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

Jamie L. Scholl, Zach R. King, Kami Pearson, Noah A. Kallsen, Erik A. Ehli, Kelene A. Fercho, Kathleen A. Brown-Rice, Gina L. Forster, Lee A. Baugh

A Basic Biomedical Sciences & Center for Brain and Behavior Research, Sanford School of Medicine, University of South Dakota, USA
b FAA Civil Aerospace Med. Inst., Oklahoma City, OK, USA
c Department of Counselor Education, College of Education, Sam Houston State University, Huntsville, TX, USA
d Department of Anatomy, University of Otago, Dunedin, New Zealand

ARTICLE INFO
Keywords:
Alcohol
Anxiety
Genetics
Methylation
C-Reactive protein

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., 2016; 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.

* Corresponding author. Division of Basic Biomedical Sciences Sanford School of Medicine University of South Dakota 414 E. Clark St. Vermillion, SD 57069, USA. E-mail address: lee.baugh@usd.edu (L.A. Baugh).

https://doi.org/10.1016/j.bbih.2022.100505
Received 22 February 2022; Received in revised form 19 August 2022; Accepted 23 August 2022
Available online 2 September 2022

© 2022 Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
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% 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 (Fuji 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 (Fuji et al., 2014) and has been related to PTSD risk (Fuji et al., 2014; Binder et al., 2008; Zhang et al., 2020; Li et al., 2019), suicidality and depression (Hernandez-Diaz et al., 2019; Perez-Ortz 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 (Fuji et al., 2014), can contribute to the regulation of inflammatory and immune responses (Zamas et al., 2016; Zamas and Binder, 2014), and mediate the relationships between circulating CRP and cortisol and epigenetic aging following adverse experiences in children (Dammeng 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.
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 co-occurring conditions 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 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, Gainesville, 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 Internationally 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 QIAasymp® instrument coupled with the QIA-symph® 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 8.5, 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
iso thermal (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; Perez-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 promoter 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 p-values for each group were compared by separate unpaired t-tests, which were corrected for multiple comparisons using a Benjamini-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.
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
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 baseline plasma CRP and anxiety measures (Fig. 2A–D). Specifically, baseline plasma CRP levels were significantly higher in hazardous alcohol users (F(2,24) = 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 (Brodman’s 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 rotations. Component 1 (MR1) encompassed approximately 39% of the variance within our original fMRI data, component 2 (MR2) encompassed 20%, and component 3 (MR3) 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.
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 TME49M (F (Reich et al., 1988; Archer et al., 2019) = 4.00, p = 0.009, adjusted R-squared = 0.349), and AUDIT scores significantly predicted TME49M (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 TME49M also significantly predicted CRP (AIM2 beta = −25.50 (6.733), t = −3.787, p = 0.001; TME49M 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...
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 predisposition 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 3**

| 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.

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.
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 alcohol-related factors and changes in the reactivity of the immune system. 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 and related genes (Archer et al., 2019; Crews, 2012) have 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 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).
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
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.

**Funding**

This work was funded by a pilot grant from the Center for Brain and Behavior Research at the University of South Dakota, a South Dakota Governor’s Team Development Grant, and a Summer Program for Undergraduate Research in Addiction (SPURA) fellowship to KP (NIH R25-DA033674).

**Declaration of competing interest**

The authors declare no conflicts of interest.

**Data availability**

Data will be made available on request.

**Acknowledgements**

The authors would like to recognize the assistance of the individuals and volunteers who participated in this research, as well as gratefully acknowledge the work of the radiology staff at Avera Sacred Heart Hospital in Yankton, SD, and the University of South Dakota Human Functional Imaging Core.

**Appendix A. Supplementary data**

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

**References**

Alca-Ferrara, L., Müller-Oehring, E., Sullivan, E., Pfefferbaum, A., Schulte, T., 2016. Brain responses to emotional salience and reward in alcohol use disorder. Brain imaging and behavior 10 (1), 136–146.

Almeida, O.P., Norman, P.E., Alcock, R., Pvy, Bockxmeer, Hankey, G.J., Jamrozik, K., et al., 2009. Polymorphisms of the CRP gene inhibit inflammatory response and increase susceptibility to depression: the Health in Men Study. Int. J. Epidemiol. 38 (4), 1049–1059.

Ancelin, M., Farre, A., Carrière, I., Ritchie, K., Chambes, i, Ryan, J., 2015. C-reactive protein gene variants: independent association with late-life depression and circulating protein levels. Transl. Psychiatry 5 (1), e499–e.

Anderson, O.S., Sant, K.E., Dolinoy, D.C., 2012. Nutrition and epigenetics: an interplay of dietary methyl donors, one-carbon metabolism and DNA methylation. J. Nutr. Biochem. 23 (8), 853–859.
