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Authors	Zabik, Nicole L;Flook, Elizabeth A;Feola, Brandee;Benningfield, Margaret M;Silveri, Marisa M;Winder, Danny G;Blackford, Jennifer Urbano
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RESEARCH ARTICLE

ALCOHOL CLINICAL & EXPERIMENTAL RESEARCH

Bed nucleus of the stria terminalis network responses to unpredictable threat in early alcohol abstinence

Nicole L. Zabik[1] | Elizabeth A. Flook[2,3,4] | Brandee Feola[5] | Margaret M. Benningfield[3,4,5] | Marisa M. Silveri[6,7] | Danny G. Winder[4,8] | Jennifer Urbano Blackford[1,4,5]

[1]Munroe-Meyer Institute, University of Nebraska Medical Center, Omaha, Nebraska, USA

[2]Department of Psychiatry, University of Pennsylvania, Philadelphia, Pennsylvania, USA

[3]Vanderbilt University School of Medicine, Nashville, Tennessee, USA

[4]Vanderbilt Center for Addiction Research, Vanderbilt University, Nashville, Tennessee, USA

[5]Department of Psychiatry and Behavioral Sciences, Vanderbilt University Medical Center, Nashville, Tennessee, USA

[6]Neurodevelopmental Laboratory on Addictions and Mental Health, Brain Imaging Center, McLean Hospital, Belmont, Massachusetts, USA

[7]Department of Psychiatry, Harvard Medical School, Boston, Massachusetts, USA

[8]Department of Neurobiology, UMass Chan Medical School, Worcester, Massachussets, USA

Correspondence
Jennifer Urbano Blackford, Munroe-Meyer Institute, University of Nebraska Medical Center, Omaha, NE, USA.
Email: jblackford@unmc.edu

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Abstract

Background: Anxiety during early alcohol abstinence, likely resulting from neural changes caused by chronic alcohol use, contributes to high relapse rates. Studies in rodents show heightened activation during early abstinence in the bed nucleus of the stria terminalis (BNST)—a neural hub for anxiety—and its extended anxiety-related corticolimbic network. Despite the clinical importance of early abstinence, few studies investigate the underlying neural mechanisms.

Methods: To address this gap, we investigated brain function in early alcohol abstinence (EA = 20, 9 women) relative to controls (HC = 20, 11 women) using an unpredictable threat task shown to engage the BNST and corticolimbic brain regions involved in anxiety and alcohol use disorder (AUD). Group, anxiety, and sex were predictors used to determine whole-brain activation and BNST functional connectivity.

Results: We found widespread interactions of group × anxiety and group × anxiety × sex for both activation and BNST connectivity during unpredictable threat. In the EA group, higher anxiety was correlated with activation in the BNST, rostral anterior cingulate cortex (ACC), insula (men only), and dorsal ACC (men only). In the HC group, higher anxiety was negatively correlated with activation in the BNST, nucleus accumbens, thalamus, and insula (men only). For connectivity, anxiety was positively correlated in EA and negatively correlated in HC, between the BNST and the amygdala, ventromedial prefrontal cortex (PFC), and dorsomedial PFC; EA men showed stronger BNST-vmPFC connectivity than HC men.

Conclusions: These novel findings provide preliminary evidence for alterations in the BNST and anxiety-related corticolimbic brain regions in early alcohol abstinence, adding to growing literature in humans supporting the BNST's role in anxiety and sex-dependent effects of chronic alcohol use.

KEYWORDS
abstinence, bed nucleus of the stria terminalis, sex differences, unpredictable threat

INTRODUCTION

Alcohol use disorder (AUD) is a common, debilitating mental health disorder. Rates of AUD have doubled since 2019, with nearly 30 million Americans having AUD in 2022 (SAMHSA, 2022). Recovery, or abstinence, from AUD is possible but remains difficult, with nearly half of individuals relapsing in the first year of abstinence (Kushner et al., 2000). One driver of high relapse rates is thought to be the heightened anxiety that emerges during early abstinence, where withdrawal symptoms have subsided and negative affect emerges. Anxiety is likely the result of neural changes in the stress response system caused by chronic alcohol exposure (Koob & Volkow, 2016). Early abstinence is a critical period of recovery, yet little is known about the neural mechanisms underlying this phase of AUD.

Numerous studies report that the bed nucleus of the stria terminalis (BNST)—a central hub for anxiety responses—exhibits heightened activation during early abstinence. The BNST is part of an extended anxiety-related corticolimbic network, termed here the "BNST network," that includes brain regions with established structural and functional connectivity with the BNST (Avery et al., 2014). The BNST network includes the amygdala, anterior hippocampus, hypothalamus, anterior insula, and ventromedial prefrontal cortex. These brain regions also have known associations with AUD and anxiety and this network has heightened activation during abstinence. For example, acute withdrawal after chronic intermittent alcohol exposure increases activation of the BNST (i.e., increased c-Fos, decreased neuronal firing threshold), and the increased BNST activation persists for 2–5 weeks in rodents (Centanni et al., 2019; Francesconi et al., 2009; Kash et al., 2009; Marcinkiewcz et al., 2016; Pleil et al., 2015; Silberman et al., 2013; Wills et al., 2012). Negative affect behaviors, like decreased time spent in open arms of an elevated-plus maze and increased immobile time in forced swim test, emerge at 2 weeks and persist for at least 4 weeks (Holleran & Winder, 2017). Inhibiting insular cortex inputs into the BNST decreases negative affect-like behaviors, as indicated by decreased latency to feed in a novelty-suppressed feeding task (Centanni et al., 2019), pointing to the importance of a BNST-corticolimbic network. BNST network alterations are also observed in humans during early alcohol abstinence; for example, we recently reported that a BNST network has stronger structural connectivity in abstinent women compared to control women; men did not exhibit group differences (Flook et al., 2021). Interestingly, an investigation of BNST intrinsic functional connectivity in early abstinence also showed weaker BNST network connectivity in abstinent men, compared to control men, and a positive correlation between BNST-amygdala connectivity and anxiety in abstinent men and women (Flook et al., 2023). Together, evidence in rodents and humans suggests that chronic alcohol exposure alters activation and connectivity of the BNST, likely contributing to anxiety symptoms during early abstinence. While structural connectivity and intrinsic functional connectivity findings in AUD suggest differences in the underlying neural circuitry, a major gap in knowledge is whether differences are observed during functionally relevant tasks, as task-based fMRI studies provide greater power for revealing differences in emotion or threat processing (Zhao et al., 2023).

Translational tasks of unpredictable threat are vital for investigating neural components of anxiety during early alcohol abstinence. Preclinical studies support unpredictable threat as a viable model for investigating anxiety, as it produces anxiety-like behavior in rodents and engages an anxiety-related network containing the BNST and prefrontal cortex (Glover et al., 2020; Goode et al., 2019; Luyten et al., 2012; Ressler et al., 2020). Previous studies of unpredictable threat in AUD show stronger startle responses to unpredictable threat (Gorka et al., 2020; Moberg et al., 2017) and greater activation of the insula and anterior cingulate cortex to unpredictable and predictable threat—regions within the BNST network (Gorka et al., 2020; Moberg et al., 2017). These data support unpredictable threat as a valuable task for probing anxiety in AUD. However, to our knowledge, no previous studies have investigated responses to unpredictable threat in early abstinence, or the role of the BNST network in these responses.

To address this knowledge gap, we investigated neural activation during unpredictable threat in adults in early abstinence compared to controls. The first aim of this study is to determine whether brain activation during unpredictable threat is altered in early abstinence. The second aim is to investigate alterations in BNST functional connectivity during unpredictable threat, as our group and others have previously demonstrated changes in structural and functional connectivity in early abstinence from AUD (Flook et al., 2021, 2023; Padula et al., 2015; Radoman et al., 2024; Rivas-Grajales et al., 2018; Ruiz et al., 2013; Sawyer et al., 2017, 2019). Sex and anxiety are included in the analyses based on previous evidence that BNST activation and connectivity are correlated with anxiety and differ by sex (Becker & Koob, 2016; Flook et al., 2021, 2023). This study is an important step toward understanding the mechanisms underlying heightened anxiety in early abstinence.

METHODS

Participants

Participants consisted of adults 21–40 years of age with an AUD in early abstinence (EA = 20) and light social drinkers with no history of AUD as controls (HC = 20). Psychiatric diagnoses of all participants (exclusions outlined below) were determined using the Structured Clinical Interview for DSM-IV (First, 1997). Interviews were conducted by trained study personnel and were confirmed by a study psychiatrist (MB).

Participants in the EA group were recruited using community advertisements, a recruitment registry, and referrals from a local treatment facility. Participants in the EA group had to meet criteria for an AUD immediately prior to the current period of abstinence and have been abstinent for 30–180 days at the interview day. While acute withdrawal typically subsides within 3–7 days in humans, we selected 30 days based on preclinical findings that affective disturbances do not emerge immediately after acute withdrawal; instead, affective disturbances emerge around 2 weeks (Holleran & Winder, 2017). We selected the upper range based on a combination of evidence from humans that (1) negative affect is often sustained (Heilig et al., 2010; Holleran & Winder, 2017) and (2) recruitment feasibility, as it is challenging to recruit people in early abstinence within a narrow window. Other studies of abstinence in AUD have similar, and often broader, windows. For example, Camchong et al. (2013a, 2013b) had a "short-term abstinence" group that was abstinent for 42–105 days, and Orban et al. (2013) included a range of 15–893 days of abstinence. Exclusion criteria for the EA group included: (1) current drug or alcohol use (except nicotine); (2) current psychiatric disorder, except anxiety or depressive disorder; (3) current use of psychoactive medications (past 6 weeks) other than a stable dose of SSRI or SNRI; (4) significant medical illness (e.g., cancer) or neurological illness (e.g., brain abnormality); and (5) any MRI safety risk (e.g., metal in body).

Controls were light social drinkers recruited using a large institutional registry and were defined as individuals with at least one standard drink in the past year, no drinking period that met criteria for AUD, and no episodes of binge drinking in the past year. Exclusion criteria for the controls included: (1) current or lifetime psychiatric disorder, including alcohol or substance use disorders (except nicotine); (2) current use of psychoactive medications (6 months); (3) significant medical (e.g., cancer) or neurological illness (e.g., brain abnormality); and (4) any MRI safety risk (e.g., metal in body).

All participants were required to screen negative on a urine drug screen (Triage Drugs of Abuse Panel, Biosite Diagnostics, San Diego, CA), an alcohol breath screen (Intoximeters, Inc, St Louis, Missouri), and a urine ethyl glucuronide (EtG) test on both study days. The sample reported here is the same as previously reported studies on structural and intrinsic functional connectivity (Flook et al., 2021, 2023). Vanderbilt University Institutional Review Board approved the study, and written informed consent was obtained after providing participants with a complete description of the study.

Alcohol and anxiety measures

Alcohol use was also evaluated using the Alcohol Use Disorders Identification Test (AUDIT) (Babor et al., 2001). Extent of lifetime drinking was assessed using the Lifetime Drinking History Scale, and timeline follow-back methods were used to establish the current period of alcohol abstinence for EA participants (Skinner & Sheu, 1982).

Anxiety scores were assessed using reliable and valid questionnaires that measured multiple domains of anxiety (e.g., social anxiety, worry). Each measure had good internal reliability in this sample (Cronbach's alphas provided in parentheses). The questionnaires included: State–Trait Anxiety Inventory ($\alpha = 0.94$) (Spielberger et al., 1983), Brief Fear of Negative Evaluation ($\alpha = 0.95$) (Leary, 1983), Beck Anxiety Inventory ($\alpha = 0.94$) (Beck et al., 1988), Intolerance of Uncertainty ($\alpha = 0.97$) (Carleton et al., 2007), Liebowitz Social Anxiety Scale ($\alpha = 0.97$) (Liebowitz, 1987), and Penn State Worry Questionnaire ($\alpha = 0.95$) (Meyer et al., 1990). The anxiety measures were correlated for both groups (average $r = 0.50$); therefore, a composite anxiety score was created by averaging standardized scores ($M = 0$, $SD = 1$; see Table 1) for each questionnaire. Therefore, the composite anxiety scores represent broad domains of anxiety. Herein, these composite scores are referred to as "anxiety scores."

TABLE 1 Demographics of participants.

	Early abstinence ($n = 20$)	Healthy control ($n = 20$)
Sex, *n* (% women)	9 (45%)	11 (55%)
Age, years (SD)	31.0 (5.8)	29.0 (4.4)
Race, *n* (%)		
White	16 (80%)	14 (70%)
Black	2 (11%)	2 (10%)
Other	1 (5%)	4 (20%)
Hispanic/Latino, *n* (%)	1 (5%)	2 (10%)
AUDIT, mean (SD)*	25.9 (9.2)	2.8 (1.8)
Length of abstinence, days (SD)	127.4 (47. 8)	–
Anxiety score, mean (SD)*	0.62 (0.76)	−0.62 (0.37)
Nicotine use (current), *n* (%)*	6 (30%)	0 (0%)

Abbreviations: AUDIT, Alcohol Use Disorder Identification Test; SD, standard deviation.

*Significant difference between groups at $p < 0.05$. Sex refers to sex assigned at birth.

Unpredictable threat task

We used an event-related cued anticipation task with unpredictable and predictable threat conditions to determine neurobehavioral responses during threat anticipation (Feola, McHugo, et al., 2021; Feola, Melancon, et al., 2021). Images were presented using Eprime software (Version 2.0, Psychology Software Tools, Sharpsburg, PA). Participants were trained to associate three different cues (colored shapes) with three different events: (1) a predictable neutral cue (purple diamond) was always followed by a neutral face; (2) a predictable threat cue (blue square) was always followed by a fear face; and (3) an unpredictable threat cue (yellow circle) was either followed by a neutral face or a fear face (Figure 1). Fear face images (i.e., threat images) and neutral face images were selected from the Karolinska Directed Emotional Faces database (40 women/40 men) (Lundqvist et al., 1998) and were presented using Eprime software (Version 2.0, Psychology Software Tools, Sharpsburg, PA). Following training, participants underwent four runs with 24 cues per run (6 min each, eight images and cues per condition); total events per condition were as follows: 32 predictable neutral cues and images;

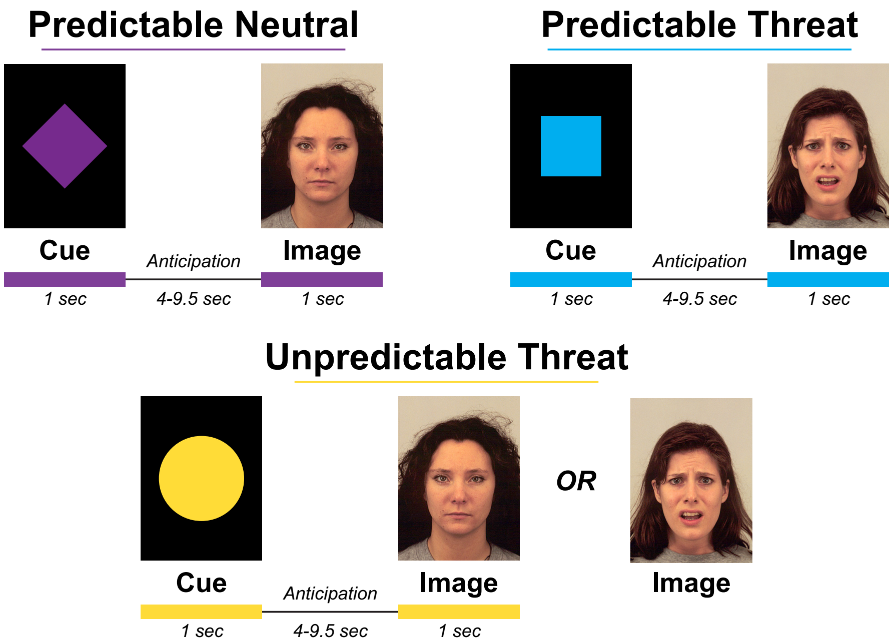

FIGURE 1 Unpredictable threat task. Participants were trained to associate three cues (colored shapes) with different events: predictable neutral face (purple diamond); predictable threat face (blue square); unpredictable neutral or threat face (yellow circle).

32 predictable threat cues and images; 32 unpredictable cues, 16 unpredictable threat images, 16 unpredictable neutral images. Cues and images were presented for 1 s each, with a 5- to 8-s jittered interstimulus interval (mean = 6 s) to evoke an anticipatory period. A fixation cross was presented for the first and last 20 s of each run to provide a baseline measurement of brain activation. To ensure attention, participants were asked to push a single button each time they saw a cue and image. Average accuracy and response time for all trials are presented in the Supporting Information.

Functional imaging

Data collection and processing

Data were collected at the Vanderbilt University Institute of Imaging Science (VUIIS) on a Phillips 3T MRI scanner with a 32-channel head coil. Functional MRI data were acquired using a sequence optimized for measuring signal in subcortical regions: 2 × 2.5 × 2.5 mm slices; an 80 × 80 matrix (reconstructed to 128 × 128); a 2 s TR; 28 ms TE; 90° flip angle, SENSE factor = 2. This sequence was designed specifically to avoid signal dropout in the amygdala and BNST and was previously used to measure amygdala and BNST activation (Feola et al., 2023). An automated higher-order shim procedure was applied to minimize possible magnetic field inhomogeneities. For anatomical verification, we collected a standard anatomical (T1-weighted) image.

Functional data were preprocessed using the following steps: slice time correction; realignment; co-registration; normalization into SPM EPI space; high pass filter; and smoothing (6 mm FWHM). For each subject, motion was assessed, and functional images were visually inspected for artifacts, signal dropout, and coverage. Individual runs were excluded for excessive motion (> 3 mm). One run was removed for two participants (2 HC), and two runs were removed for two participants (1 EA, 1 HC).

Brain activation

Since our primary goal was to characterize brain activation in early abstinence using the unpredictable threat task, we chose a whole-brain approach. Individual participant GLMs were estimated for each cue and image type (cues: predictable neutral, predictable threat, and unpredictable; images: predictable neutral, predictable threat, unpredictable neutral, unpredictable threat). Given our interest in the BNST and planned BNST connectivity analyses, we also performed analysis of BNST activation.

Functional connectivity of the BNST

Whole-brain functional connectivity analyses were performed with the bilateral BNST as the seed region using our published BNST mask (Avery et al., 2014). Functional connectivity was calculated using gPPI (Friston et al., 1997; McLaren et al., 2012). BNST location was verified via visual inspection by comparing the BNST on the T1W image and the BNST mask (Avery et al., 2014).

Power analyses

Sample size was determined *a priori* as part of the grant application that supported data collection (R21AA025385). The proposed sample size ($N=40$; 20 subjects per group) was determined to give 80% power to detect medium-large effects (Cohen's $d=0.60$) for the group comparison. We proposed exploratory analysis of sex differences and acknowledged the sample size was only powered to find large effect sizes (Cohen's $d>1.1$).

Statistical analyses

The primary analysis focused on unpredictable versus predictable contrasts for whole-brain activation and BNST functional connectivity. The comparisons included: (1) *unpredictable* versus *predictable neutral cues*, defined as unpredictable cue minus predictable neutral cue; (2) *unpredictable* versus *predictable threat cues*, defined as unpredictable cue minus predictable threat cue; (3) *unpredictable* versus *predictable neutral images*, defined as unpredictable neutral images minus predictable neutral images; and (4) *unpredictable* versus *predictable threat images*, defined as unpredictable threat images minus predictable threat images. We chose to investigate cues and images separately, as they represent unique components of threat processing: brain responses to cues reflect anticipation of an upcoming event (threat or neutral), while brain responses to images reflect processing of the images (threat/neutral). Evidence that the cues (anticipation) and images engage distinct networks has been demonstrated by others (e.g., Klumpers et al., 2017). We focused on unpredictable versus predictable contrasts, as prior research indicates that individuals with an AUD show enhanced startle during unpredictable (vs. predictable) aversive events (Gorka et al., 2016, 2020; Moberg et al., 2017). A multiple regression was performed with group (EA/HC), anxiety score, and sex (men/women) as predictors in SPM12, along with all interactions. Whole-brain analyses were investigated for these contrasts using SPM cluster-based thresholding for whole-brain $p<0.05$ ($p<0.005$ and $k>90$ for activation; $p<0.005$ and $k>100$ for connectivity). Significant group × anxiety × sex interactions in whole-brain activation were also followed up with functional connectivity analyses; these results are presented in the Supporting Information. All significant whole brain and BNST connectivity results were followed up with exploratory linear mixed model analyses in the EA group, which included days abstinent in the model; these results are presented in the Supporting Information. For the BNST activation analyses, percent signal change was extracted for the bilateral BNST, and a linear mixed model (controlling for hemisphere) was performed to test for group, sex, and anxiety for each of the planned contrasts. Effect sizes are provided for significant main effects and interactions.

RESULTS

Sample characteristics and behaviors

Participants did not differ on primary demographics (age, sex, race; Table 1). As expected, EA participants had higher AUDIT, anxiety scores, and nicotine use than HC participants. No main effects or interactions with sex were detected (p's >0.05); See Table S1 for details.

All participants had acceptable button push accuracy. There were no significant differences in accuracy or reaction time between groups (EA/HC or men/women) and no correlations with anxiety scores (see Figure S1).

Brain activation

Activation during cues

Unpredictable versus predictable neutral

For the BNST, there was a group × anxiety interaction ($p=0.05$, $d=0.74$). BNST activation was negatively correlated with anxiety in the HC group ($r=-0.68$) but not correlated in the EA group ($r=-0.08$). For the whole-brain analysis, there was a group × anxiety interaction in the left nucleus accumbens and caudate (voxels = 243, $p=0.022$, $Z=3.99$, $xyz=-12, 16, -14$, $d=1.62$) and left postcentral gyrus (voxels = 376, $p=0.006$, $Z=3.61$, $xyz=-60, -10, 38$, $d=1.43$). Post hoc analyses revealed that higher anxiety levels in the HC group were negatively correlated with activation in the left accumbens and caudate ($r=-0.74$) and left postcentral gyrus ($r=-0.75$). We also found a main effect of anxiety in the left putamen, where higher anxiety levels were negatively correlated with activation (Supporting Information).

Unpredictable versus predictable threat

There were no significant differences for the BNST. For the whole-brain, there was a group × anxiety × sex interaction in the left postcingulate cortex (voxels = 453, $p=0.002$, $Z=3.65$, $xyz=-10, -46, 32$, $d=1.45$; Figure 2), left mid-occipital lobe (voxels = 237, $p=0.020$, $Z=3.80$, $xyz=-40, -68, 24$, $d=1.53$), and right superior frontal gyrus (voxels = 174, $p=0.042$, $Z=3.70$, $xyz=18, 46, -14$, $d=1.48$; Figure 2). Post hoc analyses by sex in the postcingulate cortex showed a group × anxiety interaction in men ($t=2.941$, $p=0.010$), but not in women ($p=0.083$); the interaction in men was driven by a positive correlation between anxiety and activation in HC men ($r=0.76$), but not EA men ($r=0.12$). Post hoc analyses by sex in the mid-occipital lobe showed a group × anxiety interaction in women ($t=-3.148$, $p=0.028$), but not in men ($p=0.068$). The interaction in women was driven by a negative correlation between anxiety and activation in HC women ($r=-0.75$), but not EA women ($r=0.34$). Post hoc analyses by sex in the right superior frontal gyrus did not reveal a significant group × anxiety interaction in men ($p=0.920$) or women ($p=0.059$). Post hoc functional connectivity analysis with the postcingulate cortex as a seed is described in the Supporting Information. We also found an anxiety × sex interaction in the right calcarine sulcus (Supporting Information).

Activation during images

Unpredictable versus predictable neutral

There were no significant BNST differences. For the whole-brain, there was a group × anxiety interaction in the rostral anterior

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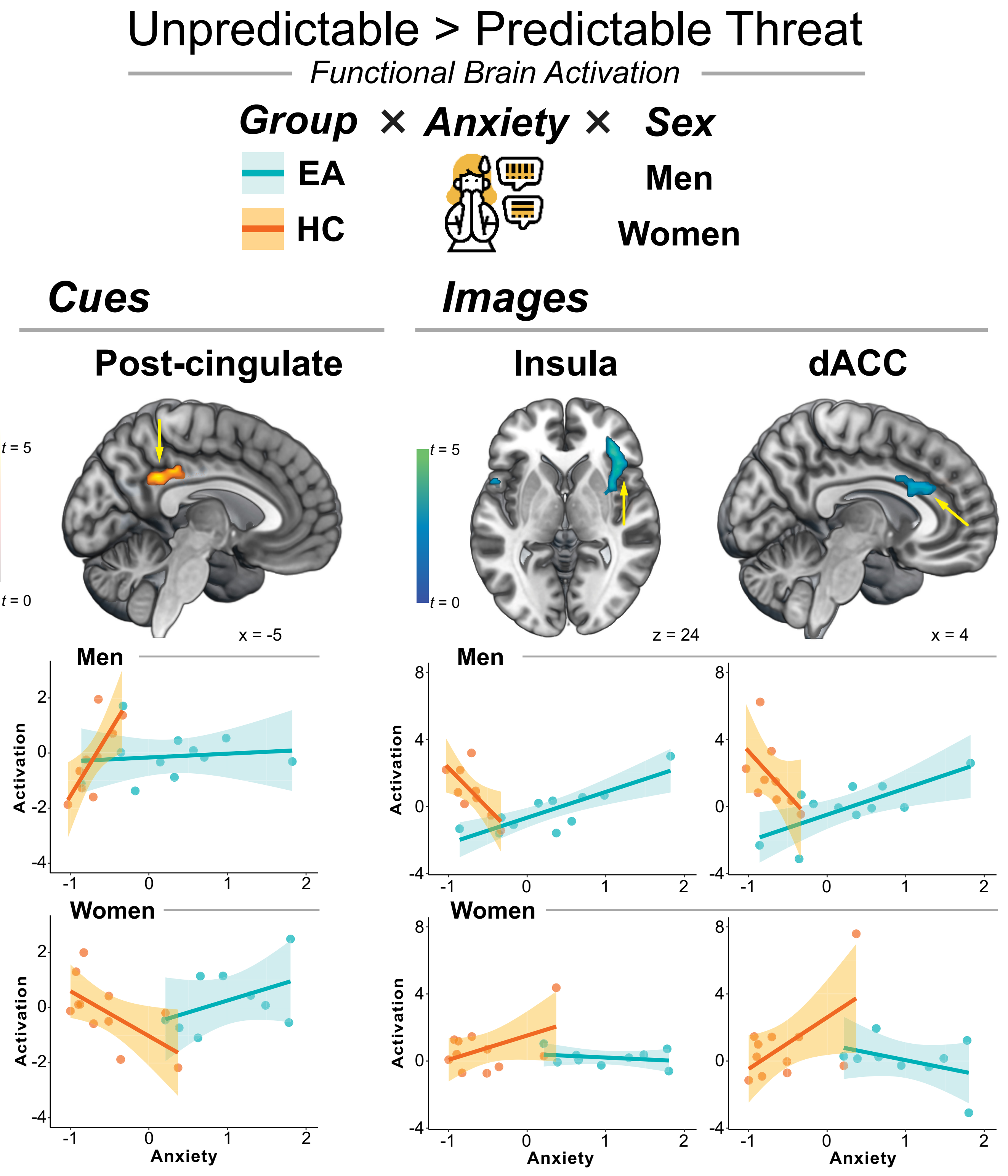

FIGURE 2 Group × anxiety × sex interaction during unpredictable versus predictable threat cues and images. HC men with higher anxiety exhibited greater postcingulate activation during cues and lesser insula and dACC activation during images. Activation refers to eigenvariate of postcingulate (*xyz* = −10, 46, 32), insula (*xyz* = 46, −4, −16), and dACC clusters (*xyz* = −2, 26, 24). EA, early abstinence; HC, healthy control; dACC, dorsal anterior cingulate cortex.

cingulate cortex (rACC) and thalamus (medial pulvinar and ventral posterolateral regions) (rACC: voxels = 245, $p = 0.013$, $Z = 3.84$, *xyz* = −14, 50, 4, $d = 1.55$; thalamus: voxels = 346, $p = 0.004$, $Z = 4.31$, *xyz* = 10, −26, 2, $d = 1.79$; voxels = 181, $p = 0.029$, $Z = 3.71$, *xyz* = −14, −24, 4, $d = 1.48$). In the EA group, higher anxiety levels were positively correlated with rACC activation ($r = 0.59$). In the HC group, higher anxiety levels were negatively correlated with activation in the rACC ($r=-0.54$) and thalamus ($r=-0.57$). We also found a group × anxiety interaction in the cerebellum, and an anxiety × sex interaction in the right calcarine sulcus and left occipital lobe/cuneus (Supporting Information).

Unpredictable versus predictable threat

For the BNST, there was a group × anxiety interaction ($p=0.005$, $d=1.09$); BNST activation was positively correlated with anxiety in the EA group ($r=0.50$) and negatively correlated in the HC group ($r=-0.59$). For the whole-brain, there was a group × anxiety × sex interaction in the insula and dorsal ACC (dACC) (insula: voxels = 627, $p<0.001$, $Z=3.96$, $xyz=46$, −4, −16, $d=1.61$; dACC: voxels = 307, $p=0.004$, $Z=3.34$, $xyz=-2$, 26, 24, $d=1.30$; Figure 2). The group × anxiety interaction was significant in men (insula: $p=0.001$; dACC: $p=0.016$), but not in women (insula: $p=0.187$; dACC: $p=0.074$). Men in the EA group showed a positive correlation between anxiety and insula ($r=0.83$) and dACC ($r=0.73$) activation, and men in the HC group showed a negative correlation between anxiety and insula activation ($r=-0.71$). Post hoc functional connectivity analyses with insula and dACC seeds are described in the Supporting Information.

Functional connectivity of the BNST

Connectivity during cues

Unpredictable versus predictable neutral

There was a group × anxiety interaction in the ventromedial prefrontal cortex (vmPFC), amygdala, and dorsomedial PFC (dmPFC) (vmPFC: voxels = 287, $p=0.003$, $Z=3.82$, $xyz=-6$, 52, −16, $d=1.53$; amygdala: voxels = 246, $p=0.006$, $Z=4.05$, $xyz=26$, 0, −14, $d=1.65$; dmPFC: voxels = 475, $p<0.001$, $Z=4.22$, $xyz=-12$, 50, 36, $d=1.74$; voxels = 171, $p=0.018$, $Z=3.59$, $xyz=22$, 52, 22, $d=1.42$; Figure 3). In the EA group, anxiety was positively correlated with BNST connectivity with the vmPFC ($r=0.30$), amygdala ($r=0.29$), and dmPFC ($r=0.44$). In the HC group, anxiety was negatively correlated with BNST connectivity (BNST-vmPFC: $r=-0.26$, BNST-amygdala: $r=-0.37$, BNST-dmPFC: $r=-0.41$).

There was also a group × sex interaction in the vmPFC, mediodorsal thalamus, and dorsolateral PFC (dlPFC) (vmPFC: voxels = 319, $p=0.002$, $Z=3.57$, $xyz=-6$, 52, −14, $d=1.41$; thalamus: voxels = 156, $p<0.001$, $Z=4.06$, $xyz=-6$, −24, 2, $d=1.48$; dlPFC: voxels = 215, $p=0.009$, $Z=4.01$, $xyz=-18$, 28, 42, $d=1.63$). The BNST-vmPFC interaction was driven by differences in men; EA men had stronger BNST-vmPFC connectivity than HC men (Figure 3). The group × sex differences in BNST connectivity with the mediodorsal thalamus and dlPFC were driven by differences in women; EA women had weaker BNST-mediodorsal thalamus and stronger BNST-dlPFC connectivity than HC women (Figure S2). We also found an anxiety × sex interaction in several brain regions including the hippocampus, amygdala, and vmPFC (Table S2).

Unpredictable versus predictable threat

There was a group × anxiety interaction for BNST-vmPFC connectivity (voxels = 456, $p=0.001$, $Z=4.41$, $xyz=8$, 52, −10, $d=1.95$; Figure 4), with a positive correlation in the EA group ($r=0.41$) and a negative correlation in the HC group ($r=-0.15$).

There was a group × sex interaction in the vmPFC and ventrolateral thalamus (vmPFC: voxels = 418, $p=0.001$, $Z=4.55$, $xyz=8$, 52, −10, $d=1.93$; thalamus: voxels = 159, $p=0.032$, $Z=4.04$, $xyz=16$, −2, −2, $d=1.65$; Figure 4). Among men, EA men had stronger BNST-vmPFC and weaker BNST-ventrolateral thalamus connectivity than HC men. Among women, EA women had weaker BNST-vmPFC and BNST-ventrolateral thalamus connectivity than HC women.

We detected an anxiety × sex interaction in several brain regions, including the thalamus and vmPFC; see Table S3 for details.

Connectivity during images

Unpredictable versus predictable neutral

We found a main effect of sex in the anterior cingulate cortex; see Supporting Information Results Section for details.

Unpredictable versus predictable threat

There were no significant main effects or interactions in BNST functional connectivity during unpredictable versus predictable threat images.

DISCUSSION

The goal of the present study was to determine whether people in early abstinence from alcohol use disorder (AUD) have different neural responses to unpredictable threat compared to healthy adults. We found that activation and functional connectivity of the BNST network is altered during early abstinence, and that anxiety and sex play a role in these alterations. Thus, our findings from a small sample of alcohol abstinent adults reveal the significant impact of anxiety and sex on neural activation during early abstinence from alcohol, extending decades of research in animal models and recent studies in humans.

The first major study finding was that adults in early abstinence from an AUD displayed altered activation to unpredictable threat in multiple regions of the BNST network, and that these relations were dependent on anxiety and sex. The BNST and its anxiety-related corticolimbic network coordinate responses to stress and anxiety, and are also implicated in stress-induced reinstatement in rodents and risk of relapse in humans (Alvarez et al., 2015; Avery et al., 2016; Flook et al., 2020; Huang et al., 2010; Paulus et al., 2001; Torrisi et al., 2015). Group differences in several brain regions were dependent on anxiety, while group differences in the cingulate cortex and insula were dependent on both anxiety and sex. For unpredictable threat cues, anxiety was negatively correlated with activation in the BNST, nucleus accumbens, and caudate in the healthy controls,

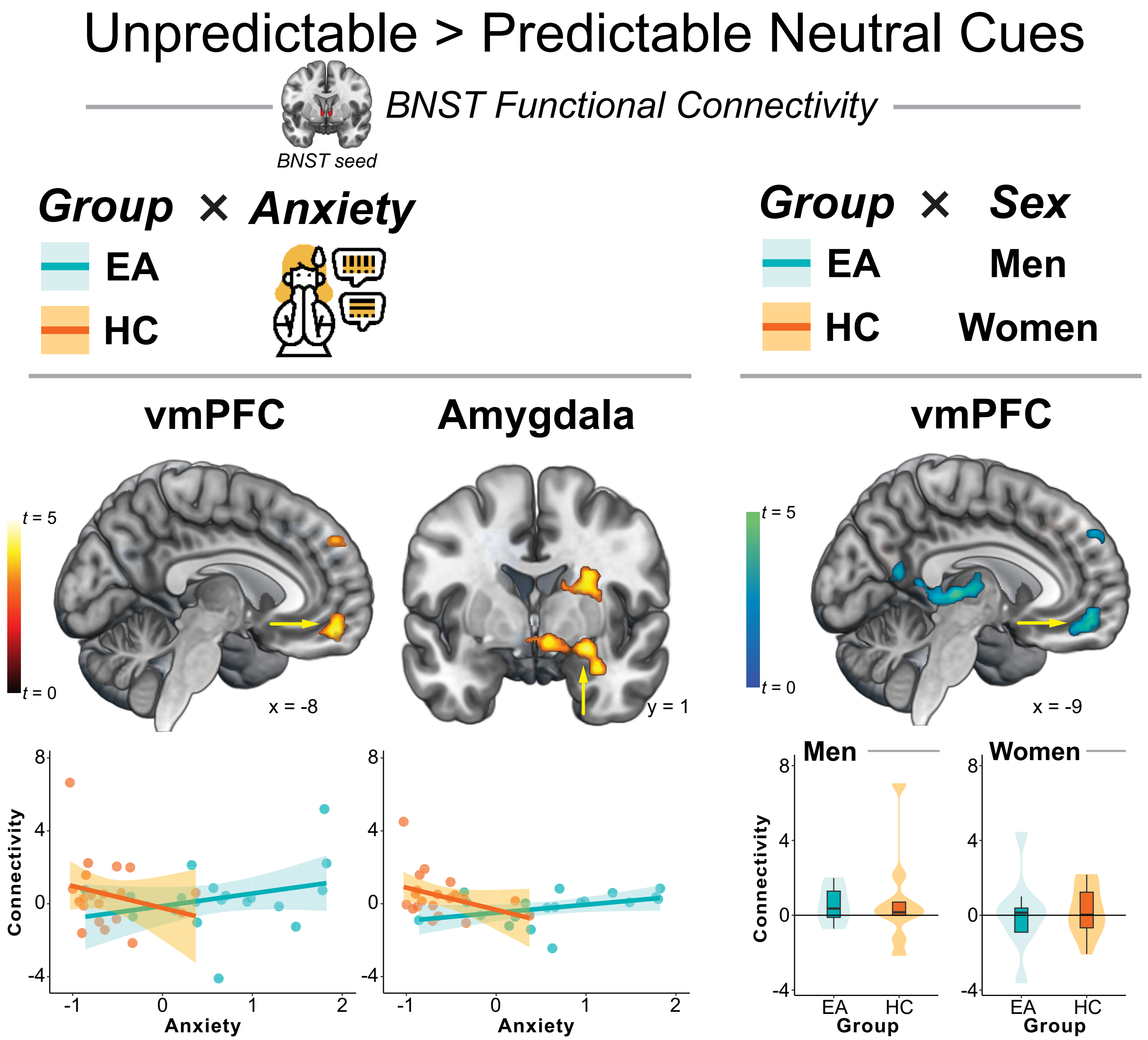

FIGURE 3 Group × anxiety and group × sex interactions in BNST functional connectivity during unpredictable versus predictable neutral cues. HC with higher anxiety exhibited weaker BNST-vmPFC and BNST-amygdala functional connectivity. EA men had stronger BNST-vmPFC connectivity than HC men. Connectivity refers to average eigenvariate of the vmPFC (Group × Anxiety *xyz* = −6, 52, −16; Group × Sex *xyz* = −6, 52, −14) and amygdala clusters. BNST, bed nucleus of the stria terminalis; EA, early abstinence; HC, healthy control; vmPFC, ventromedial prefrontal cortex.

whereas the EA group failed to show this pattern. Additionally, men in the HC group with higher anxiety exhibited lesser posterior cingulate activation than EA men. For unpredictable threat images, anxiety was positively correlated in HC and negatively correlated in EA in the BNST, rACC, and thalamus. EA men with higher anxiety also exhibited greater insula and dACC activation than HC men. A prior study in humans with a current AUD reported greater insula and dACC activation during unpredictable threat, which also predicts future problematic drinking in young adults (Gorka et al., 2023). In addition, insula activation during unpredictable threat was also associated with using alcohol to cope with negative affect (Gorka et al., 2020, 2023). The consistent elevation of insula and dACC activation during unpredictable threat across different phases of AUD may represent both the consequence of brain changes associated with heavy alcohol use and a biological risk factor for AUD. Additionally, the sex differences in brain activation were specific to men—to our knowledge, no other studies have investigated the effect of sex on unpredictable threat processing in early abstinence from an AUD. The findings of the current study may give insight into the neural mechanisms responsible for a myriad of sex differences that exist in AUD: drinking patterns, time to escalation to an AUD, and stress-related relapse (Barker & Taylor, 2019; Flores-Bonilla & Richardson, 2020; Peltier et al., 2019). Future longitudinal studies investigating sex differences during the transition from AUD to

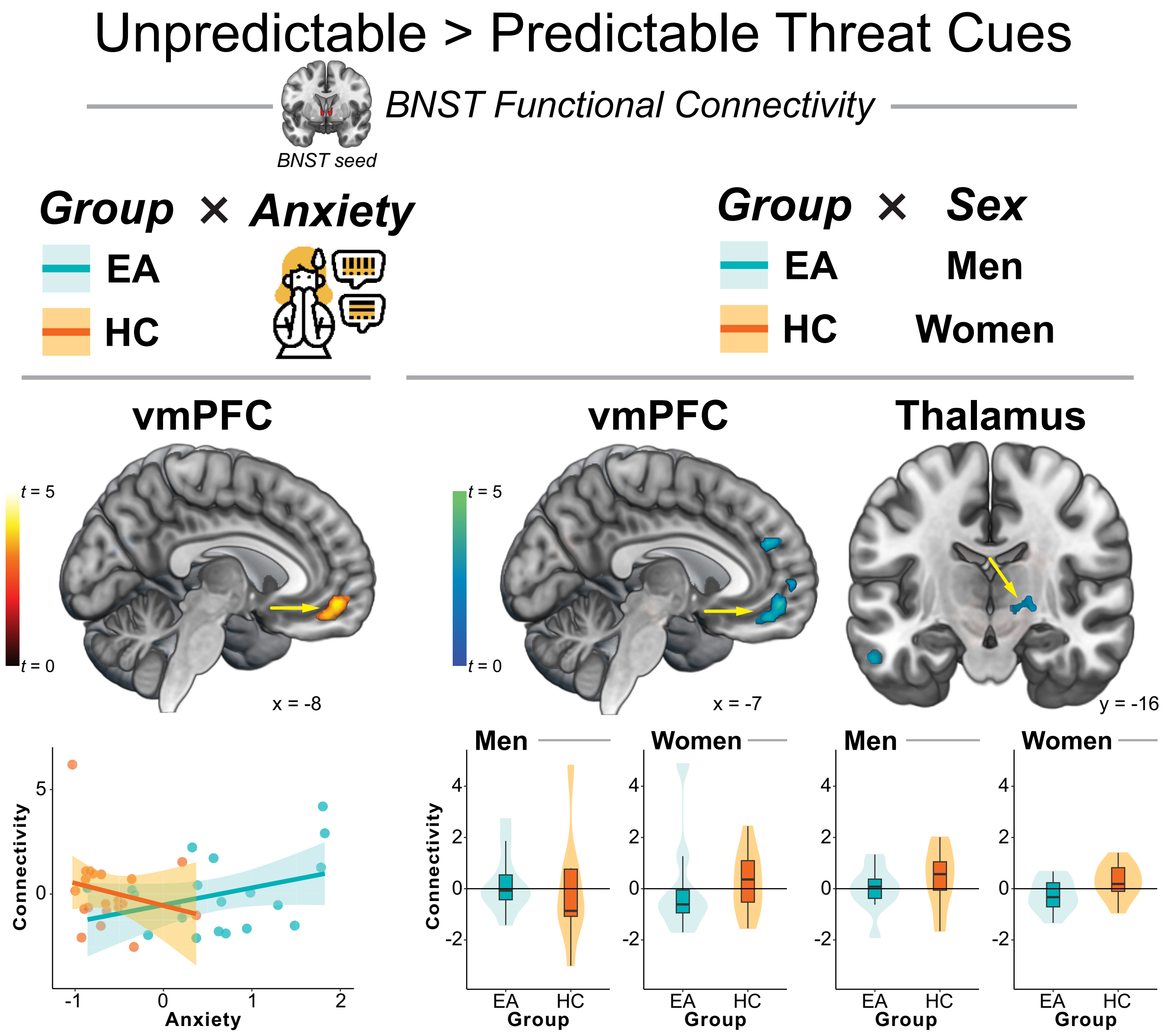

FIGURE 4 Group × anxiety and group × sex interactions in BNST functional connectivity during unpredictable versus predictable threat cues. HC with higher anxiety exhibited weaker BNST-vmPFC and BNST-amygdala functional connectivity. EA men had stronger BNST-vmPFC connectivity than HC men. Connectivity refers to average eigenvariate of the vmPFC (Group × Anxiety *xyz* = −6, 52, −16; Group × Sex *xyz* = −6, 52, −14) and amygdala clusters. BNST, bed nucleus of the stria terminalis; EA, early abstinence; HC, healthy control; vmPFC, ventromedial prefrontal cortex.

early alcohol abstinence will be critical for understanding BNST network alterations that may contribute to clinically meaningful outcomes.

The second major study finding was that adults in early abstinence displayed altered BNST functional connectivity during unpredictable threat cues, which was dependent on anxiety and sex. EA women had weaker BNST-vmPFC functional connectivity to unpredictable threat cues, while EA men had stronger BNST-vmPFC functional connectivity during unpredictable neutral cues. We also found that during unpredictable (vs. predictable) neutral and threat cues, higher anxiety scores in the HC group showed weaker BNST-vmPFC functional connectivity than in the EA group. These findings are consistent with our previous report on intrinsic connectivity (Flook et al., 2023). BNST-vmPFC connectivity regulates stress responding and anxiety, and its consistent relation across imaging modalities may represent a critical feature of alcohol abstinence in men that decreases their likelihood of stress-induced relapse (Erol & Karpyak, 2015; Motzkin et al., 2015). Interestingly, EA women in our study displayed weaker BNST network functional connectivity. Weaker BNST network functional connectivity may account for greater stress-induced relapse in women, as EA women may lack significant regulatory connections from the BNST that are critical for managing anxiety. Taken together, we propose that the connectivity findings, which highlight emotional regulatory connections from the BNST, may be critical neural nodes for tracking treatment response and progress.

Several study limitations should be noted. First, our study has a modest sample size. While many investigations of AUD typically use large, publicly available datasets—to our knowledge, the available datasets rarely include individuals in this critical phase of early abstinence and do not include an unpredictable threat task. Thus, this sample and the use of the unpredictable threat paradigm are unique and fill a critical gap in our understanding of early abstinence. Additionally, the sample sizes within group and sex are relatively small; therefore, the sex-differences findings should be replicated using a larger sample. An important future direction is to investigate the impact of hormone levels (estradiol, progesterone) in women to determine whether differences are specific to menstrual cycle phases. Second, a limitation of the cued anticipation task is that there are half as many threat and neutral images that follow the unpredictable cue, relative to the predictable threat and neutral cues, which might result in less reliable estimates of the unpredictable image activations. While this is a natural consequence of this study design and similar to other cued anticipation tasks (e.g., Williams et al., 2015), it is different from some other unpredictable threat tasks (e.g., N-P-U tasks; Schmitz & Grillon, 2012).

Overall, these data extend prior research in animal models that show the BNST network is altered during early abstinence and contributes to increased anxiety during this period. This small study also supports longstanding data that chronic alcohol use affects men and women differently, and that these differences continue during abstinence. Taken together, anxiety severity and sex play a critical role in early abstinence from alcohol, emphasizing the need for a greater understanding of the neural mechanisms that occur during alcohol abstinence to improve treatment outcomes.

AUTHOR CONTRIBUTIONS

JUB and DGW conceptualized the project and JUB designed the project; MS consulted on project design. Both DGW and JUB acquired funding for the study. EF, BF, and MB collected data for the project. MB provided oversight for clinical interviews. NLZ and JUB analyzed and interpreted data for the manuscript. NLZ and JUB wrote the first draft of the manuscript. NLZ, EF, BF, MS, MB, DGW, and JUB additionally edited and reviewed the study.

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CONFLICT OF INTEREST STATEMENT

The authors have nothing to disclose.

DATA AVAILABILITY STATEMENT

The data generated and analyzed during the current study are not currently publicly available but can be requested from the corresponding author prior to public availability. Data will be uploaded to a public repository after publication of this manuscript.

ORCID

Nicole L. Zabik https://orcid.org/0000-0003-2433-9597
Marisa M. Silveri https://orcid.org/0000-0001-9668-8548
Danny G. Winder https://orcid.org/0000-0002-6185-4769
Jennifer Urbano Blackford https://orcid.org/0000-0002-0261-4845

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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