings, including sexually-distinct mechanisms of increased CeA GABA synaptic transmission and cytokine profiles of stress history, provide future opportunities to identify sex-specific biomarkers/mechanisms of vulnerability vs. resistance to comorbid drinking, and will help to define these previously neglected characteristics for females. Finally, translational use of this paradigm to identify the molecular and neurocircuitry bases of AUD-PTSD comorbidity and test targeted interventions is needed since no FDA-approved treatments for PTSD/AUD comorbidity exist⁸⁶.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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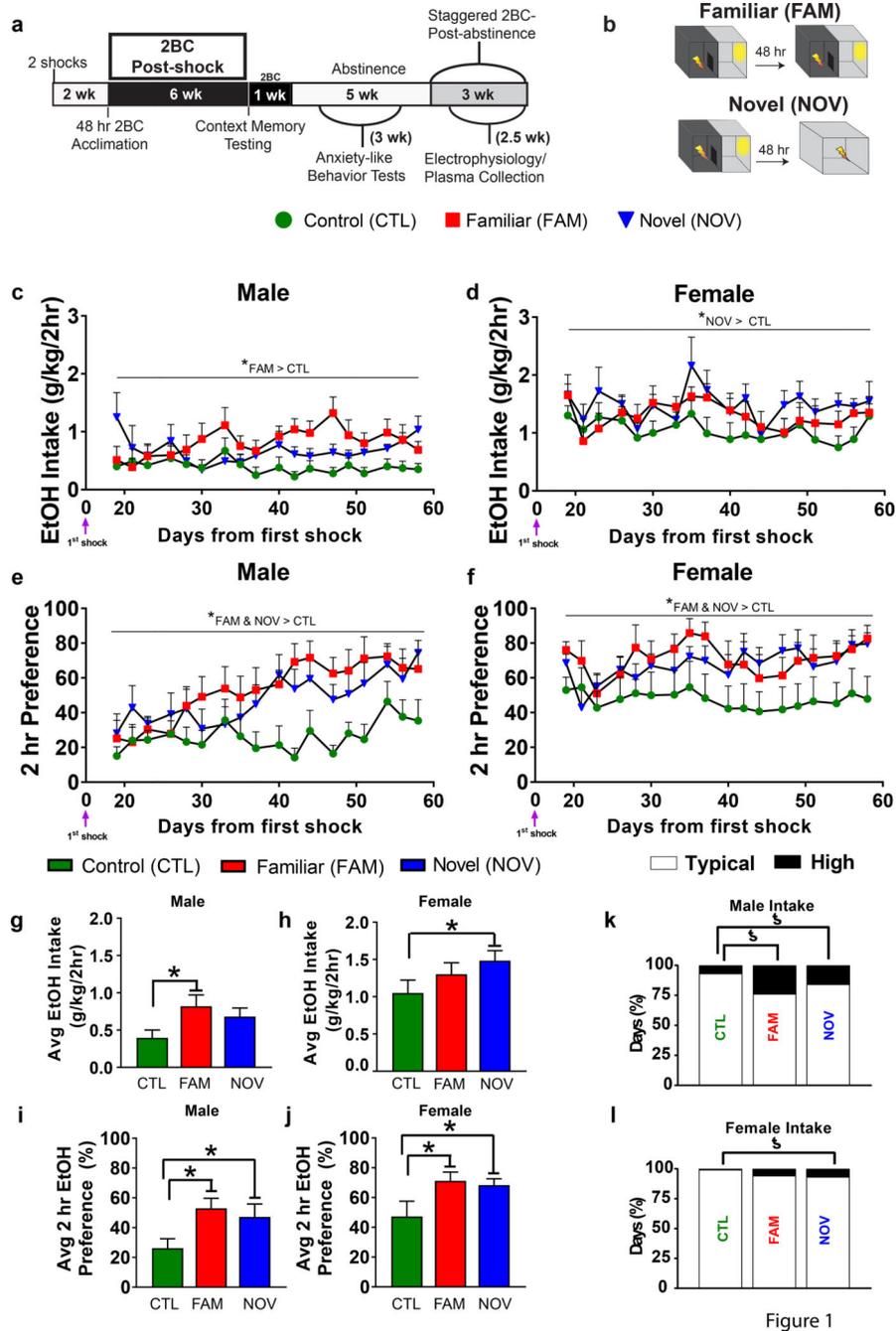


Fig. 1. The “2-hit” stress paradigm increased ethanol intake in rats with intermittent, limited ethanol access.

(a) Experimental timeline with post-shock limited access (2-hr) 2BC sessions. (b) Rats were naive to stress (CTL) or administered “2-hit” stress with the second shock in a familiar (FAM) or novel (NOV) context. (c,d) Two-way ANOVA with repeated measures (Session) indicated a main effect of Stress on post-shock ethanol intake ($F_{2,42}=4.119$, $P=0.023$). Within-sex planned comparisons revealed that the increase was significant in FAM males ($P=0.039$) and NOV females ($P=0.034$), but not NOV males ($P=0.159$) or

FAM females ($P=0.363$). Females drank more ethanol (g/kg) than males ($F_{1,42}=31.717$, $P<0.001$, main effect of Sex). Intraclass correlations demonstrated strong, stable individual differences in post-shock ethanol intake ($ICC(2,41)=0.92$ for males, 0.9 for females). **(e,f)** Ethanol preference was increased in stressed males **(e)** and females **(f)** during the post-shock phase, regardless of the stress context (Stress main effect: $F_{2,41}=6.894$, $P=0.003$). Within-sex planned comparisons confirmed that all stress-treated males and females showed significantly elevated ethanol preference (all $P's<0.05$). Females had greater ethanol preferences than males (Sex main effect: $F_{1,42}=11.383$, $P=0.002$, without Stress*Sex interaction: $F_{1,42}=0.026$, $P=0.974$). Intraclass correlations revealed strong, stable individual differences in post-shock ethanol preference ($ICC(2,41)=0.95$ for males, 0.94 for females). **(g,h)** Bar graphs summarizing mean male **(g)** and female **(h)** post-shock intakes from the line graphs in previous panels. To investigate whether 2-hit stress increased ethanol intake without regard to second stressor context, we also combined FAM and NOV groups (yellow bars) and found a significant increase in males ($P=0.044$) and a trend for an increase in females ($P=0.051$). **(i,j)** Increased ethanol preference also was seen for males ($P=0.009$) and females ($P=0.014$) in the combined Stress group. **(k,i)** Contingency graphs. The percentage of high drinking (>1.96 standard deviations above the respective CTL mean) vs. typical intake days (144 observations/group). **(k)** FAM and NOV males as well as **(l)** NOV females had increased rates of high drinking days. $n=8$ rats/group. * $P<0.05$, within-sex planned comparisons; $^{\ddagger}P<0.05$, two-sided Fisher's exact test; $^{\textcircled{a}}P=0.051$.

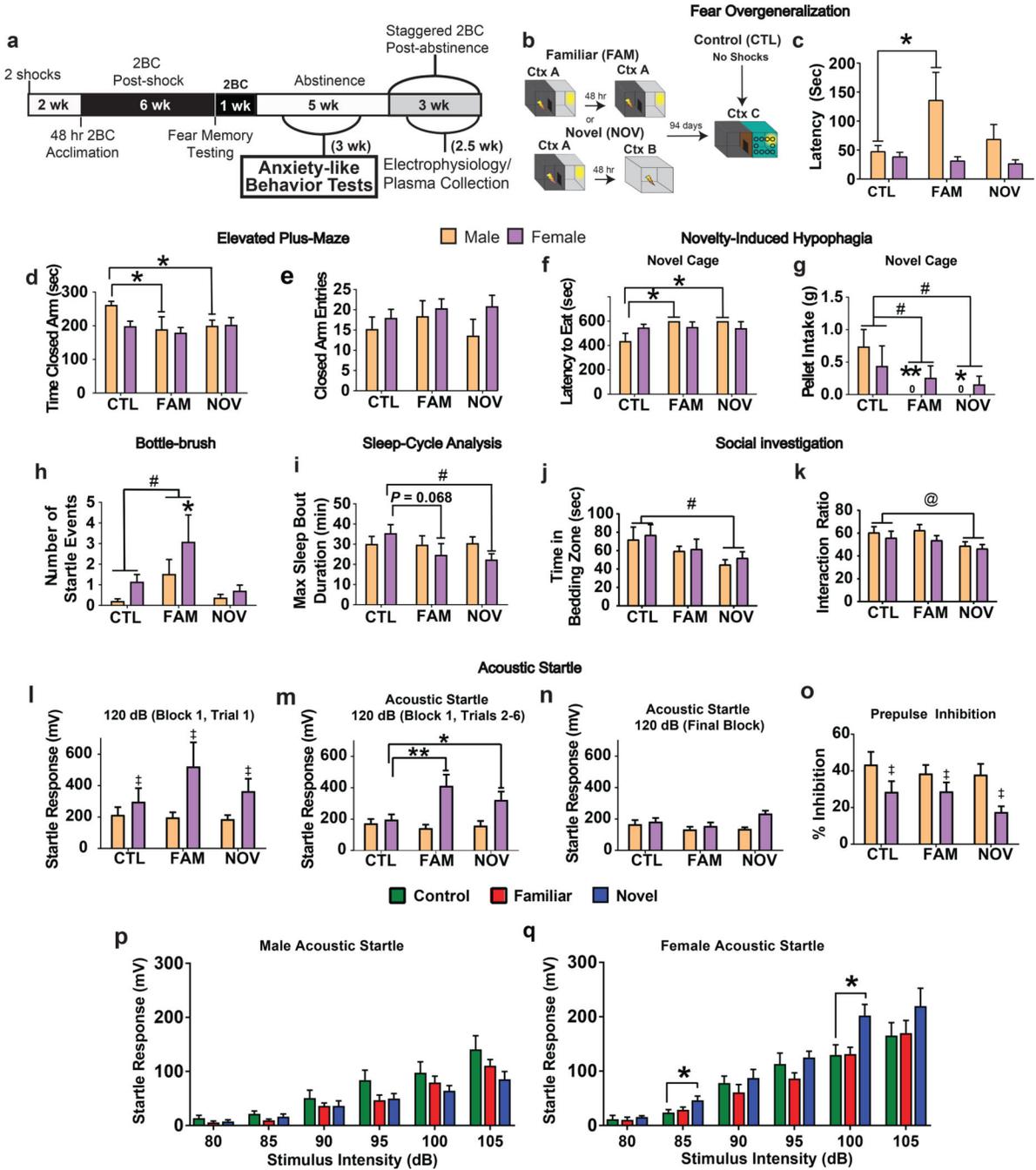


Fig. 2. Effects of prior “2-hit” stress with familiar (FAM) versus novel (NOV) context on anxiety-related behaviors during abstinence.

Rats were tested across 3 weeks in the following order beginning after at least 1 week of abstinence: elevated plus-maze, novelty-induced hypophagia, bottle-brush irritability, and then in counter-balanced order, by CLAMS and social investigation, and then acoustic startle and finally fear overgeneralization. **(a)** Timeline illustrating behavioral tests relative to two-bottle choice limited access. **(b)** Diagram of fear overgeneralization testing. Stress rats received a second footshock in a familiar context (Ctx A) or novel (Ctx B) box. Ninety-four

days later, latency to cross to the dark compartment of a distinct, novel box (Ctx C) was measured. (c) FAM males showed longer latencies ($P=0.009$, planned comparisons). (d) Both FAM males ($P=0.019$, within-sex planned comparisons) and NOV males ($P=0.049$, within-sex planned comparison) spent significantly less times in the closed arms of the elevated plus-maze. There was no interaction with Sex ($F_{1,42}=1.376$, $P<0.264$) and a trend for a Sex main effect ($F_{1,41}=2.455$, $P=0.098$). (e) We did not observe any significant effects of Stress or Sex on the number of entries to the closed arms (all $P's>0.05$). (f) NOV and FAM males did not consume any pellets in novelty-induced hypophagia testing, producing maximum and significantly greater intake latencies ($P's$ 0.008, planned comparisons). (g) A main effect of Stress ($F_{2,41}=4.309$, $P=0.02$), reflected reduced food intake in NOV and FAM rats ($P's$ 0.037, Dunnett's t -test) during novelty-induced hypophagia testing. Planned comparisons suggest males ($P's$ 0.011) rather than females ($P's$ 0.3) drive the effect. (h) In bottle-brush testing, there was a significant main effect of stress ($F_{2,42}=4.324$, $P=0.02$) wherein FAM rats showed increased startle compared with controls ($P=0.034$, Dunnett's t -test). Within-sex planned comparisons showed that the effect was driven by females ($P=0.045$) vs. males ($P=0.168$). (i) Stress reduced the longest bout of sleep in females (NOV, $P=0.028$; FAM, $P=0.068$; planned comparisons) during the first 11-hr of the light phase of a Comprehensive Lab Animal Monitoring System testing. (j) For social investigation/avoidance, NOV rats spent less time in the zone containing soiled bedding from unfamiliar same-sex conspecifics ($P=0.019$, Dunnett's t -test; significant main effect of Stress, $F_{2,41}=3.695$, $P=0.033$). (k) There was also a main effect of Stress ($F_{2,41}=3.329$, $P=0.046$) on percentage of time in the bedding zone (interaction bedding zone time/(interaction zone time+acclimation zone time)*100), and NOV rats favored the unsoiled bedding ($P=0.05$, Dunnett's t -test). * $P<0.05$, ** $P=0.01$ within-sex planned comparisons, # $P<0.05$, Dunnett's t -test, @ $P=0.05$, Dunnett's t -test, † $P<0.05$, main effect of Sex. $n=8$ rats/group, except NOV males where $n=7$ as one NOV male was removed before behavior studies due to illness. All data shown as mean±SEM. (l-q) Acoustic startle response was also assessed. (l) The very first trial of acoustic startle testing was considered separately, as is commonly done, and did not yield significant Stress ($F_{2,41}=0.867$, $P=0.428$) or interaction effects ($F_{1,41}=1.046$, $P=0.361$) in two-way ANOVA. Females showed an overall greater response than males ($F_{1,41}=7.914$, $P=0.007$, main effect of Sex). (m) During the first block of 120-dB stimuli, NOV females ($P=0.045$, within-sex planned comparisons) and FAM females ($P=0.001$, within-sex planned comparisons), but not males of either stress group ($P's>0.6$) exhibited significantly increased startle responses (Stress*Sex interaction: $F_{2,41}=4.126$, $P=0.023$, two-way ANOVA). Females still had higher responses (Sex: $F_{1,41}=18.457$, $P<0.001$), with no main effect of Stress ($F_{2,41}=2.345$, $P=0.109$). (n) Groups did not differ in their responses to the final block of 120-dB stimuli (all $P's>0.05$, two-way ANOVA with within-sex planned comparisons). (o) There were no Stress or interaction effects on prepulse inhibition (all $P's>0.05$, two-way ANOVA with within-sex planned comparisons), but females showed a lower prepulsed response (Sex: $F_{1,41}=11.992$, $P=0.001$). (p) Whereas NOV and FAM males also showed normal responses to less intense acoustic stimuli (all within-sex planned comparisons $P's>0.05$), (q) within-sex planned comparisons showed that NOV females also exhibited exaggerated startle to 85-dB ($P=0.005$) and 100-dB stimuli ($P=0.003$), yielding Stress*Sex interactions (85-dB: $F_{2,41}=3.326$, $P=0.046$; 100-dB: $F_{2,41}=5.740$, $P=0.006$). * $P<0.05$, ** $P=0.001$, within-sex planned comparison, # $P<0.05$, Dunnett's t -test, @ $P<0.07$,

Dunnett's t -test, $\ddagger P < 0.05$, main effect of Sex. $n = 8$ rats /group, except NOV males where $n = 7$. All data are shown as mean \pm SEM.

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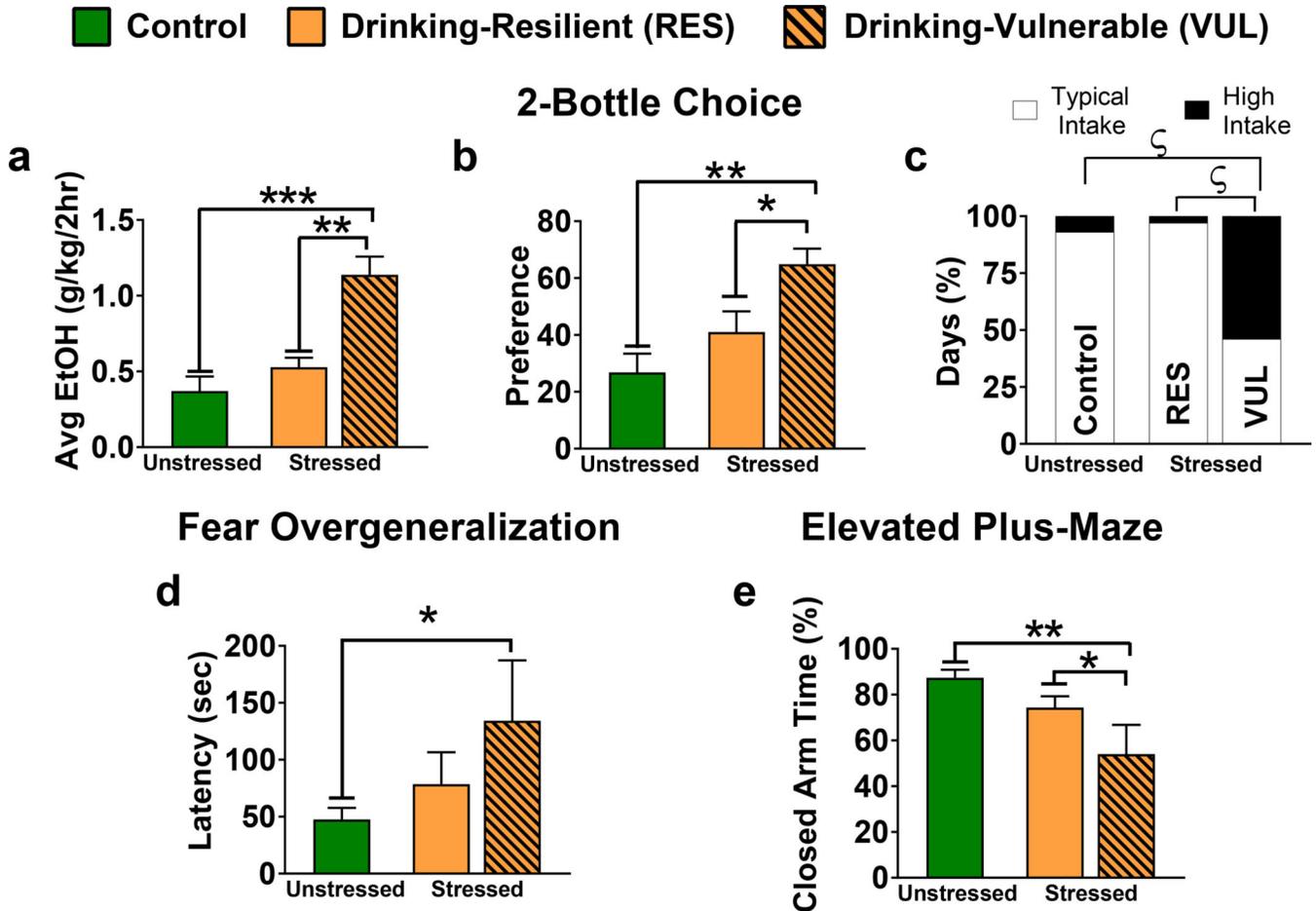


Fig. 3. Drinking-Vulnerable males exhibit increased two-bottle choice (2BC) drinking with heightened fear overgeneralization.

Drinking-Vulnerable rats were defined as those with average ethanol intakes equal to or exceeding 0.8g/kg/2-hr over 4 wk^{44, 45} and ethanol preferences above 50%. For analyses, 7 Stressed males were Vulnerable, 8–9 were Resilient (1 Resilient rat was removed before anxiety-related behavior testing due to illness) and 8 were controls. Only 2 females met criteria for vulnerability, so we focused within-sex planned comparisons to contrast Vulnerable males with control (CTL) and Resilient males. **(a)** Vulnerable males had higher ethanol intakes and preferences than Resilient males ($P=0.002$) and CTL males ($P<0.001$). **(b)** Vulnerable males also had a significantly higher ethanol preference than Resilient ($P=0.021$) and CTL counterparts ($P=0.001$). **(c)** Graphical depictions of contingency tables for percentage of drinking days that were high intake days (>1.96 standard deviations from the mean of the CTL group for the respective sex) and typical intake days per treatment. Due to differences in sample size among groups, the total number of observation days differ (CTL, total days=96; Resilient, total days=108; Vulnerable, total days=84) thus results are standardized as percent to allow comparison. Vulnerable males had a significantly greater rate of high intake days than Resilient ($P<0.001$, 2-sided Fisher's exact test) and CTL counterparts ($P<0.001$, 2-sided Fisher's exact test), whereas CTL and Resilient males did not differ. **(d)** Males were tested in a highly modified, and novel inhibitory avoidance box

to assess fear overgeneralization (i.e. potentially inappropriate generalization of fear to a novel context). Vulnerable males generalized fear to the novel context as demonstrated by a significant increase in latency to cross to the dark compartment ($P=0.014$). (e) Just as Vulnerable males overgeneralized in the novel box by avoiding the dark compartment, they also spent a significantly lower percentage of time during elevated plus-maze testing in the dark, closed arms than control ($P=0.001$) or Resilient ($P=0.043$) counterparts. * $P<0.05$, ** $P<0.01$, *** $P<0.001$; planned comparisons; [§] $P<0.001$, Fisher's exact test. All data are shown as mean±SEM.

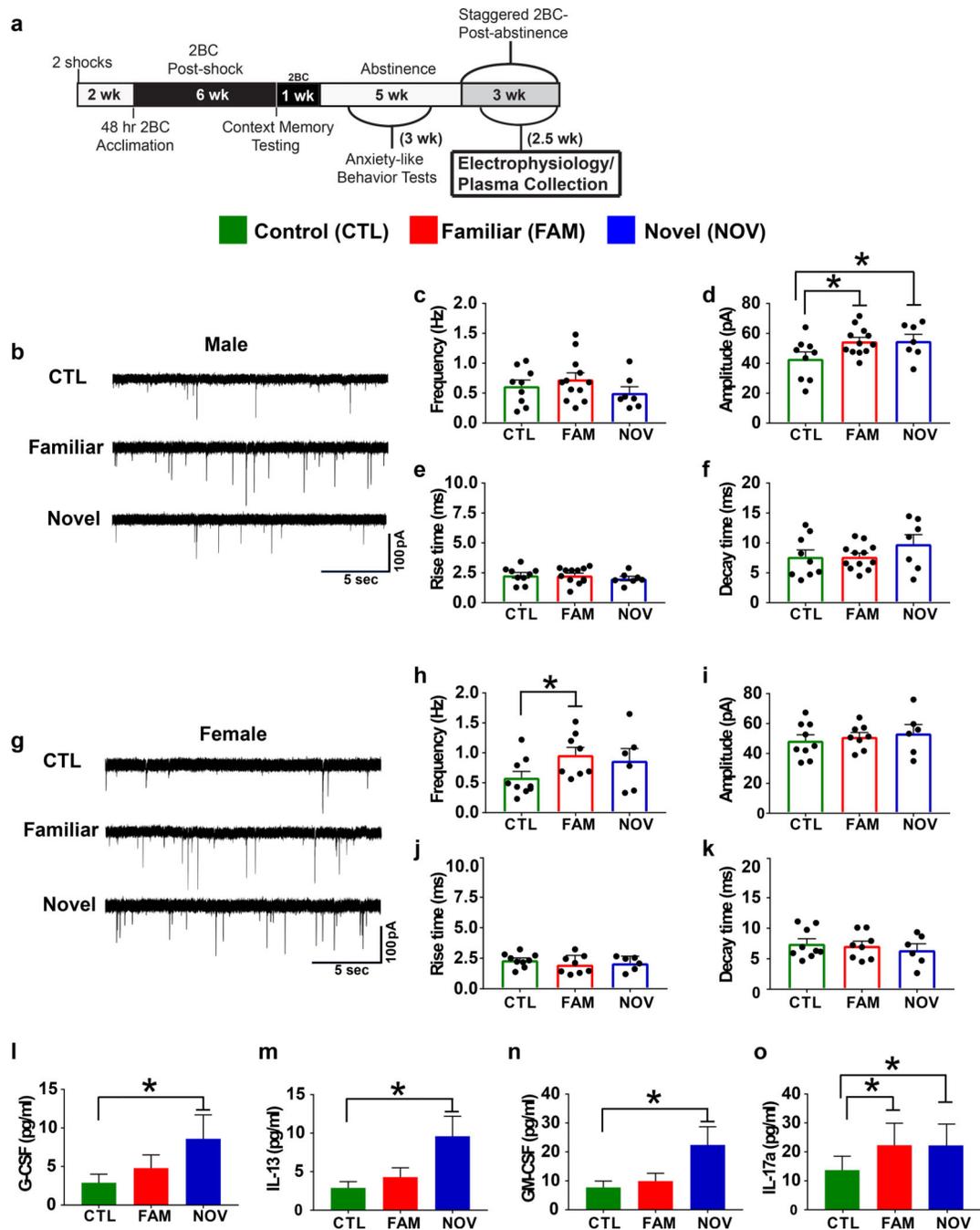


Fig. 4. Stress increased central amygdala (CeA) inhibitory GABAergic transmission and elevated peripheral cytokines.

(a) Timeline showing when physiological endpoints were measured (electrophysiology and plasma collection for cytokine analysis). (b–k) We examined CeA miniature inhibitory postsynaptic currents (mIPSCs). Representative mIPSCs for males (b) and females (g) performed 24-hr into abstinence, 104–123 days post-first stressor ($n=3-4$ rats/group, # cells/group=6–12 (see scatter plots)). (d) Within-sex planned comparisons demonstrated that among males, FAM ($P=0.025$) and NOV ($P=0.044$) stress significantly increased mIPSC

amplitude. **(h)** Conversely, FAM females exhibited increased mIPSC frequency ($P=0.034$). Frequency was not affected in males **(c)** and amplitude was not affected in females **(i)**, and there were no differences in rise **(e,j)** or decay **(f,k)** times in either sex (all P 's >0.05 , planned comparisons). **(i-o)** Graphs showing backlog transformed cytokine and chemokine concentrations with Luminex plate ($n=2$) as a covariate ($n=7-8$ rats/group; 1 FAM and 1 CTL male removed as outliers, $n=7$ NOV males). Least significant difference analyses indicated that NOV males showed significantly elevated plasma concentrations of G-CSF, IL-13 and GM-CSF compared with controls (P 's >0.04), while plasma IL-17a levels were elevated in both NOV ($P=0.034$) and FAM ($P=0.041$) males. * $P<0.05$, within-sex planned comparisons. All data are shown as mean \pm SEM.

Table 1.

Cytokine stress and resilience profiles from linear discriminant analysis (LDA)

"Stress" LDA Profile			"Resilience" LDA Profile		
Cytokine	<i>r</i> -value	<i>P</i> -value	Cytokine	<i>r</i> -value	<i>P</i> -value
G-CSF	0.617	0.003	G-CSF	0.647	0.002
IL-13	0.586	0.005	Leptin	0.473	0.03
GM-CSF	0.561	0.008	IL-13	0.467	0.033
IL-6	0.470	0.032	TNF α	0.413	
IL-17A	0.468	0.032	RANTES	0.382	
Leptin	0.467	0.033	IL-17A	0.350	
IL-4	0.451	0.04	IL-6	0.348	
TNF α	0.406		Eotaxin	0.343	
IL-5	0.394		GM-CSF	0.337	
IP-10	0.373		IP-10	0.310	
MIP2	0.330		MIP-2	0.309	
Eotaxin	0.305		EGF	0.306	
EGF	0.291		IL-4	0.219	
IL-1 β	0.273		IL-12p70	0.219	
MCP1	0.198		IL-1 α	0.211	
IL-10	0.186		LIX	0.197	
IL-1 α	0.166		MCP-1	0.185	
IL-12p70	0.138		Fractalkine	0.156	
RANTES	0.111		IL-5	0.120	
LIX	0.110		VEGF	0.116	
VEGF	0.051		IL-1 β	0.033	
Fractalkine	0.050		IL-10	-0.007	
MIP-1 α	0.009		IL-18	-0.007	
IL-18	0.031		MIP-1 α	-0.011	
IL-2	0.079		IL-2	-0.149	

Note: Values show the strength of correlation (Pearson's *r*) of each cytokine to separate LDA functions that discriminated male rats according to either their Stress history (left) or drinking-Resilience (right). Significant correlations ("loadings") are indicated with their corresponding *P*-values. Note that significance vs. non-significance shown for a given cytokine (vs. $P < 0.05$) was the same whether calculated within the LDA from each individual cytokine's Wilks' lambda or externally as the correlation of the cytokine to the discriminant function score. Stress LDA scores differed significantly across stress groups with NOV>FAM>CTL. Drinking-Resilience associated with significantly higher Resilience LDA scores. See text and Fig.4 for univariate group differences for the significantly predictive cytokines (**shown in bold**). $n=21$ male rats.