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Methylation of genes and regulation of inflammatory processes on emotional response in young adults with alcoholic parents

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ABSTRACT

Many Americans are adult children of an alcoholic parent (ACoA), which can confer an increased risk of trauma and hazardous alcohol use, as well as heritable and environmental genetic influence. Psychological health and related neural activity can be influenced by inflammation responses, but it is not clear how these factors interact regarding risk or resilience to hazardous alcohol use. The goals of this study were to better understand the relationships between current alcohol use and inflammation, how these are modified by single nucleotide polymorphisms (SNPs) and/or epigenetic modifications of inflammation-associated genes; and how these alter neural reactivity to emotionally-salient stimuli. To do so, ACoA participants were dichotomized as resilient (not engaged in hazardous alcohol use) or vulnerable (currently engaged in hazardous alcohol use). Measures of blood-oxygen-level-dependent (BOLD) activity within regions of interest (ROIs), SNPs and DNA methylation of specific inflammation regulatory genes, and biological markers of inflammation were compared between these groups. Vulnerable ACoAs exhibited higher plasma C-reactive protein (CRP) and greater BOLD activity in the right hippocampus and ventral anterior cingulate cortex in response to emotional cues as well as reduced methylation of CRP and glucocorticoid-related genes. Path analysis revealed significant relationships between alcohol use, SNPs, DNA methylation of inflammatory-related genes, CRP levels, and BOLD activity to emotional stimuli. Taken together, these findings suggest a complex association related to hazardous alcohol use in ACoAs that may predict current inflammation and neural reactivity to emotional stimuli. A better understanding of these associations could direct the future of individual treatment options.

1. Introduction

One in 35 children lived in households with at least one alcoholic parent, resulting in nearly 17 million American adults identifying as children of alcoholics (NAfCo, 2018; Lipari and Van Horn, 2017), and one in four college students meet the criteria to be an adult child of an alcoholic parent (ACoA) (Kelley et al., 2010). Being raised by an alcoholic parent confers an increased risk of trauma exposure, not limited to abuse (Mackrill and Hesse, 2011), neglect (Reich et al., 1988; Haverfield and Theiss, 2014) as well as dysfunction in social development and attachment (Reich et al., 1988; Lander et al., 2013). ACoA's are significantly more likely to report difficulties in psychological, cognitive, and social functioning (Haverfield and Theiss, 2014; Klostermann et al., 2011; Brown-Rice et al., 2018), and are 3–4 times more likely to develop a substance use disorder (SUD) in their lifetime (Yoon et al., 2013; Eddie et al., 2015), due to interactions between genetic and environmental factors (Crane, 2019). Trauma exposure can influence mental health outcomes directly (Khoury et al., 2010; Carr et al., 2013) through the alteration of epigenetic factors (Klengel et al., 2014; Kader et al., 2018). Combined, genetic and environmental components account for a significant role in the individual's increased risk for not only developing a SUD (Kranzler et al., 2019; Verhulst et al., 2015; Edenberg and Foroud, 2013) but depression and anxiety disorders (Kelley et al., 2010; Klostermann et al., 2011), as well as autoimmune and inflammatory diseases

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(Ellinghaus et al., 2016; Zhao et al., 2016; Goldfinger, 2009). The relationships between inherited genetic predisposition and environmental influence on epigenetic modulation combined with an increased risk of prior trauma exposure could all contribute to an individual's emotional processing and overall mental health. The exact relationship between these factors, however, is unknown.

Consumption of alcohol is related to inflammation and immune response activation (Archer et al., 2019; Bishehsari et al., 2017; Crews et al., 2006), and biomarkers of inflammation can be used in conjunction with alcohol-use status to assess psychological health (Archer et al., 2019). Biomarkers of inflammation have also been related to risk for addiction and alcohol dependence (Crews, 2012). Psychological health is closely linked to neural and stress hormone function and inflammation responses, which can include circulating CRP. Circulating CRP can have an effect on mental health, including depression (Felger and Lotrich, 2013; Das, 2016; Eraly et al., 2014; Köhler-Forsberg et al., 2017), anxiety disorders (Khandaker et al., 2016; Pierce et al., 2017; Michopoulos et al., 2017), and PTSD (Eraly et al., 2014; Michopoulos et al., 2015). Higher levels of CRP have also been associated with decreased cognitive function and brain volume (Bettcher et al., 2012). Circulating CRP can be influenced by a variety of inputs, including preexisting genetic factors (Carlson et al., 2005; Wang et al., 2018; Veen et al., 2011; Halder et al., 2010; Almeida et al., 2009; Ancelin et al., 2015) and epigenetic modifications such as DNA methylation (Myte et al., 2019; Miller et al., 2018; Ligthart et al., 2016).

Alcohol Use Disorders (AUD) are thought to be partially inherited, with estimates of 49% heritability and around 10% of variance due to environmental factors (Verhulst et al., 2015). Genetic predisposition towards risky alcohol use can be inherited by an alcoholic parent (Heath et al., 1997; Pickens et al., 1991), with single nucleotide polymorphisms (SNPs) related to addiction, mental health, and inflammation genes potentially playing a role in the underlying mechanism dictating risk or resilience (Kranzler et al., 2019; Edenberg and Foroud, 2013; Tabakoff et al., 2009; Ducci and Goldman, 2008). Several genes have been identified in their relationship between mental health, inflammation, and addiction with *C-Reactive Protein* (*CRP* (Wang et al., 2018; Almeida et al., 2009; Ancelin et al., 2015; Gaysina et al., 2011);), the *Absent in Melanoma 2* (*AIM2* (Miller et al., 2018);), and the *FK506 binding protein 51* (*FKBP5* (Fujii et al., 2014; Binder et al., 2008);) co-chaperone genes of particular interest. Polymorphisms in the *CRP* gene have been implicated in depression (Wang et al., 2018; Almeida et al., 2009; Ancelin et al., 2015) PTSD (Ancelin et al., 2015), and emotional control (Gaysina et al., 2011). *AIM2* is a mediator of an inflammatory response (Freeman and Ting, 2016) and modifications of *AIM2* can predict plasma CRP levels (Ligthart et al., 2016) and are associated with an increased risk of PTSD (Miller et al., 2018). *FKBP5* is involved in the regulation of glucocorticoid feedback and suppressed cortisol response (Fujii et al., 2014) and has been related to PTSD risk (Fujii et al., 2014; Binder et al., 2008; Zhang et al., 2020; Li et al., 2019), suicidality and depression (Hernandez-Diaz et al., 2019; Pérez-Ortiz et al., 2013), and alcohol consumption and withdrawal severity (Huang et al., 2014; Qiu et al., 2016; Nylander et al., 2017), including increased risk of heavy drinking following trauma in college-aged students (Lieberman et al., 2016). Polymorphisms on the *FKBP5* gene have also been related to structural changes in the dorsal anterior cingulate cortex (dACC), cognitive function (Fujii et al., 2014), can contribute to the regulation of inflammatory and immune responses (Zannas et al., 2016; Zannas and Binder, 2014), and mediate the relationships between circulating CRP and cortisol and epigenetic aging following adverse experiences in children (Dammering et al., 2019).

The influence of current alcohol-use status can also play a role in mental and physical health, as well as modulate epigenetic regulation of gene function (Heberlein et al., 2015; Vadigepalli and Hoek, 2018; Ponomarev, 2013). The specific phenotype of the ACoA, risky use of alcohol compared to those not currently engaged in alcohol use can aid in the understanding of the role each factor plays in the development of negative mental health outcomes, and may have a direct effect on epigenetic changes. Many factors can influence DNA methylation, such as environmental factors, stress (Thayer and Kuzawa, 2011), diet (Anderson et al., 2012; Niculescu and Zeisel, 2002; Lim and Song, 2012), as well as current alcohol use (Ponomarev, 2013; Voisey et al., 2019). Differing methylation levels in genes that regulate the HPA axis and inflammatory response have been reported in AUD (Gatta et al., 2019; Dogan et al., 2016), veterans with PTSD (Miller et al., 2018; Kim et al., 2017; Yehuda et al., 2015; Kang et al., 2019), as well as suicide deaths (Palma-Gudiel et al., 2015; Labonte et al., 2012). Differing methylation of the glucocorticoid receptor gene (Nuclear Receptor Subfamily 3 Group 1 Member 1; NR3C1) gene has been found in survivors of abuse and childhood maltreatment as well as rodent models of early life stress (Palma-Gudiel et al., 2015; Labonte et al., 2012; Bockmühl et al., 2015). Hyper- or hypo-methylation of shore and promoter CpG islands on the NR3C1 gene, the absent in melanoma 2 (AIM2), and other CRP-related genes can differentially affect the regulation of the HPA axis (Bockmühl et al., 2015; de Kloet et al., 2005) leading to dysregulation of cortisol and CRP feedback and mediate overall inflammatory responses (Walton et al., 2018). It has not been determined if differing methylation at these regulatory sites has a direct relationship with genetic polymorphisms or neural network regulation.

To better understand the relationships between genetic factors and AUD, current alcohol use, and the role of inflammation and how it affects neural activity requires the use of a multi-stage model. We sought to determine the causal relationship as well as the predictive capacity of inherited genetic SNPs and environmental factors on epigenetic modulation circulating CRP, and how these factors relate to the neural activity involved in emotional processing as revealed in an fMRI task. To accomplish this task, we constructed a model to determine whether a combination of hazardous drinking and genetic pre-disposition had an impact on markers known to be associated with levels of inflammation. We also hypothesized that these previous steps in the model would be associated with both elevated levels of CRP and cortisol, which would have an impact on brain activity during an emotional valence task and levels of anxiety. Lastly, we believed that levels of anxiety may feed back onto levels of alcohol use. This model, thusly, tested whether CRP levels will be increased in the ACoAs currently engaged in alcohol use (hazardous), with the assumption that those engaging in alcohol consumption experience low-level chronic inflammation. Further, we anticipated a causal relationship between alterations of SNPs on CRP-related genes, inflammation, and current alcohol use which further influenced methylation at promoter regions on influential genes, and that this methylation could underlie (or at least contribute) to the relationship between alcohol use and negative mental health outcomes.

To accomplish these goals, we examined common genetic factors in an ACoA population of students currently enrolled at the University of South Dakota, and determined the role of current alcohol use on functional epigenetic changes and circulating CRP, and how those effects interacted with generalized anxiety and neural activity within emotional centers of the brain.

2. Materials and methods

2.1. Participants

All procedures were approved by the Institutional Review Board of the University of South Dakota. Twenty-nine participants were enrolled in the study from advertisements posted on campus at the University of South Dakota as part of a larger study (Brown-Rice et al., 2018). An initial screening was completed to determine eligibility, which included a score of 6 or more on the Children of Alcoholics Screening Test (CAST (Jones, 1983),), indicating that the participant was more than likely a child of an alcoholic (Brown-Rice et al., 2018), as well as for contraindications to functional magnetic resonance imaging (fMRI) and possible psychotic or psychological symptoms excluding them from testing.

Table 1
Allele Frequency for single nucleotide polymorphisms associated with plasma CRP and inflammation.

					Allele Frequency (%)			
Polymorphism	Gene	p-Value	Alleles	Major	Non-Hazardous Major Allele	Non-Hazardous Minor Allele	Hazardous Major Allele	Hazardous Minor Allele
rs2518564	AIM2	0.80	A:G	A	82.14	17.86	83.33	16.67
rs1205	CRP	0.72	C:T	C	75.00	25.00	66.67	33.33
rs2808630	CRP	0.46	C:T	T	57.14	42.86	70.83	29.17
rs1417938	CRP	0.83	A:T	T	71.43	28.57	72.73	27.27
rs3093059	CRP	0.54	A:G	A	96.43	3.57	95.83	4.17
rs1360780	FKBP5	0.85	C:T	C	60.71	39.29	54.17	45.83
rs3800373	FKBP5	0.67	G:T	T	67.86	32.14	58.33	41.67
rs9380524	FKBP5	0.40	A:C	C	100.00	0.00	91.67	8.33
rs9394314	FKBP5	0.85	A:G	A	60.71	39.29	54.17	45.83

Participants ranged in age from 18 to 29, average age 21, with 44.83% male and 55.17% female. For full demographics, see (Brown-Rice et al., 2018). Comprehensive methods related to recruitment, psychological assessment, and substance use are detailed in the parent study (Brown-Rice et al., 2018) and are only presented here in brief.

2.2. Psychological, physical, and alcohol use assessments

To assess hazardous alcohol use, participants completed the Alcohol Use Disorders Identification Test (AUDIT (Babor, 1992),). An AUDIT score of 8 or above was considered to represent hazardous alcohol use (Heitzeg et al., 2008), with 14 participants within the hazardous, and 15 within the non-hazardous groups. AUDIT scores for the enrolled participants were 15 ± 1.866 for hazardous and 2.8 ± 0.634 for non-hazardous groups. The Beck Anxiety Inventory (BAI (Beck and Steer, 1993);) was used to assess anxiety (15 ± 2.8 and 7.9 ± 1.8 for the hazardous and non-hazardous groups respectively.

2.3. fMRI procedures and blood sampling

Full fMRI and blood sampling methods are presented in detail in (Brown-Rice et al., 2018), briefly, participants were screened for contraindications to the fMRI procedures and consent to plasma sampling during initial screening, resulting in 24 of the 29 participants completing the fMRI portion of the study (13 non-hazardous and 11 hazardous), and 27 of the 29 participants completing the blood sampling portion (14 non-hazardous and 13 hazardous). Participants had a 5 ml blood sample taken both before and immediately following the fMRI scanning, with all baseline samples collected between 12:00–3:15 p.m., and post-test samples collected within 1.5 h of baseline. Blood samples were collected into heparin-coated tubes and placed on ice until processing. Participants were briefed on the tasks they were to complete in the scanner (details in (Brown-Rice et al., 2018) with the Ranking Emotion Task presented here).

2.4. Apparatus and Ranking Emotion Task

All participants received training and instructions for the Ranking Emotion Task outside of the scanner via a prepared set of slides illustrating the procedure before being placed within the bore of the magnet. For the full description of the apparatus and detailed methods for image acquisition, see (Brown-Rice et al., 2018). Briefly, participants were instructed to lay head-first supine in the bore, and their heads were stabilized by noise-canceling headphones and foam padding. The images for the ranking task were presented on an MR-compatible LCD screen (Invivo, Gainseville, FL) at the head of the scanner and reflected in a mirror box affixed to the head coil. An MR-compatible 4-button response box (Lumina LP-400, Cedrus Corporation, San Pedro, CA) was used to collect ranking responses. The response pad consisted of buttons that moved the scale bar to the left or right using their dominant (right) hand. Task image presentation and data recording were accomplished using custom LabVIEW software on a dedicated PC (LabVIEW, 2015; National Instruments, Austin, TX). Participants were presented with 90 International Affective Picture System (IAPS (Brown-Rice et al., 2018; Lang and Bradley, 2007);) images consisting of neutral, positive, and negative images (30 of each). Images were presented over 3 runs for 500 ms each, and participants were given an additional 5500 ms to rank the images by moving the scale bar towards a happy or sad face on a horizontal plane at the bottom of each image, starting at neutral (Brown-Rice et al., 2018). The rankings were determined on a scale of 0–7, with 0 being the most positive (happy face) and 7 being the most negative (sad face).

2.5. Analysis of plasma cortisol and C-reactive protein

Plasma samples were collected from a total of 27 participants (14 non-hazardous and 13 hazardous). Measurement of plasma cortisol was performed using an enzyme-linked immunoassay kit (Enzo Life Sciences, Farmingdale, NY). Briefly, 20 µl of plasma was diluted with 640 µl of assay buffer for a 1:32 part dilution. Duplicates of each sample were treated with 0.2 µl of Steroid Displacement Reagent, and duplicates of each sample, standard, and control were assayed. Cortisol was detected using an automatic plate reader (Bio-Tek Instruments, Winooski, VT, USA) at an absorbance of 405 nm with a wavelength correction set at 570 nm. Samples were compared to known standards and absorbance values were used to calculate maximum binding percent (28.78%), and percent non-specific binding (4.34%). The detection limit sensitivity of the assay was 56.72 pg/ml.

Measurement of plasma CRP was performed using an enzyme-linked immunoassay kit (Enzo Life Sciences, Farmingdale, NY). Briefly, 20 µl of plasma was diluted with 320 µl of assay buffer for a 1:16 part dilution. Duplicates of each sample, standard, and control were assayed and optical density detected using an automatic plate reader (Bio-Tek Instruments, Winooski, VT, USA) at an absorbance of 450 nm. Samples were compared to known standards and absorbance values were used to calculate non-specific binding (range 0.047–0.055). The sensitivity of the assay was 12.685 ng/ml.

2.6. Analysis of single nucleotide polymorphisms (SNP)

DNA Extraction: DNA from a total of 27 participant samples was extracted using the QIAsymphony® instrument coupled with the QIAsymphony® DSP DNA midi kit version 1.0 according to the manufacturer's instructions (QIAGEN Inc, Germantown, MD). The extracted DNA was eluted into ATE buffer composed of 10 mM Tris-Cl pH 8.3, 0.1 mM EDTA, and 0.04% NaN3 (sodium azide) with a final eluate volume of 60 µl.

SNP Genotyping: Illumina's Infinium® Global Screening Array-24 v1.0 BeadChip was used to generate SNP genotypes on the study participants. This array interrogates over 650,000 genetic markers with an added 50,000 custom genetic markers, providing an extensive range of applications. A total DNA mass of 200 ng per sample, from normalized DNA, was introduced into the assay, and samples were carried through the protocol per the manufacturer's explicit instructions. The main steps for genome-wide SNP genotyping on Illumina Infinium arrays include an

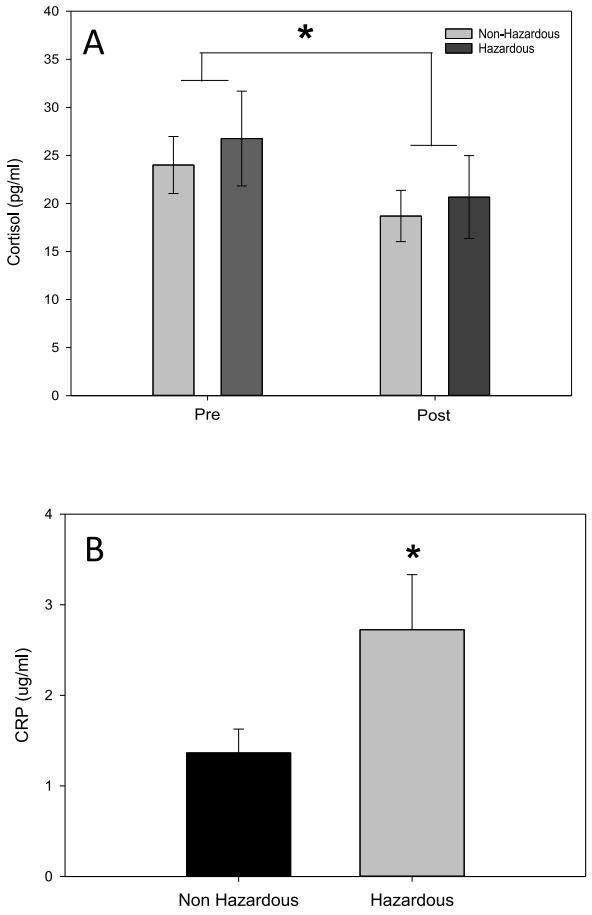

Fig. 1. Cortisol (baseline and post-task) and baseline C-Reactive Protein (CRP) Levels in ACoAs show that plasma cortisol levels were significantly reduced in both vulnerable and resilient ACoAs following fMRI scanning compared to pre-task baseline levels (**A**), and no significant differences were found between ACoA groups at either time point. However, ACoAs currently engaged in hazardous alcohol use (vulnerable) exhibited significantly higher baseline plasma CRP compared to non-hazardous (resilient) groups (**B**). Data represent mean ± SEM. *significant difference, $p < 0.05$.

isothermal (37 °C) whole genome amplification step, fragmentation of the amplified product, PCR product purification, hybridization to array probes, single-base primer extension, and scanning. Sample performance, including call rate, and all internal controls were assessed utilizing GenomeStudio 2.0 software.

From the approximately 700,000 SNP markers present on the arrays, nine different polymorphisms associated with inflammation, addiction, or mental health were selected from candidate genes (e.g. CRP, AIM2, and FKBP5) for analyses from the data generated on each participant (data and allele frequencies are summarized in Table 1). SNPs were chosen for the following genes *C-Reactive Protein* (*CRP* (Wang et al., 2018; Almeida et al., 2009; Ancelin et al., 2015; Gaysina et al., 2011);), the *Absent in Melanoma 2* (*AIM2* (Miller et al., 2018);), and the *FK506 binding protein 51* (*FKBP5* (Fujii et al., 2014; Binder et al., 2008);). SNPs residing in these genes have been implicated in depression, PTSD (Wang et al., 2018; Almeida et al., 2009; Ancelin et al., 2015; Miller et al., 2018; Fujii et al., 2014; Binder et al., 2008; Zhang et al., 2020; Li et al., 2019; Hernandez-Diaz et al., 2019; Pérez-Ortiz et al., 2013), and emotional control (Gaysina et al., 2011). Variation in these genes can also mediate inflammatory and immune responses as well as modulate plasma CRP levels (Ligthart et al., 2016; Freeman and Ting, 2016; Zannas et al., 2016; Zannas and Binder, 2014) and regulate glucocorticoid feedback and cortisol response (Fujii et al., 2014). SNPs in these genes have also been implicated in risk for increased alcohol consumption and withdrawal severity (Huang et al., 2014; Qiu et al., 2016; Nylander et al., 2017).

2.7. DNA methylation

DNA methylation was assessed using the Illumina Infinium Methylation EPIC Beadchip Array following the manufacturer's instructions. This platform interrogates over 850,000 methylation sites across the genome at single-nucleotide resolution. Briefly, the isolated DNA is treated with sodium bisulfite, converting unmethylated cytosines into uracil via deamination. Subsequently, methylated cytosines remain unaffected during treatment and are protected from conversion to uracil. Bisulfite conversion was carried out by utilizing the Zymo EZ-96 DNA Methylation™ Kit. Following bisulfite conversion; whole-genome amplification, fragmentation, hybridization, staining, and scanning were carried out according to the protocol. Sample performance including bisulfite conversion and all internal controls was assessed by The GenomeStudio Methylation (M) module.

Twenty-nine methylation sites of shore and promoter CpG islands on the NR3C1 gene, the *absent in melanoma 2* (AIM2), *brain-derived neurotrophic factor* (BDNF), and other CRP-related genes were assayed for each participant. Island regions were selected based on known associations with inflammation and stress responses, mental health outcomes, and alcohol use disorders. Methylation of the NR3C1 gene has been linked to PTSD, depression, and suicide (Palma-Gudiel et al., 2015; Na et al., 2014). Differential methylation of shore and exon regions of the NR3C1 gene is also involved in negative long-term effects of early-life stress (Palma-Gudiel et al., 2015; Bockmühl et al., 2015). Hyper- and hypomethylation of promoter regions of the BDNF gene are found at varying stages of alcohol withdrawal (Heberlein et al., 2015), and hypermethylation of BDNF promoter regions is also associated with PTSD as well as AUD (Kim et al., 2017). Hypermethylation of the promotor region cg10636246 on the AIM2 gene is associated with decreased serum CRP and mediates the inflammatory response (Miller et al., 2018; Ligthart et al., 2016), as well as being significantly associated with the severity of PTSD (Miller et al., 2018). DNA methylation at encoding regions of CRP-related genes such as *transmembrane protein 49* (TMEM49), *B-Cell lymphoma 3 protein* (BCL3), and *microRNA 21* (MIR21) are also thought to be involved in negative feedback mechanisms of CRP regulation (Walton et al., 2018).

2.8. Data analysis

All plasma, SNP, and methylation analyses were performed using SigmaPlot for Windows v13.0 with the significance level set at $p < 0.05$ (Systat Software, Inc., San Jose, California, USA). Plasma cortisol levels were compared between hazardous and non-hazardous alcohol use groups in both the pre-scan and post-scan conditions, as well as across both time points using two-way ANOVA with one repeated measure. Plasma CRP levels were compared between hazardous and non-hazardous groups using an unpaired *t*-test. Allele frequencies for each SNP were compared between the hazardous and non-hazardous alcohol groups with separate Chi-squared tests. Separate one-way ANOVAs were used to determine whether specific allele combinations for each single nucleotide polymorphism contributed to alcohol use, anxiety, cortisol, or CRP levels, with significant differences further analyzed by Student-Neuman-Keuls (SNK) posthoc test for multiple comparisons. DNA methylation results were collated by CpG sites, and β-values for each group were compared by separate unpaired t-tests, which were corrected for multiple comparisons using a Benjamin-Hochberg correction (McDonald, 2009).

Anatomical and functional images for ROI analysis were preprocessed using Brain Voyager v20.2 (Ranier Goebel, Brain Innovation, Maastricht, The Netherlands) as detailed by us previously

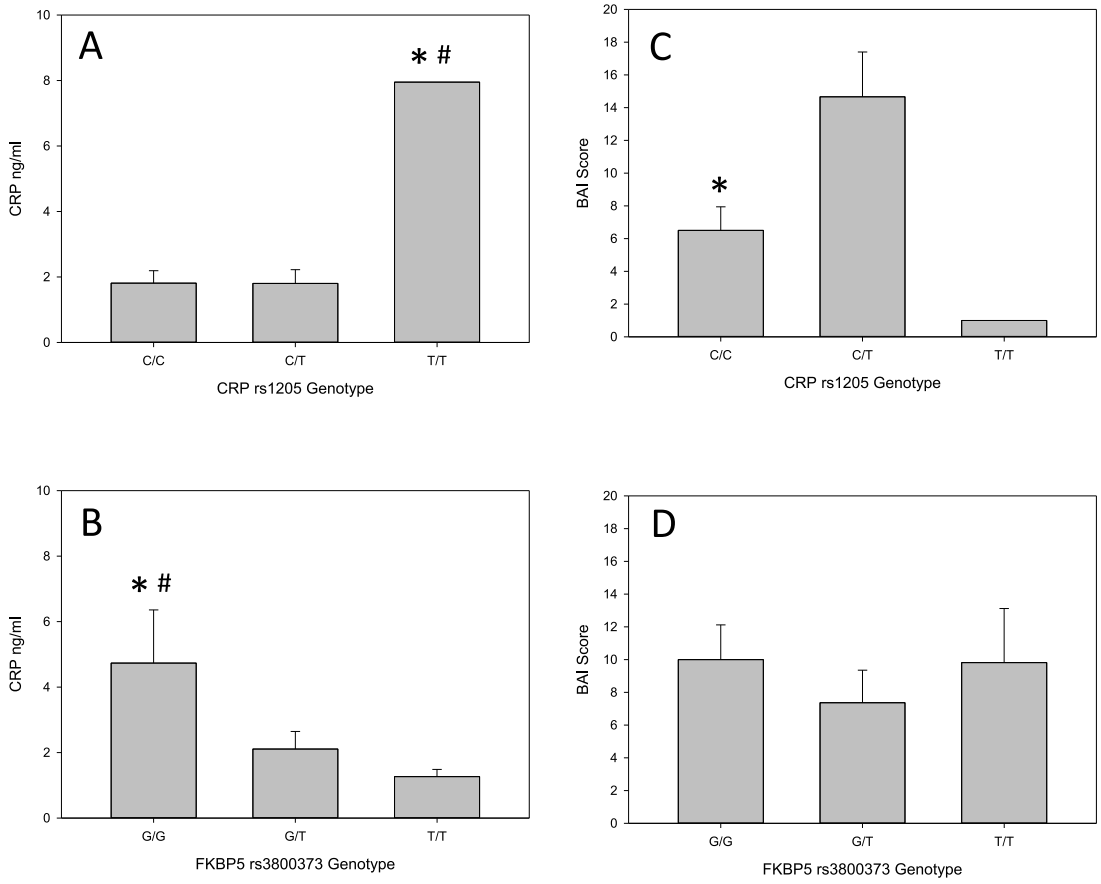

Fig. 2. Baseline C-Reactive Protein (CRP) Levels and Beck Anxiety Inventory (BAI) Scores (mean ± SEM) across genotypes for the SNPs associated with *CRP* and *FKBP5* genes. (**A, C**) rs1205 *CRP*, C-Reactive Protein; (**B,D**) rs3800373 *FKBP5*, FK506 binding protein 51. *significantly different from heterozygous genotype; #significantly different from other homozygous genotypes ($p < 0.05$).

(Brown-Rice et al., 2018). Preprocessing included brain extraction, slice scan time correction (cubic spline interpolation with an ascending/interleaved scanning order), 3D motion correction (trilinear/sinc interpolation referencing the first volume of each run), and high-pass GLM-Fourier temporal filtering. The ROIs for this study consisted of the right and left hippocampus and the ventral anterior cingulate cortex (vACC; Brodman's area 24), given their role in the appraisal of emotional salience (Taylor et al., 2011; Leech and Sharp, 2014; Alba--Ferrara et al., 2016). Each ROI was defined by the MNI152 template and converted to Talairach coordinates to generate an ROI mask within Brain Voyager. The left/right hippocampus included 2536 voxels centered at −/+30.19, −45.25, 4.37 (x,y,z), and the left/right vACC encompassed 410 voxels centered at −/+4.93, 20.61, −4.79 (x,y,z). Beta-weights were extracted for neutral, negative, and positive conditions of the Ranking Emotion Task for each ROI. Analyses consisted of separate two-way ANOVA with one repeated measure performed using GraphPad Prism 9 version 9.2.0 for macOS, with the significance level set at $p < 0.05$ (GraphPad Software, San Diego, California, USA). Post-hoc comparisons were made, where warranted, using Sidak *posthoc* tests for multiple comparisons.

To create a combined brain activity variable for Pathway Analysis, the ROI responses to each stimulus condition were analyzed in R v3.5.1 (R Core Team, Vienna, Austria) using the factor analysis tool (fa) in the R psych package (Revelle, 2021). Factor Analysis is commonly used to find latent variables in data and is employed here to find the latent combination of brain activity that best predicts our outcomes of interest. Using this method, the created model attempts to explain the most variance in the fMRI data with the minimum number of variables and separates the activity in these regions based on similar activity patterns (function) rather than structure or stimulus condition. Scree Plot was used to determine the optimal number of components (Cattell, 1966).

The Pathway Analysis Model was hierarchically constructed with multiple linear regressions. Each independent variable from the previous leg of the pathway analysis was added as a dependent variable for the next leg to analyze any mediation or partial mediation effects within the data. Partial correlation was calculated for each significant dependent variable for each leg of the pathway to determine how much variance within the independent variable is uniquely predicted by that significant dependent variable.

3. Results

3.1. Circulating plasma cortisol and CRP

Baseline cortisol levels were not significantly different between hazardous and non-hazardous alcohol users (F (NAfCo, 2018; Archer et al., 2019) $= 0.464$, $p = 0.502$), although levels did significantly decrease from pre to post sampling F (NAfCo, 2018; Archer et al., 2019) $= 6.057$, $p = 0.022$; Fig. 1A). CRP levels, however, were significantly increased in ACoAs currently engaged in hazardous alcohol use ($p = 0.048$; Fig. 1B) compared to non-hazardous users.

3.2. Single nucleotide polymorphisms associated with CRP and inflammation

Table 1 summarizes the allele frequencies for genes related to CRP

Table 2
DNA methylation sites associated with CRP and inflammation.

CpG Site	Chr	Position	Gene	p-Value	Non-Hazardous β-value	Hazardous β-value
cg03906910	5	142,794,581	NR3C1	0.64	0.196 ± 0.004	0.186 ± 0.007
cg23776787	5	142,794,508	NR3C1	0.21	0.068 ± 0.004	0.058 ± 0.004
cg08818984	5	142,795,020	NR3C1	0.23	0.047 ± 0.004	0.036 ± 0.004
cg26720913	5	142,795,127	NR3C1	0.81	0.031 ± 0.011	0.023 ± 0.011
cg18146873	5	142,763,020	NR3C1	0.81	0.074 ± 0.009	0.068 ± 0.009
cg20753294	5	142,762,984	NR3C1	0.74	0.123 ± 0.042	0.088 ± 0.042
cg01967637	5	142,764,212	NR3C1	0.67	0.111 ± 0.010	0.100 ± 0.010
cg13648501	5	142,765,451	NR3C1	0.64	0.077 ± 0.010	0.067 ± 0.010
cg04111177	5	142,763,800	NR3C1	0.83	0.051 ± 0.002	0.053 ± 0.002
cg11152298	5	142,763,576	NR3C1	0.83	0.049 ± 0.001	0.048 ± 0.001
cg15645634	5	142,763,832	NR3C1	**0.02**	0.022 ± 0.001	0.017 ± 0.001
cg15910486	5	142,763,814	NR3C1	0.54	0.091 ± 0.008	0.078 ± 0.008
cg17860381	5	142,763,762	NR3C1	0.64	0.039 ± 0.007	0.031 ± 0.007
cg18068240	5	142,764,036	NR3C1	0.64	0.021 ± 0.004	0.016 ± 0.004
cg21702128	5	142,764,914	NR3C1	0.42	0.090 ± 0.002	0.086 ± 0.002
cg01068621	1	157,327,993	AIM2	0.81	0.768 ± 0.017	0.777 ± 0.017
cg10636246	1	157,313,597	AIM2	**0.02**	0.241 ± 0.013	0.180 ± 0.013
cg01565803	1	157,951,288	CRP	0.83	0.726 ± 0.045	0.745 ± 0.045
cg12054453	17	55,270,499	TMEM49	0.71	0.258 ± 0.010	0.277 ± 0.010
cg03823539	17	55,139,821	TMEM49	0.81	0.012 ± 0.004	0.009 ± 0.004
cg11251069	17	55,139,824	TMEM49	0.64	0.013 ± 0.005	0.008 ± 0.005
cg11698899	17	55,139,790	TMEM49	0.81	0.055 ± 0.005	0.052 ± 0.005
cg17544904	17	55,139,869	TMEM49	**0.04**	0.044 ± 0.001	0.038 ± 0.001
cg23838308	17	55,139,838	TMEM49	0.77	0.018 ± 0.008	0.012 ± 0.008
cg05371867	17	55,139,636	TMEM49	0.23	0.039 ± 0.001	0.036 ± 0.001
cg06747916	17	55,139,617	TMEM49	0.06	0.061 ± 0.003	0.049 ± 0.003
cg07852793	17	55,139,576	TMEM49	0.81	0.022 ± 0.007	0.018 ± 0.007
cg20202881	17	55,139,561	TMEM49	0.64	0.028 ± 0.006	0.022 ± 0.006
cg25953464	17	55,139,594	TMEM49	0.83	0.151 ± 0.003	0.150 ± 0.003
cg26470501	19	45,252,955	BCL3	0.23	0.458 ± 0.011	0.430 ± 0.008
cg27023597	17	57,918,262	MIR21	0.23	0.595 ± 0.019	0.644 ± 0.014

and inflammation. No significant differences were found between hazardous and non-hazardous alcohol users in specific allele frequencies, and no differences were found between specific polymorphisms and AUDIT scores. However, one-way ANOVA revealed relationships between specific polymorphisms and baseline plasma CRP and anxiety measures (Fig. 2A–D). Specifically, baseline plasma CRP levels were significantly higher in TT carriers of *CRP* rs1205 (Fig. 2A; F (NAfCo, 2018; Archer et al., 2019) = 17.678, $p < 0.001$, SNK $p = 0.001$ vs CT and CC), and in GG carriers of *FKBP5* rs3800373 (Fig. 2B; $F_{(2,24)} = 5.913$, $p = 0.009$, SNK 0.007 vs TT and 0.016 vs GT). Differences in BAI scores between C/C and C/T groups were found in *CRP* rs1205 (Fig. 2C; F (NAfCo, 2018; Bishehsari et al., 2017) = 6.589, $p = 0.017$, SNK 0.032), however no differences were found between any genotype of FKBP5 and anxiety (Fig. 2D; F (Lipari and Van Horn, 2017; Archer et al., 2019) = 0.271, $p = 0.765$).

3.3. Changes in DNA methylation of CpG sites associated with CRP and inflammation

CpG sites that were related to CRP and inflammation regulation were investigated, with β-values from 32 promoter regions and island regions, compared between hazardous and non-hazardous groups using separate unpaired t-tests. Results are summarized in Table 2, and three CpG promoter regions showed differential DNA methylation following corrections for multiple comparisons. Mean methylation of promotor-associated regions of the BDNF gene showed no differences between groups and is not included in Table 2. ACoAs exhibiting hazardous alcohol use had decreased DNA methylation in two CRP related genes (AIM2 gene, cg10636246, $p = 0.026$; Fig. 3A) and TMEM49 (cg1754490, $p = 0.040$; Fig. 3B) as well as decreased methylation in a glucocorticoid receptor gene, NR3C1 (cg15645634, $p = 0.026$; Fig. 3C).

3.4. BOLD activity during a Ranking Emotion Task

No participants exceeded 1 mm of translation movement of 1 degree of rotations movement necessitating their removal from further analysis. ACoAs reporting hazardous alcohol use exhibited greater BOLD activity in the right hippocampus (Fig. 4A and B) regardless of emotional condition (F (NAfCo, 2018; Goldfinger, 2009) = 7.97, $p = 0.010$) when compared to ACoAs with non-hazardous alcohol use. An interaction was found between hazardous alcohol use and emotional condition for the right ventral anterior cingulate cortex (vACC; F (Lipari and Van Horn, 2017; Ligthart et al., 2016) = 6.53, $p = 0.003$; Fig. 4C and D), with negative stimuli resulting in increased activity in hazardous users when compared to non-hazardous alcohol users (*Sidak* $p = 0.018$). After adjusting for multiple comparisons, none of the additional interaction terms were significant (p's > 0.10).

3.5. Factor analysis of ROI activity during the Ranking Emotion Task

Hippocampal and vACC (Brodmans' Area 24) activity during the three emotional stimuli conditions resulted in 6 combined brain activities for each participant. Scree Plot Analysis suggested two principal components to be optimal. A third was calculated as a control variable for the pathway analysis. Using the minimum residual factor analysis with "oblimin", oblique transformations. Component 1 (fMRI1) encompassed approximately 39% of the variance within our original fMRI data, component 2 (fMRI2) encompassed 20%, and component 3 (fMRI3) encompassed 10% resulting in 69% variance accountancy. The mean item complexity was 1.7. Factor loading scores for each component can be found in Table 3. Factor scores for each component and each participant were calculated and passed through to the pathway analysis.

3.6. Pathway analysis model

All tables of regression results for each leg of the pathway analysis can be found in Table 4 and the final model is illustrated in Fig. 5. A visual outline of the proposed model demonstrating the pathway between alcohol use and negative mental health outcomes is illustrated in Fig. 6.

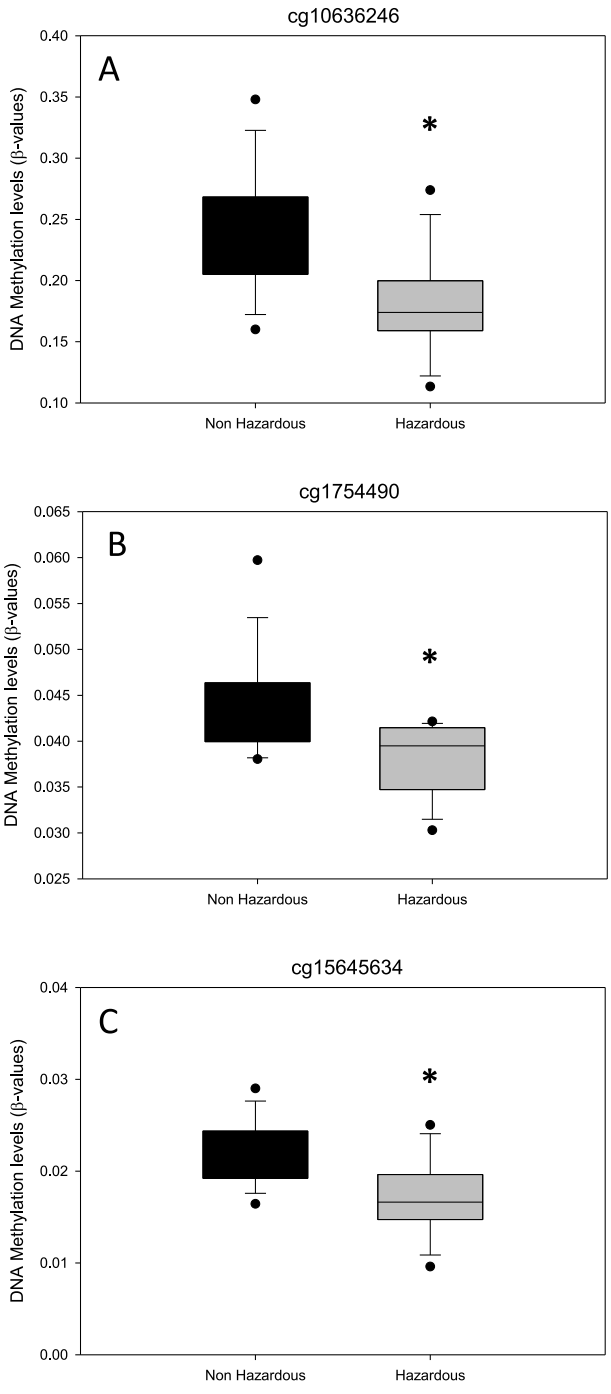

Fig. 3. Box plots showing differences in gene methylation for the identified promoter regions in hazardous and non-hazardous alcohol users. Upper and lower limits represent the 75th and 25th percentile respectively, and data points in the 95th and 5th are represented by a dot. ACoAs exhibiting hazardous alcohol use have decreased methylation in promoter regions of two CRP-related genes; absent in melanoma 2 (*AIM2*) gene (**A**), transmembrane protein 49 (*TMEM49*) gene (**B**), and one glucocorticoid receptor gene; nuclear receptor subfamily 3 group C member 1 (*NR3C1*) gene (**C**), when compared to the non-hazardous group. *significant difference between hazardous and non-hazardous groups, $p < 0.05$.

3.6.1. Methylation

Overall model fit was not significant for AIM2 (F (Reich et al., 1988; Archer et al., 2019) = 2.10, p = 0.102, adjusted R-squared = 0.164). AUDIT significantly predicted AIM2 (beta = −0.003 (0.001), t = −2.43, p = 0.023) with a partial R-squared of 0.204. Overall model fit was also significant for TMEM49 (F (Reich et al., 1988; Archer et al., 2019) = 4.00, p = 0.009, adjusted R-squared = 0.349), and AUDIT scores significantly predicted TMEM49 (beta = −0.0003 (0.0001), t = −2.89, p = 0.008) with a partial R-squared of 0.266. For NR3C1, overall model fit was significant (F (Reich et al., 1988; Archer et al., 2019) = 3.94, p = 0.010, adjusted R-squared = 0.344). With AUDIT significantly predicting NR3C1 (beta = −2.136e-4 (8.375e-5), t = −2.55, p = 0.018) with a partial R-squared of 0.221. Additionally, SNP rs1205 significantly predicted NR3C1 levels (beta = −8.644e-3 (4.004e-3), t = −2.841, p = 0.009) with a partial R-squared of 0.260.

3.6.2. CRP

Overall model fit was significant for CRP (F (Klostermann et al., 2011; Ellinghaus et al., 2016) = 4.681, p = 0.002, adjusted R-squared = 0.513). Both SNPs rs1205 and rs3800373, significantly predicted CRP (beta = −3.225 (1.236), t = −2.610, p = 0.017; beta = −3.796 (1.401), t = −2.710, p = 0.013) with partial R-squared values of 0.254 and 0.269, respectively. Methylation sites AIM2 and TMEM49 also significantly predicted CRP (AIM2 beta = −25.50 (6.733), t = −3.787, p = 0.001; TMEM49 beta = −166.89 (74.622), t = −2.236, p = 0.037) with a partial R-squares of 0.418 and 0.200.

3.6.3. *Cortisol: overall model fit was not significant for cortisol (F (Klostermann et al., 2011; Ellinghaus et al., 2016) = 1.107, p = 0.399, adjusted R-squared = 0.0297)*

3.6.4 Brain Activity: Overall model fit was significant for fMRI1 (F (Yoon et al., 2013; Verhulst et al., 2015) = 3.509, p = 0.010, adjusted R-squared = 0.473). NR3C1 significantly predicted fMRI1 (beta = −88.303 (30.494), t = −2.896, p = 0.010) with a partial R-squared of 0.318. Overall model fit was also significant for fMRI2 (F (Yoon et al., 2013; Verhulst et al., 2015) = 5.05, p = 0.001, adjusted R-squared = 0.591). rs1205 significantly predicted fMRI2 (beta = −0.532 (0.232), t = −2.292, p = 0.034) with a partial R-squared of 0.226. rs2808630 predicted fMRI2 (beta = −0.570 (0.174), t = −3.27, p = 0.004) with a partial R-squared of 0.373. CRP significantly predicted fMRI2 (beta = −0.124 (0.037), t = −3.371, p = 0.003) with a partial R-squared of 0.387. Cortisol significantly predicted FMRI2 (beta = 0.008 (0.003), t = 2.532, p = 0.021) with a partial R-squared of 0.263. AUDIT significantly predicted fMRI2 (beta = 0.013 (0.006), t = 2.24, p = 0.038) with a partial R-squared of 0.216. Overall model fit was not significant for fMRI3 (F (Yoon et al., 2013; Verhulst et al., 2015) = 0.933, p = 0.527, adjusted R-squared = −0.025), as would be expected for the control variable.

3.6.4. Anxiety

Overall model fit was not significant for BAI (F (Khoury et al., 2010; Klengel et al., 2014) = 1.391, p = 0.268, adjusted R-squared = 0.154). fMRI1 significantly predicted BAI (beta = 9.609 (4.500), t = 2.135, p = 0.050) with a partial R-squared of 0.233.

3.6.6 Alcohol Use: Overall model fit was significant for AUDIT (F (Crane, 2019; Kader et al., 2018) = 2.468, p = 0.047, adjusted R-squared = 0.386). fMRI2 significantly predicted AUDIT (beta = 14.942 (6.988), t = 2.138, p = 0.048) with a partial R-squared of 0.222.

4. Discussion

Adult children of alcoholics that are currently engaged in hazardous alcohol use have previously been linked to poorer mental and physical health outcomes, including anxiety and depression measures (Brown--Rice et al., 2018). The current study sought to explore the association between hazardous alcohol use and inflammation, and how this relates

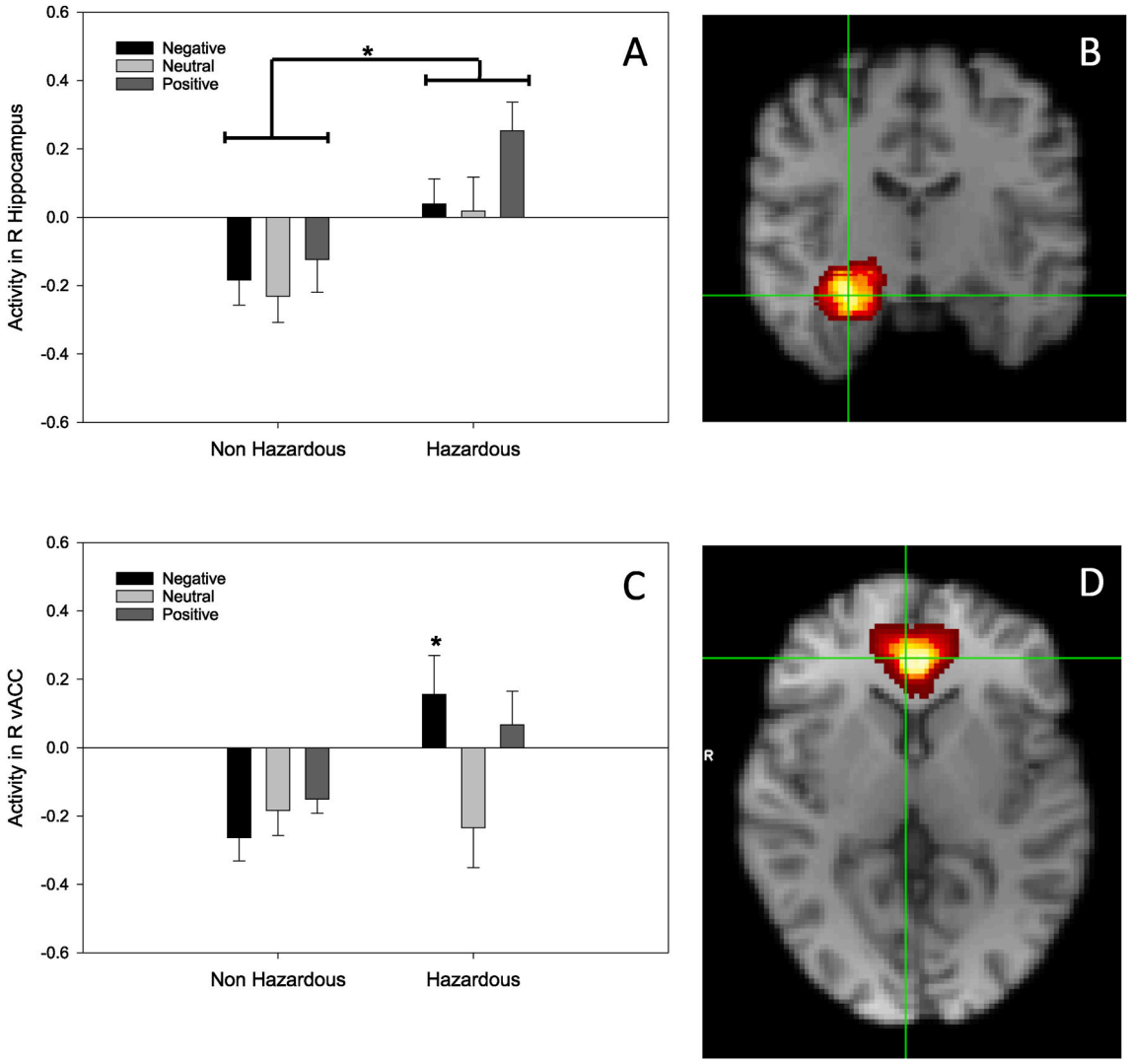

Fig. 4. ACoAs reporting hazardous alcohol use exhibited greater blood-oxygen level dependent (BOLD) activity in the right hippocampus across all emotional conditions (**A**), with the region of interest (ROI) mask in the coronal view depicted in (**B**). ACoAs reporting hazardous alcohol use also exhibit greater activity in the right ventral anterior cingulate cortex (vACC; Brodman's area 24) (**C**) in response to positive and negative conditions compared to non-hazardous ACoAs, with the bilateral ROI mask and crosshair on the right vACC depicted in (**D**). *significantly different from the non-hazardous group, $p < 0.05$.

Table 3
Factor Analysis Components of Factor Loading Scores indicating influence on each Principal Component.

	Principal Component		
Region and Stimulus	**fMRI1**	**fMRI2**	**fMRI3**
Hippocampus Negative	0.72	−0.42	0.12
Hippocampus Neutral	0.59	−0.08	0.06
Hippocampus Positive	0.93	−0.26	0.19
vACC Negative	0.66	0.43	−0.61
vACC Neutral	−0.05	0.37	0.32
vACC Positive	0.40	0.80	0.31
Proportion Variance Explained	0.39	0.20	0.10
Sum of Squared Loadings	2.33	1.21	0.63

vACC: ventral anterior cingulate cortex.

to brain activity in areas known for emotional regulation. Circulating CRP, a marker of inflammation, can affect a variety of mental health outcomes, including depression (Felger and Lotrich, 2013; Das, 2016; Eraly et al., 2014; Köhler-Forsberg et al., 2017), anxiety disorders (Khandaker et al., 2016; Pierce et al., 2017; Michopoulos et al., 2017), and PTSD (Eraly et al., 2014; Michopoulos et al., 2015), but a direct association between inflammation and hazardous alcohol use, and outcomes such as anxiety or neural activation during emotional responses, had not previously been explored. We, therefore, constructed a model to determine whether a combination of hazardous drinking and genetic pre-disposition had an impact on markers known to be associated with levels of inflammation. We also hypothesized that these previous steps in the model would be associated with both elevated levels of CRP and cortisol, which would have an impact on brain activity during an emotional valence task and levels of anxiety. Lastly, we believed that levels of anxiety may feed back onto levels of alcohol use.

When examining cortisol levels, there was a significant decrease in plasma cortisol levels from baseline sampling to post-task sampling (Fig. 1A). This result is most likely a result of elevated levels of anxiety prior to the experimental methods, such as the MRI task which then reduced following completion of the experimental tasks, with a concurrent reduction in cortisol levels. As the timing of both the baseline samples and post-test samples were controlled (see Methods), we do not believe timing of the blood sampling further interfered with the measurement. As predicted, CRP levels were increased in the ACoAs currently engaged in alcohol use, which suggests that at baseline, those engaging in alcohol consumption experience low-level chronic

Table 4
Pathway Analysis Regression Coefficients. Table represents the individual regression coefficients (betas) for each of the variables used in the pathway analysis. Regressions are covariate adjusted. Significant beta values are presented in bolded text.

	Independent Variables															
Dependent variables	AIM2		TMEM49		NR3C1		CRP		Cortisol		fMRI1		fMRI2		fMRI3		BAI	
	Beta Value (sd)	p-Value	Beta Value (sd)	p-Value	Beta Value (sd)	p-Value	Beta Value (sd)	p-Value	Beta Value (sd)	p-Value	Beta Value (sd)	p-Value	Beta Value (sd)	p-Value	Beta Value (sd)	p-Value	Beta Value (sd)	p-Valu
rs1205	-0.022 (0.041)	0.594	-3.250e-4 (0.0034)	0.932	-0.009 (0.003)	**0.009**	-3.230 (1.240)	0.017	-7.670 (14.400)	0.600	-0.667 (0.594)	0.276	-0.532 (0.232)	**0.034**	0.016 (0.227)	0.943	-5.600 (12.600)	0.66
rs2808630	-0.037 (0.038)	0.337	-0.004 (0.003)	0.298	-0.005 (0.003	0.068	-1.650 (1.050)	**0.131**	2.420 (12.200)	0.844	-0.447 (0.445)	0.329	-0.570 (0.174)	**0.004**	0.037 (0.170)	0.830	2.650 (10.440)	0.80
rs1360780	0.024 (0.052)	0.655	-9.840e-4 (0.005)	0.838	-0.001 (0.004)	0.715	1.370 (1.360)	0.324	8.430 (15.800)	0.599	0.581 (0.567)	0.319	-0.072 (0.222)	0.748	0.092 (0.217)	0.677	-10.980 (10.400)	0.30
rs3800373	0.017 (0.050)	0.731	0.008 (0.005)	0.098	0.005 (0.004)	0.163	-3.800 (1.400)	**0.014**	14.400 (16.300)	0.387	-0.246 (0.655)	0.712	-0.393 (0.256)	0.143	0.279 (0.250)	0.280	8.280 (13.200)	0.54
AUDIT	-0.003 (0.001)	**0.023**	-2.980e-4 (1.030e-4)	**0.008**	-2.140e-4 (8.380e-5)	**0.018**	-0.010 (0.036)	0.779	-0.363 (0.416)	0.394	0.023 (0.015)	0.087	0.013 (0.006)	**0.038**	-0.007 (0.006)	0.244	0.164 (0.342)	0.63
AIM2							-25.500 (6.730)	**0.001**	-50.400 (78.300)	0.527	-0.108 (3.720)	0.380	-0.761 (1.450)	0.607	0.394 (1.420)	0.785	-74.600 (66.900)	0.28
TMEM49							166.000 (74.600)	**0.037**	-1010.8 (867.8)	0.258	43.700 (33.500)	0.134	-5.600 (13.100)	0.674	-20.500 (12.800)	0.127	-259.30 (696.40)	0.71
NR3C1							-45.900 (75.400)	0.550	-37.700 (877.2)	0.966	-88.300 (30.500)	0.977	9.820 (11.900)	0.421	4.120 (11.600)	0.728	1255.8 (686.1)	0.08
CRP											0.171 (0.094)	0.209	-0.124 (0.037)	**0.003**	-0.008 (0.034)	0.837	-3.530 (2.240)	0.13
Cortisol											-0.007 (0.008)	**0.010**	0.008 (0.003)	**0.021**	-0.005 (0.003)	0.103	0.016 (0.182)	0.93
fMRI1																	9.610 (4.500)	**0.05**
fMRI2																	-11.200 (10.900)	0.31
fMRI3																	-23.300 (11.900)	0.06

inflammation (Fig. 1B). This finding is to be expected, as it is known that chronic alcohol use can lead to both inflammation (Bishehsari et al., 2017; Crews, 2012) and changes in the reactivity of the immune system and related genes (Archer et al., 2019; Crews, 2012). A recent study estimated the inheritance of circulating plasma CRP to be moderately heritable (around 50%) from a study using over 6000 twin participants and that CRP heritability is stable across the life span (Sas et al., 2017). As a result, approximately one-half of the variation in CRP plasma levels is due to environmental factors and we observe in our study that CRP levels are higher in vulnerable ACoAs which could be explained by hazardous alcohol use or it may act as an endophenotype/biomarker for an undetermined environmental influence. Gene association studies with anxiety behavior were not selected as a focus of this study but are an excellent avenue for future directions. Increased baseline CRP levels were found to be associated with TT carriers of the *CRP* gene (rs1205) (Fig. 2A) and in participants homozygous for the G allele in the *FKBP5* gene (rs3800373) (Fig. 2B), which in addition to being associated with increased inflammation, have also been linked to increased depression (Almeida et al., 2009) in men, and increased risk for PTSD (Xie et al., 2010) and AUD (Qiu et al., 2016) in Asian populations. The minor (G) allele of rs3800373 has been implicated in both PTSD and AUD as possibly playing a protective role against development or symptoms, however, this association has not been found in Caucasian populations specifically (Qiu et al., 2016; Xie et al., 2010). Whether that lack of risk protection is related to CRP levels has yet to be determined. We also found that decreased methylation of AIM2 and TMEM39 was significantly associated with CRP levels, with decreased methylation levels being observed in the hazardous group (Fig. 3A–C). This is supportive of previous work which has shown that decreased methylation of the AIM2 gene has been associated with increased CRP (Miller et al., 2018; Ligthart et al., 2016), and PTSD severity (Miller et al., 2018), and decreased methylation of the NR3C1 gene has been linked to increased PTSD risk (Palma-Gudiel et al., 2015), however, hypermethylation has been reported in AUD (Gatta et al., 2019). Also, in the currently reported data, NR3C1, AIM2, and TMEM49 were all associated with AUDIT scores, with increased AUDIT scores being associated with decreased methylation values (Fig. 5, Table 4), largely supporting the previously referenced works showing links between alcohol use and DNA methylation of inflammatory markers (see Introduction).

When examining the fMRI ROI data, we observed an increase in BOLD activity in the right hippocampus within ACoAs reporting hazardous alcohol in comparison to those that are not (Fig. 4A). Further, it was found that the right vACC activity was increased upon viewing negative stimuli in hazardous users (Fig. 4B). It is important to note that this pattern of activity is substantively different from what was reported in our previous work (Brown-Rice et al., 2018), which demonstrated a significant Group (hazardous vs. non-hazardous) x Condition (positive vs. neutral vs. negative) for a component of frontopolar cortex (BA10; lateral middle frontal gyrus). Specifically, hazardous alcohol participants exhibited greater BA10 activity when viewing negative visual stimuli when compared to the non-hazardous participants. In comparison to the previous study that utilized a whole-brain analysis technique, for the presently reported data we specifically focused on regions of interest that are known to be associated with the processing of emotionally salient stimuli and that have shown differential responses or structural changes in studies of alcohol use (De Bellis et al., 2000; Schweinsburg et al., 2010; Luciana et al., 2013; Nagel et al., 2005; Claus et al., 2011). This approach was adopted to both constrain the analysis to theoretically driven regions of the brain, while also reducing the number of potential variables to be included in the model – an important consideration with the low participant numbers presently utilized.

When factor analysis was performed on the functional MRI data, two distinct components were found to best explain the recorded brain activity. The first (fMRI1) was predictive of anxiety levels with increased activity being associated with increased BAI scores. fMRI1 was predicted by the methylation marker NR3C1. The second fMRI component (fMRI2) activity was predicted by AUDIT scores and cortisol levels, where higher scores were significantly correlated with increased component activity. Additionally, fMRI2 was also predicted by levels of CRP with lower levels of CRP being associated with increased activity, as were rs1205

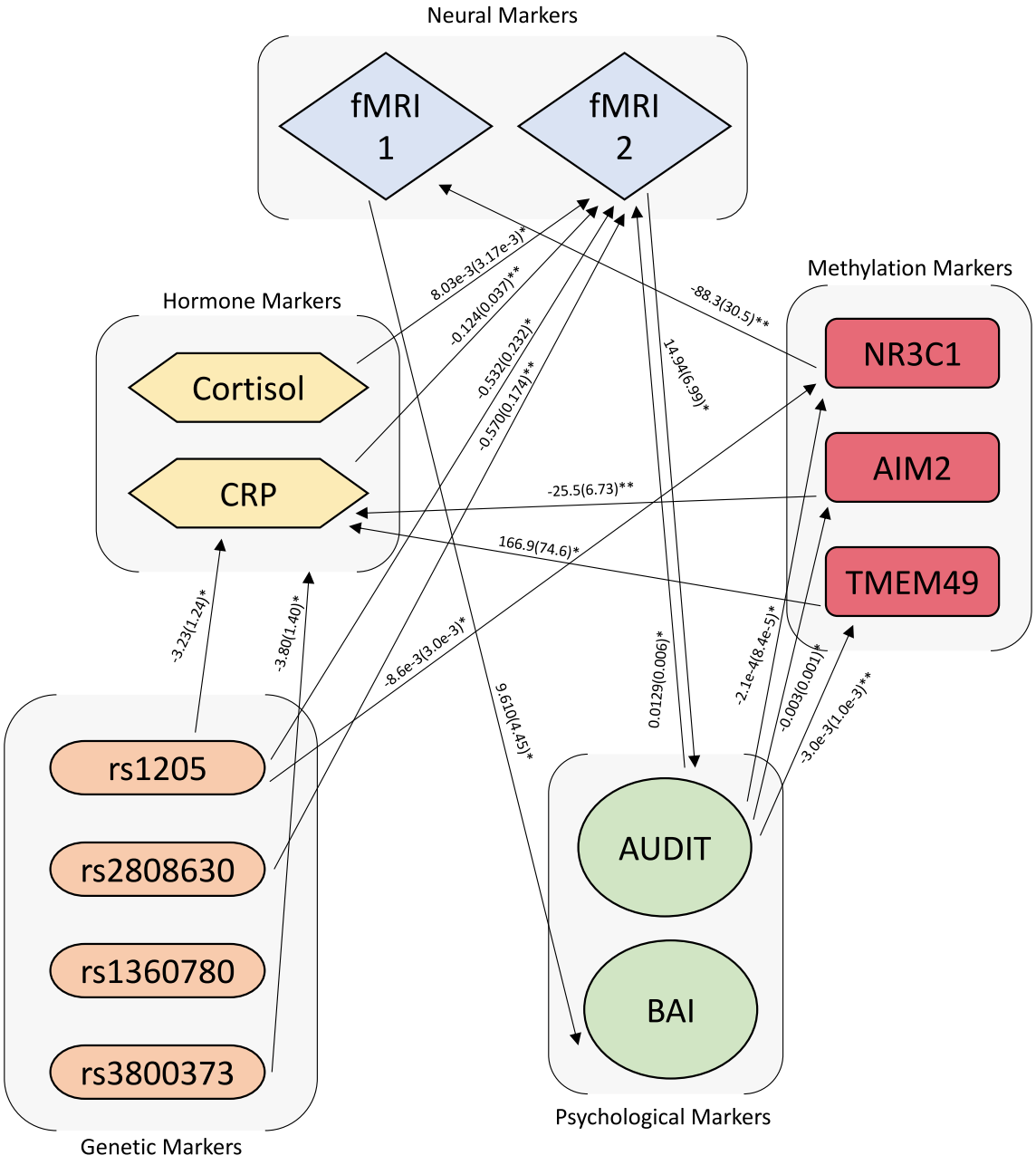

Fig. 5. Pathway analysis model. Each pathway represents a significant regression result with the beginning of the arrow being the dependent variable and the end of the arrow being the independent variable. Beta values and their standard deviations are presented next to each pathway. *significant difference, $p < 0.05$, **significant difference, $p < 0.01$.

and rs2808630 (Table 3).

The potential for a causal relationship between alterations of SNPs on CRP-related genes, and current alcohol use to increase inflammation, which can influence methylation at promoter regions on influential genes could underlie the pathway between alcohol use and negative mental health outcomes. The role that this pathway could play on neural activity and the overall influence on emotional regulation was tested in the presented model (Fig. 5). Specifically, this model suggests that CRP SNP rs1205 has both a direct and indirect effect (through CRP) on the neural activity observed during the Ranking Emotion Task used in the present study. This increased activity is, in turn, associated with alcoholism in what can be conceived as an escalating circular pathway. This provides evidence for how continued alcohol use and genetic predispositions might interact to modify behavior such that an individual's alcohol consumption will increase over time. A second potential relationship is observed in the model as both alcohol use and methylation predict activation of brain regions following the presentation of emotional cues, and methylation is associated with baseline CRP levels. These CRP levels are then correlated to activation of BA24 and hippocampus, which then link back to alcohol use. This pathway suggests that alcohol use can modify CRP levels and inflammation which, in turn, influences neural regulation of emotional input. Further, our model shows this altered regulation can have a positive feedback effect on alcohol use, which continues the cycle of negative affect which can play a role in overall mental health.

Our research uniquely demonstrates that although anxiety and alcohol use are often found to be comorbid and are associated with similar brain regions (NIDA, 2021; Castillo-Carniglia et al., 2019), the biological pathways that produce these mental health outcomes are likely different. Although the same systems are involved, the pathways

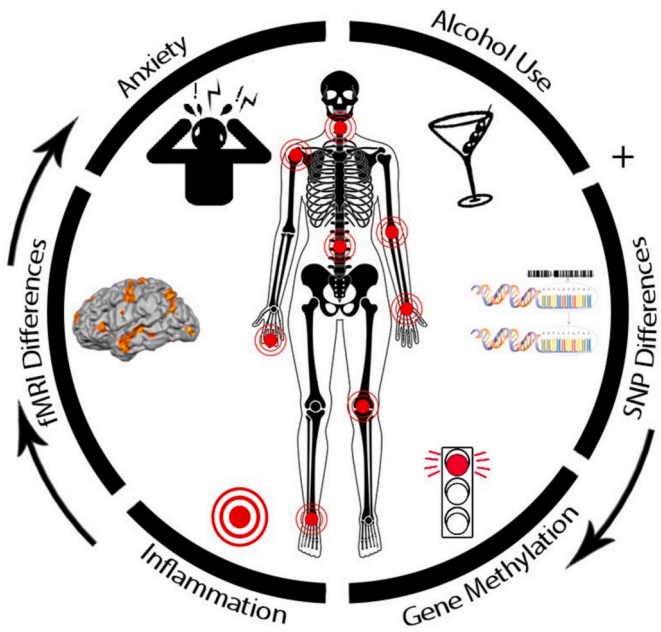

Fig. 6. Visual outline of the proposed model demonstrating the pathway between alcohol use and negative mental health outcomes. Based on the reported model, alterations of SNPs on CRP-related genes, and current alcohol use can act to increase inflammation, which can in turn influence methylation at promoter regions on influential genes. Predicted outcomes of this pathway are increased anxiety and alterations in neural activity in regions responsible for emotional regulation.

within those systems can be related to different mental health outcomes. Within our model, alcohol use is associated with decreases in NR3C1 which is associated with the specific brain activity in component fMRI2, which finally predicts anxiety. A novel conclusion from our research is that although anxiety and alcohol use are both associated with increased Hippocampal and BA24 activity while performing an emotional ranking task, the pathways through which these mental health outcomes arrive are unique. Each principal component within the factor analysis (fMRI1, fMRI2, and fMRI3) is orthogonal to each other, highlighting how each of these components explains unique portions of variance within the brain activity of these areas. This suggests that these brain areas activate differently for participants with increased alcohol use, and increased levels of anxiety. Further, even though the activity in the regions related to alcohol use are also those that we see increases in with increased anxiety, this only occurs after increased NR3C1 activity. Taken together, what this pattern of results suggests is that the comorbidity among these two mental health disorders is not as simple as increased alcohol and increased anxiety increasing brain activity in these regions. Instead, a complex interaction of genetics, methylation, behavior, inflammation, and brain activity all combine to produce this common comorbidity. Additionally, it was found that CRP emerged to play a central role in both methylation and genetic predisposition effects on observed brain activity, whereas cortisol appears to be an independent factor that significantly predicts brain activity for alcohol use but was not significantly related to other factors within the model.

To emphasize a key finding, while the pathway of factors predicting alcoholism is an escalating circuit, the pathway that predicts anxiety is terminal. Specifically, increased AUDIT scores are associated with increases in fMRI2 component activity, which in turn is significantly correlated with AUDIT scores – as drinking levels increase, fMRI2 component activity would be predicted to increase as well, which is positively associated with increased AUDIT scores, completing a circuit. In comparison, BAI scores are associated with increased activity of component fMRI1, but the converse is not true.

5. Limitations

Limitations of this study include the low sample size of participants, as well as a lack of a control comparison sample without a familial history of AUD. These limitations can restrict the number of genes targeted for SNP analysis and methylation, as well as can influence the conclusions drawn from the outcomes; however, the findings point to several important variables that provide evidence of the pathways involved in the complex etiology of AUD, heritability and mental health outcomes that can be used to inform future work and improve the scope of intervention measures.

In summary, our results provide empirical evidence of the unique pathways that create the multifaceted activity observed within emotional response regions of the brain during an emotional evaluation task (simplified visual overview in Fig. 6). Specifically, although both the anxiety and alcoholism pathways studied here are affected by both genetic predisposition and methylation sites, the specific sites, and the way they interact with other biological effects, such as inflammation, produce unique activity in emotional valence regions of the brain. These results help further differentiate comorbid mental health measures and could lead to further individualized treatments based on the known etiology of the aberrant psychiatric response.

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Declaration of competing interest

The authors declare no conflicts of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbih.2022.100505.

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