Alcohol Cue Reactivity and Salivary Alpha-Amylase Response in Binge Drinkers

by

Clayton Michael Ridner

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Approved by

Sara Blaine, Chair, Assistant Professor, Department of Psychological Sciences
Richard Macatee, Assistant Professor, Department of Psychological Sciences
Jennifer Robinson, Professor, Department of Psychological Sciences

Abstract

Alcohol Use Disorders (AUDs) are a leading cause of mortality and morbidity in the world and binge drinking is a known risk factor for the development of AUDs. Blood-level-oxygen-response (BOLD) signal during alcohol cues and cortisol, a hormone measuring the bodies hypothalamic-pituitary-adrenal stress system, have been correlated with time to relapse in individuals with AUD. But it is yet to be seen if these results are etiological factor of AUD or a result of prolonged drinking. The ventromedial prefrontal cortex (vmPFC), anterior insular cortex (AIC) and amygdala (AMYG) have been linked to cue responsivity through their roles in autonomic regulation, interoception, and craving/arousal, respectively. Furthermore, salivary alpha amylase (sAA), a digestive enzyme which correlates with sympathetic nervous system activation, an enzyme correlated with the bodies noradrenergic response to stress, has been linked with AUD. Yet, at this time a research study connecting sAA and alcohol cue reactivity has yet to be conducted. Forty-six MD (n = 20) or BD (n = 26) participants completed two functional magnetic resonance imaging (fMRI) scans during which alcohol or water cues were presented. Saliva samples were collected before and after each scan to measure sAA. We found group differences in that BD had greater levels of sAA at baseline suggesting heightened SNS arousal. We examined group differences in the vmPFC, AIC and AMYG and correlated them with sAA change scores (sAA Δ). Group neural alcohol cue activity differences were not found, but trends between BOLD signal and sAA Δ across participants were. AIC BOLD signal during alcohol cues was positively correlated with sAA Δ, suggesting increased craving/arousal associated with SNS arousal. We found that the LAMYG and LvmPFC were both positively correlated with sAA Δ scores while the RAMYG and RvmPFC were negatively correlated with sAA Δ. Functional asymmetries such as these can be plastic or task dependent. Thus, this lateralization warrants further exploration to discover if a relationship between unilateral BOLD activity, SNS arousal and problem drinking contributes to BD.

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Introduction

Alcohol Use Disorders (AUDs) are the 5th leading preventable cause of mortality and morbidity in the world (Whiteford et al. 2013; Grant et al. 2015). Relative to healthy individuals, participants with AUD demonstrate higher levels of negative affect (Park et al., 2016), a stronger likelihood of displaying anger (Giancola et al. 2002; George & Marlatt, 1986), greater risk behavior, (Korlakunta & Reddy, 2019) and increased suicidal ideation (Dillon et al. 2019). These factors make AUD a primary cause for health concern in the United States, where in 2010 over $249 billion dollars was spent on alcohol misuse costs (Sacks et al., 2015). Binge drinking is a known risk factor for the development of AUDs, yet 25.8% of US adults regularly drink to excess (Brumback et al., 2015; Jones et al., 2018). Binge drinking is defined as 5 or more drinks over a two-hour period for men, and 4 or more drinks over a two-hour period for women (White et al., 2018). Binge drinking can transition to AUD symptomology and understanding the initial stages of AUD could help arrest problem drinking at an earlier time.

Incentive salience

The predominant theory surrounding substance use disorder research for some time has been that of the “addiction cycle” (Koob & Volkow, 2010). The addiction cycle model consists of three stages: binge/intoxication, preoccupation/anticipation, and withdrawal/negative affect. In parallel, the Alcohol and Addiction Research Domain Criteria (AARDoC) posits that three domains are present in the formation of an AUD: incentive salience, executive control dysfunction, and negative emotionality, each corresponding with the previously described stages in the addiction cycle (Al-Khalil et al., 2021). Specifically, incentive salience corresponds with the binge/intoxication phase, executive control dysfunction with preoccupation/anticipation phase, and negative emotionality with withdrawal/negative affect phase. Incentive salience refers to the sensitization of physiological response and previously learnt associations towards reward cues. It is through increased sensitization that individuals with AUD become more responsive to alcohol related stimuli (Robinson & Berridge, 1993). This corresponds with

neuroadaptations in the corticostriatal network, including dopaminergic tracts from the nucleus accumbens/ventral striatum to the cortex, that take place during binge/intoxication. The altered neurobiology is associated with dysregulation of the hypothalamic-pituitary-adrenal (HPA) system, one of the body's primary facilitators of stress response.

The HPA axis and Alcohol cue reactivity

HPA axis activity originates in the paraventricular nucleus of the hypothalamus where corticotropin releasing hormone (CRH) is secreted. Following this secretion, the pituitary gland is stimulated causing the release of adrenocorticotropic hormone (ACTH). Once this biofactor has been released, it enters the blood stream where it is delivered to the adrenal glands. ACTH causes the adrenal glands to release cortisol which impacts autonomic nervous system function. Cortisol is one of the principal stress hormones in the body that increases the availability of blood glucose in the brain. Cortisol is increased to allow the body to process stressful situations in which increased awareness is needed to consistently operate on high alert. If the sympathetic nervous system (SNS) is stimulated for a prolonged period through the amygdala (AMYG), then the HPA axis is consistently stimulated, ultimately leading to an increase in the allostatic load. The allostatic load, also known as "wear and tear" on the body caused by stress, occurs as a result of this persistent hyper-processing of glucose (McEwen, 2000).

Individuals with AUD show altered response patterns to cue reactivity which have been linked to the HPA axis. Sinha et al. 2009 initially demonstrated altered autonomic activity in alcohol dependent (AD) individuals following exposure to stress and alcohol cues. Seo et al. 2013 were able to further expound upon this research to show that abnormal neural and HPA axis reactivity during personalized, auditory, stress and alcohol cues was a predictive factor of time to relapse. Thus, differential cue and stress reactivity was linked to increased relapse probability through abnormalities in the autonomic nervous system and the HPA axis. Continuing this research, Blaine and Sinha 2015 examined alcohol and stress blood-oxygen-

level dependent (BOLD) reactivity's correlation with cortisol:ACTH ratio in AD treatment seeking participants. BOLD neutral cue hyperactivity in the ventromedial prefrontal cortex (vmPFC) and cortisol:ACTH ratio were found to be predictive factors of time to relapse. Through hierarchical regression modeling, they showed the vmPFC was the sole mediator of increased relapse risk through cortisol:ACTH ratio (Blaine et al., 2017). Prior research had suggested the vmPFC as an indicator of HPA activity (Thayer et al., 2012) and Dager et. al 2013 found group differences between transitioning drinkers and controls during functional cue reactivity in the vmfPFC and left insula. The vmPFC has both afferent and efferent connections to the paraventricular nucleus (PVN) of the hypothalamus, it has been hypothesized HPA axis activity is regulated through this pathway.

As a continuation of this research, Blaine et al 2019. examined blood cortisol levels in moderate drinking (MD) and binge drinkers following alcohol and stress cues to elucidate if HPA axis abnormalities are etiological contributors to the development of AUDs or rather a resultant from prolonged risky drinking behavior. Indeed, the results indicated that prior to developing an AUD or alcohol dependence, BD displayed increased basal cortisol levels and blunted cue reactivity relative to SD. Further bolstering the connection between the two phenomena, Blaine et al. 2020 found that AUD participants had vmPFC hyperactivation during neutral cues but blunted responsivity to alcohol cues. This mirrors other the prior studies indicating HPA resting state hyperreactivity but blunted responsivity in individuals with AUD but translates the reactivity to a paradigm designed around incentive salience.

Abnormal activation to alcohol cues not only occurs the vmPFC, but the insular cortex (IC) as well. The IC lies within the lateral sulcus, separating the frontal, temporal and parietal lobes (Naidich et al., 2004). The insula has bidirectional connections with the each of these lobes as well as with subcortical structures including the cingulate, amygdala, brainstem, thalamus and basal ganglia (Flynn et al., 1999). The IC is subdivided into the anterior insular cortex (AIC), middle insula, and posterior insula (Gu et al., 2013). The posterior insula receives

afferents from the spinal cord and is involved in the integration of the somatosensory cortex and thalamus (Flynn et al., 1999). The AIC connects cortical and subcortical areas, integrating autonomic and interoceptive attention (Flynn et al., 1999). The AIC encodes subjective feelings and is recruited while anticipating changes in physiological sensation intensity (A. D. Craig, 2002; A. D. B. Craig, 2009; Flynn et al., 1999; Naqvi & Bechara, 2009). The AIC's role in processing awareness of physiological signals arising from the body will make it the focus of this study in relation to alcohol cues (Wang et al., n.d.). The sensation of arousal caused by alcohol cue reactivity stimulates the AIC, causing physiological arousal from memory, thus further activating the HPA axis and sympathetic nervous system (Blaine et al., 2017; Campbell et al., 2019).

Additional evidence suggests AIC structural abnormalities and alcohol cue reactivity patterns in at risk drinkers. For example, during an alcohol cue exposure paradigm in which subjective alcohol expectancy scores were correlated with brain grey matter volume (GMV) in social drinkers (SD), but decreased GMV of the right insula in women was associated with increased alcohol expectancy in women (Ide et al., 2017). Therefore, loss of or the lack of GMV might support the transition from binge drinking to AUD. While the abnormal AIC GMV might be suggestive of a precursor to AUD, it is also a region highly susceptible to the effects of acute alcohol intoxication. Zhu et al. 2003 found after acute alcohol intoxication, the largest decreases in activity were found in the occipital cortex and basal ganglia. Running connectivity analyses using these two regions as seeds, the basal ganglia's variance was associated with activity in the insula and the occipital cortex's variance was associated with activity in the vmPFC. Therefore, it can be hypothesized that acute alcohol administration may lead to changes in the reward response via the insular connections to the nucleus accumbens, caudate and putamen of the basal ganglia. In addition, Schacht et al. 2013's meta-analyses implicated the AIC and vmPFC as regions of abnormal activity during alcohol cue reactivity tasks in heavy drinking/AUD positive individuals. This meta-analysis found the bilateral insula and the vmPFC to be two of

the regions most prone to differential activation in 679 heavy drinking/AUD positive individuals compared with 174 controls.

It has been hypothesized the AIC mediates top-down processing of the vmPFC along with bottom-up signaling from the AMYG during increased arousal to form interoceptive states (Gu et al., 2013). There is a robust body of literature linking the amygdala to the fear/startle response and the associated sympathetic arousal in humans (Davis, 1997; Etkin et al., 2011; Öhman, 2005; Thayer et al., 2012; Yoshihara et al., 2016). This connection directly links the AMYG to the sympathetic nervous system, but it is also closely related to the consolidation of salient memories. This connection could indicate a linkage between the AMYG, abnormal stress responding and incentive salience in at risk drinkers. Specifically, provocation of the hypothalamus by way of the amygdala leads to the release of norepinephrine stimulating the sympathetic nervous system (Talarovicova et al., 2007; Tanaka et al., 2000). After the initial phase of the stress response, the AMYG will recruits the hypothalamus via the PVN to begin the HPA axis stress response (Smith & Vale, 2006). Persistent stimulation from the HPA axis causes glutamate signaling abnormalities in the N-methyl-d-aspirate (NMDA) and a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptor subtypes. Hyperstimulation of these receptor subtypes have been theorized to contribute to excitotoxicity through both increased glutamate release and increased pro-stress neuropeptides (Roberto et al., 2012). Thus, we can see both the increase in allostatic load through the peripheral nervous system, i.e. increases in sympathetic noradrenergic activity as well as through the central nervous system via glutamate and pro-stress neuropeptides. It is therefore not surprising that the AMYG, which influences activity between the PVN and vmPFC, might play a role in the transition from binge drinking to AUD. These phenomena place the AMYG as a central region of focus during the transition from BD to AUD.

ANS and Salivary Alpha-Amylase

The autonomic nervous system is comprised of the sympathetic-adrenal-medullary (SAM) system and parasympathetic system, which consistently modulate the activity of one another (Ali & Nater, 2020). HPA axis activation co-occurs with SAM activation. One particularly robust biomarker of this activity is cortisol. And yet, the HPA axis and ANS are distinct parts of the bodies' stress response system. So, while one influences the other, Salivary alpha amylase (sAA) is a better biomarker for the SAM during the transition from BD to AUD. If sAA levels show hyperactivity at resting state and hypoactive cue responsivity in BD, this may indicate SAM dysregulation as a potential etiological factor in the development of AUD.
Patients with AUD have greater resting autonomic nervous system activation but blunted SAM alcohol cue responsivity (Sinha et al., 2009), mirroring that of cortisol and the vmPFC.

A more commonly used metric of abnormal autonomic activity in individuals with AUD is heart rate variability (HRV). HRV measures the interplay of the sympathetic and parasympathetic divisions of the autonomic nervous system through fluctuations in heart responsiveness (Acharya et al., 2006). In consonance with protracted stress, decreased HRV has also been linked to negative health outcomes like increased inflammation, immune dysfunction, cardiovascular disease, and mortality (Kemp & Quintana, 2013). Increases in sympathetic nervous system activity cause the interval between heart beats to become shorter, resulting in decreased HRV during acute stress (Thayer et al., 2012). During a grip task promoting sympathetic arousal through stress, individuals had increased BOLD AMYG and vmPFC activity corresponding with decreased HRV (Napadow et al., 2008). Thus, HRV has been suggested as a metric of understanding the brain-body connection via vagal nerve stimulation and the autonomic response.

Similar correlations have been made using HRV as an indicator of autonomic activity in individuals with AUD, correlating these metrics with fMRI BOLD signal. For example, Ingjaldsson et al. 2003 focused on HRV and alcohol cue reactivity in alcoholic subjects. These

participants were asked to visualize alcoholic imagery. Participants who were able to later able to successfully reduce craving had increased HRV during the visualization. Based on their meta-analysis, Thayer et al. 2012 postulates this occurs through reduction of the sympathetic response through vmPFC inhibition. Indeed, these results appear to be supported similarly by Wang et al. 2020, whose study examined HRV and the BOLD response in relation to alcohol cues. Using HRV measured as the root-mean-squared difference (RMSSD), an indicator of parasympathetic activity, AUD participants were found to have increased sympathetic activity. This decreased RMSSD activity also correlated with increased AUDIT scores and blunted vmPFC activity during alcohol cues. These results echo a similar blunted stress and vmPFC response to alcohol cues as seen by Blaine et al. 2017 when examining cort:ACTH ratio in AUD participants.

Similarly, sAA has been used as a biomarker of response to stress (Ali & Nater, 2020; Chatterton et al., 1996; Ditzen et al., 2014; Muehlhan et al., 2017; Petrakova et al., 2017). For example, when corticotropin releasing hormone, a hormone that stimulates the plasma noradrenergic response through downstream HPA axis activity was administered. sAA, plasma noradrenaline, and self-reported stress perception were significantly elevated relative to placebo (Petrakova et al., 2017). In addition, clinical studies found that after the administration of a "stress test", individuals had increased levels of sAA (Alsalman et al., 2016; de Vente et al., 2015). sAA and cortisol were both found to increase following acute alcohol administration in healthy males (Magrys et al., 2013). The matching directionality of these responses suggests that there might be a mirrored response in the HPA and SAM stress systems. Further, this might indicate that dysregulation of the SAM occurs in a pattern like that of the HPA axis. Indeed, AUD participants were found to have blunted sAA responsivity compared to healthy controls after completing a "stress test". This occurred despite AUD participants' higher reports of subjective stress (Muehlhan et al., 2017). Blaine et al. 2015 showed a similar blunted cort:ACTH stress response in individuals with AUD following simulated stress. Therefore, it would make sense

that there is a similar associative learning pattern to alcohol cues related to the stress response (Blaine et al., 2019). Because of the connection between the SAM and HPA axis, as well as evidence indicating there might be a co-occurring response pattern, I propose sAA is examined in response to alcohol cues in BD.

New contribution to the study of stress and cue reactivity in AUDs

Therefore, in this fMRI study, the specific relationship between the BOLD and the sAA responses to alcohol cues were explored to better understand the underlying biobehavioral mechanisms contributing to abnormal alcohol cue reactivity in BD. Participants were assigned to MD and BD groups. Two scans were performed for each participant to allow both within and between subject analyses. One scan will be performed with alcohol pictures as the active stimuli and an additional scan will be performed with water pictures as the active stimuli (see Figure 1). Utilizing two participant groups and two conditions allows us to control for baseline differences in groups and to isolate the effects of alcohol cues. In doing so, an accurate measurement of BD cue reactivity can be recorded. sAA levels were measured before and after each scan. It was hypothesized binge drinkers would show higher baseline sAA levels and lower sAA Δ following presentation of alcohol cues. Furthermore, I hypothesized that during alcohol cue presentation, BD would have less BOLD signal in the vmPFC and AMYG but greater signal in the AIC relative to MD. To fully explore this effect, water imagery was used to control for non-alcohol related BOLD signal. Lastly, we hypothesized sAA Δ would be positively correlated with AIC BOLD signal and negatively correlated with vmPFC and AMYG BOLD signal.

Purpose

The purpose of this study was to examine the relationship between the BOLD signal during alcohol cue reactivity and sympathetic nervous system responses in BD vs MD.

Hypothesis

H1: BD will have greater BOLD activity in the AIC relative to MD during alcohol cues.

H2: BD will have less BOLD activity in the vmPFC and AMYG relative to MD during alcohol cues.

H3: sAA levels will be higher in BD than MD at baseline.

H4: BD will show less sAA response following alcohol cue observation relative to MD.

H5: AIC increased responsivity will be negatively correlated with sAA Δ

H6: vmPFC and AMYG reduced responsivity will be positively correlated with sAA Δ

Methods

Screening and intake procedures

Participants were recruited from the greater Auburn-Opelika area via flyers calling for individuals who "like beer." In addition, advertisements were posted on Facebook, Instagram, Reddit, and Craigslist in the Auburn-Opelika area to further recruit the community. Auburn University's SONA recruiting system was also utilized to recruit undergraduate students on Auburn's campus. Undergraduates were offered 1 unit of extra credit for each hour participating. Participants who did not request course credit received $400 in compensation for completing a larger overall study being conducted by Dr. Sara Blaine at Auburn University. Compensation was $25 for initial intake interview, $75 for first functional scan, $100 for second functional scan and $200 for additional self-report questionnaires completed for research outside of the current project.

Participants were healthy, non-substance using, beer drinking men and women, that medical, demographic, substance abuse and interview-driven psychiatric health assessments. Binge Drinker (BD) status was characterized using the National Institute of Alcoholism and Alcohol Abuse (NIAAA) criteria for hazardous drinking, with 8 or more drinks/week in women and 15 or more drinks/week in men with weekly binge drinking episodes (five or more drinks in men; four or more drinks in women per drinking episode) for BD. Participant drinking history was determined on the basis of their responses during intake interviews and self-report

questionnaires, including an alcohol intake screening that assesses past and last 30 days drinking using items from the Addiction Severity Index (McLellan et al., 1992), as well as current alcohol intake on the Cahalan Quantity Frequency Variability Index (QFVI) (Cahalan et al., 1969) and Alcohol Use Disorders Identification Test (AUDIT) (Bohn et al., 1995). The MD group was comprised of those who reported less than 8/week for women and less than 15/week for men with no episodes of binge drinking (*Alcohol Facts and Statistics | National Institute on Alcohol Abuse and Alcoholism (NIAAA)*, n.d.). Participants were excluded if they met current DSM-V criteria for any other substance use or psychiatric disorders, or if they took any psychiatric medications. These recruitment criteria and all subsequent experimental features were approved by the Office of Human Research at Auburn University. After participants were screened through an online recruiting questionnaire created with QualtricsXM, participants were invited to participate in an intake interview conducted over Zoom Video Chat.

During the Zoom intake interview, participants provided written informed consent with digital signatures. After, participants reviewed the Auburn Magnetic Resonance Imaging (MRI) Center's MRI Pre-Screening form, in which potential participants were asked about prior surgery and potential ferromagnetic implants which might prevent participation in MRI research. Next, participants were administered the Structured Clinical Interview for the Diagnostic and Statistical Manual V (First, 2015), QFVI and AUDIT. Upon completion, if the participant did not meet any exclusionary criterion, they were scheduled for their first and second scan appointments. Participants were reminded not to consume alcohol for 24 hours prior to each appointment. Transportation was provided to the MRI center, as participants were drinking alcohol during their appointment. Participants were completing an alcohol taste test (ATT) (Marlatt et al., 1973) as a result of participating in a larger study at Auburn University.

The study was conducted as a within-subjects design, with participants undergoing two separate cue presentation conditions. Each participant was assigned to the "Alcohol" or "Water" condition randomly based upon an online list generator. From this software, "1" and "0"

represented “Alcohol” and “Water” conditions respectively. Condition assignments were assigned in the order of intake completion.

Cue Image Selection and Evaluation

Images were selected from royalty free searches through the internet. Pictures were divided into three separate subsets for the experimental design and evaluation. Alcohol cues included images of wine, beer, cocktails, champagne, and hard liquor. Water cues included pictures of drinking water in cups, bottles, and fountains. Neutral images of mountains, grass, trees, hygienic items (toothbrush, dental floss etc.), alarm clocks, and athletic items (baseballs, soccer balls, etc.) were selected to provide baseline signal. Pictures were evaluated for arousal and valence using the International Affective Picture System Technical Manual (Bradley & Lang, 2017).

Undergraduate students were given extra credit points to complete a picture evaluation survey. During this survey, participants rated alcohol, water, and neutral images (192 alcohol, 196 water, 67 neutral) on a continuous scale with 1 indicating low arousal/valence and 5 indicating high/arousal and valence. 162 students completed the survey and mean scores for each picture were created. A one-way ANOVA was run to determine if there were significant differences in arousal and valence between alcohol and water images. Model significance was confirmed $F(2,454)$, $p < .05$ and individual t-tests were performed to determine group differences. Valence scores on t-tests showed significant differences between alcohol and water cues ($p = 2.5 * 10^{-14}$), alcohol and neutral cues ($p = 0.024$) as well as water and neutral cues ($p = 0.0024$). Arousal scores on t-tests did not show significant differences between alcohol and water cues ($p = 0.84$). However, alcohol and neutral cues ($p = 0.023$) as well as water and neutral cues ($p = 0.023$) did show significant differences. These results are keeping with the desired context of the experiment. Overall, the participants found alcohol images to be more pleasurable than water images, but their arousal remained consistent regardless of image type.

Breath Alcohol Content, Drug Screen and Pregnancy Test

Upon arrival at the Auburn University MRI Research Center, participants gave breath alcohol testing and urine toxicology screens to confirm sobriety at each study appointment. Breath alcohol content was be measured on a Drager Alcotest 6820 to ensure a breath alcohol content of 0.000. iCup drug screens were used to test for 15 commonly used drugs of abuse. iCup drug screens test in 2-5 min while minimizing the collector's exposure to urine. Positive test results cancelled the scan for the day. This occurred for one participant. This participant was rescheduled for a day in which they provided a drug free urine sample. Female participants also took urine pregnancy tests before each scan. Pregnancy tests were conducted using Medline hCG 25mlU/mL pregnancy tests. Positive pregnancy tests would have resulted in exclusion from the study.

Salivary Alpha-Amylase Collection Procedure

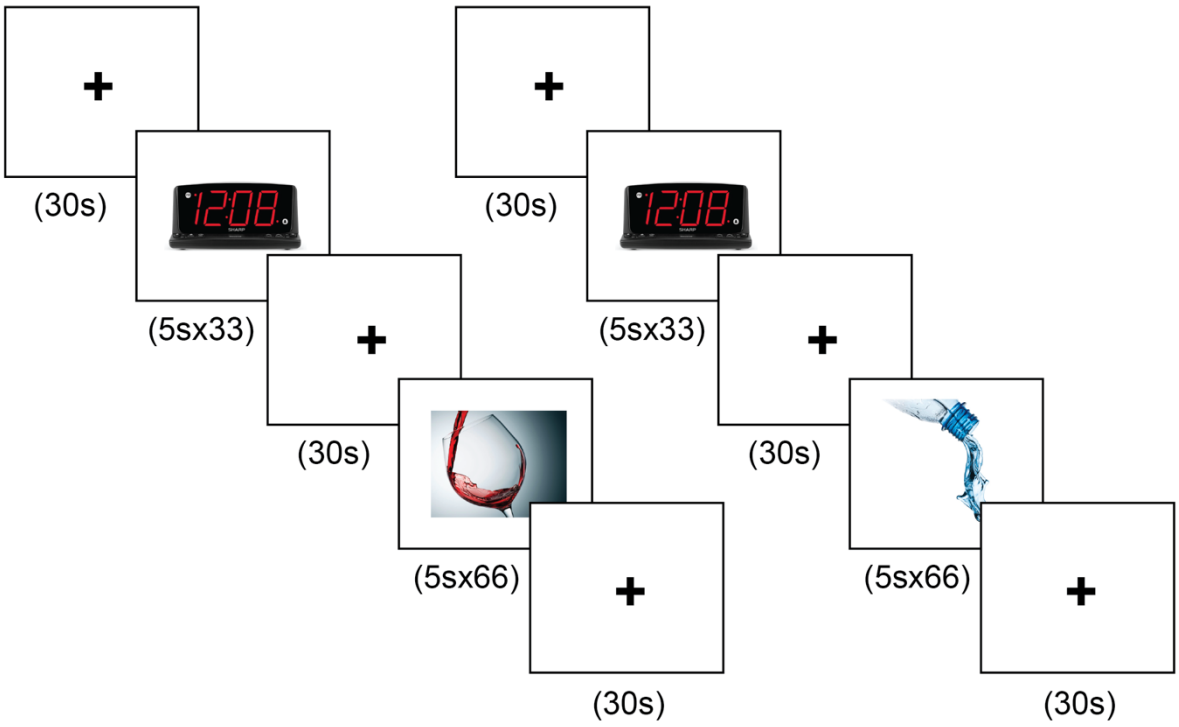
Salivary Alpha-Amylase was collected using SalivaBio Oral Swabs and SalivaBiO Swab Storage Tubes. Participants were instructed to place the SalivaBio Oral Swab under their tongue for one minute without movement or swallowing during sample collection. This helped maintain analyte consistency across participants. After collection, saliva samples were transferred to a -20^{o} C freezer. Saliva samples were collected twice at each scan with a total of four for each participant. The first, baseline collection will occur immediately after the participants arrival. The second sAA sample will be collected immediately following cue exposure to water or alcohol pictures in the MRI. Processing was conducted off site by Salimetrics (Carlsbad, CA). Transfer to the off-site processing facility utilized coolers lined with dry ice to keep temperature stable during transit. Salimetrics processed the saliva samples using an enzymatic alpha-amylase assay kit. This assay kit uses a substrate which allows spectrophotometrical measurement at 405nm. The fluorescence at this wavelength indicates sAA present within the sample.

fMRI Data Acquisition and Analysis

Imaging Parameters and Image Presentation

Scanning was performed in a 7 Tesla (7T) Siemens MAGNETOM MRI system equipped with a standard 32 channel head coil, using the T_1 magnetization-prepared rapid gradient-echo (MPRAGE) sequence for structural scanning. High resolution structural images were acquired with the following parameters (TR=2200 ms, TE=2.89ms, TI=1050ms, bandwith=240Hz/pixel, flip angle =7°, field of view = 190x190mm, matrix = 256x256, slice thickness = .7mm, gap = .35mm, 256 sagittal slices). A echo planar (T_2^*) sequence was used to collect functional images. Two-hundred twenty-eight volumes (TR=3000 ms, TE=2.8 ms, bandwith=1124Hz/pixel, flip angle=70^o, field of view=200x200mm, matrix = 234x234, slice thickness=1.5mm, gap = .9mm, 37 axial slices parallel to the anterior commissure-posterior commissure line) were collected for functional blocks.

During each session, there were 3 functional blocks consisting of “Alcohol” or “Water” task stimuli. Prior to the first run, a 30-second fixation point appeared on screen to record baseline activity levels. After this, a 3-minute neutral image run was be presented, with each image lasting 5 seconds interspersed with a 1-second interstimulus fixation point (33 images per block; 33 fixations points per 3-minute run). The neutral run was presented first regardless of condition. Following the neutral run, “Alcohol” or “Water” runs were presented. Images appeared on screen for 6 seconds with a 1-second interstimulus fixation point (66 images per block; 66 fixations per 7-minute run).



Fig, 1: Participants in two groups (MD, BD), will give saliva samples before and after each scan. Participants will complete both scan designs in a counterbalanced order. Following structural scanning, participants will undergo three functional blocks consisting of neutral + ALC or neutral + WAT images. Each block will begin with a 30s fixation point, followed by 33 neutral images, each presented 5 seconds. After an additional 30s fixation point is presented, participants will observe 66 ALC or WAT images, each presented 5 seconds. Each block will conclude with a 30s fixation point. Following completion of the three blocks, participants will undergo a 10-minute resting state scan and then be removed from the scanner.

MRI Data Processing

The following steps were performed twice for each participant, once for each scan. Scan data was exported to the local network server at the Auburn MRI Center. Cyberduck was used to download MRI image data as Digital Imaging and Communication (DICOM) files. DICOM files were converted to Neuroimaging Informatics Technology Initiative (NIFTI) format using MRICRON. NIFTI files were added to Brain Imaging Data Structure (BIDS) folders to be processed by the fMRI Prep (Esteban et al., 2017). The BIDS folder comprised of three sessions corresponding to each image block. fMRI Prep used the anatomical images to create spatially normalized image and performed brain tissue segmentation. In addition, fMRI Prep created head-motion and field inhomogeneity estimates using functional images. These were output as a list of potential confounds/nuisance regressors in a .csv file. These potentially non-neuronal

signals were used to correct for motion and inhomogeneity errors. fMRI Prep also created a brain mask which was later used in FSL first level processing. Following image preprocessing, our functional images were aligned onto our anatomical images (Greve & Fischl, 2009). FMRIprep output html reports which were visually inspected for spatial normalization, distortion, and functional/anatomical image alignment. Following this, brain extraction was performed on our functional images which allowed us to perform first level analysis in FSL.

Confounds/nuisance regressors were then input to FSL by importing the csv from fMRI Prep. Block-by-block contrasts were created in which neutral stimuli were subtracted from active stimuli. Times specifying active or neutral stimuli are included based upon our task design. FSL subtracted the neutral activation from the active activation and modeling during these conditions were created using the general linear model implemented by FSL FEAT (Smith et al., 2004). For our second level processing the water session activation was subtracted from alcohol session activation. The BOLD signal output after second level analysis was used as our BOLD signal indicating neural activity during alcohol cue presentation. Once we had created these scores for each participant, whole-brain group analyses were performed. There were not significant differences between our groups, so ROI analyses were performed to increase power in our analysis. ROI coordinates for the vmPFC, AIC and AMYG will be assigned based on Montreal Neurological Institution coordinates provided from prior literature (Blaine et al., 2020; Wang et al., 2019) with instructions from Andy's Brain Book (Jahn, 2022. Doi:10.5281/zendo.5879293). Following the creation of individual contrasts, t-tests were performed in Jamovi (*The Jamovi Project (2021). Jamovi (Version 1.6) [Computer Software]. Retrieved from Https://Www.Jamovi.Org*, n.d.) to compare activation between groups.

Statistical Plan of Analysis

H1: BD will have greater BOLD activity in the AIC relative to MD in response to alcohol cues.

H2: BD will have less BOLD activity in the vmPFC and AMYG relative to MD in response to alcohol cues.

Participants were assigned to their respective groups. T-tests were performed to assess group differences between MD and BD during alcohol cue presentation. This statistical test was repeated for each of the three ROI (AIC, vmPFC, and AMYG).

H3: sAA levels will be higher in BD drinkers than MD at baseline.

H4: BD will show less sAA response following alcohol cue observation relative to MD.

After sAA data was received from Salimetrics, it was cleaned using R Studio (RStudio Team, 2020) and checked for normality using a Shapiro-Wilk test (SHAPIRO & WILK, 1965). Once data was cleaned and inspected it was transferred to Jamovi. A three-factor repeated measures ANOVA was run with group, time, and condition as independent variables and sAA scores as the dependent variable. Tukey's honest significant difference (HSD) test (Haynes, 2013) was used to evaluate group differences at baseline (H3). To evaluate H4, the group * time * condition effect was examined.

H5: AIC increased responsivity will be negatively correlated with sAA Δ

H6: vmPFC and AMYG reduced responsivity will be positively correlated with sAA Δ

For this portion of statistical analyses, three linear regression were performed for each ROI. AIC BOLD signal, vmPFC BOLD signal and AMYG BOLD signal were included as predictors and sAA Δ scores were included as our dependent variable.

Power Analysis

An a priori G* Power (Faul et al., 2007) analysis indicated that to detect a moderate effect (*f* = .4), fixed effects linear regression with three predictors (AIC BOLD, vmPFC BOLD, AMYG BOLD), 48 participants would be needed for the study giving a power of .95. Ultimately, a total of 53 (30 BD, 23 MD) participants were collected for this study, but 46 (26 BD, 20 MD) participants were included. 7 participants were excluded because of head motion.

Results

H1: Binge drinkers (BD) will have greater activity in the AIC during alcohol cues relative to moderate drinkers (MD).

Independent samples T-tests were run for ROIs assigned to the left and right insula. 5mm spheres were placed at (-38, 20, -2) and (40,14,0) for the left and right insula respectively. There were not significant group differences in the L AIC, t(46) = -0.92, *p* = .362, where despite BD (*M* = .067, *SD* = .79) having lower scores there was not a significant difference to those of MD (*M* = .29, *SD* = .87). This was also true for the R AIC, where despite BD (*M* = -0.018, *SD* = 0.74) having lower overall scores than MD (*M* = .29, *SD* = .84) there was not a group difference, t(46) = -1.30, *p* = .202.

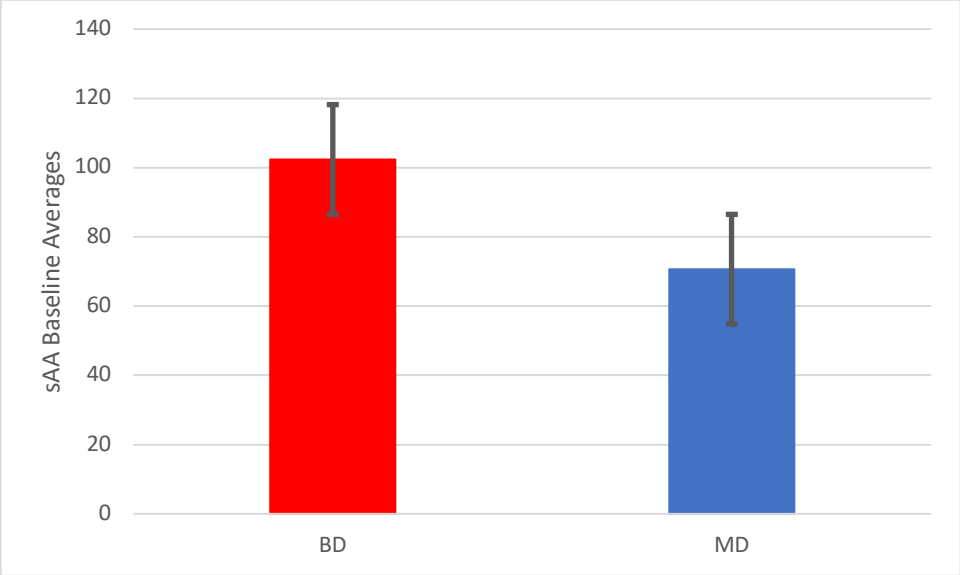
H2: Binge drinkers (BD) will have less activity in the vmPFC and AMYG during alcohol cues relative to moderate drinkers (MD).

Independent samples T-tests were run for ROIs assigned to the left and right vmPFC. ROI spheres were placed at (-17, 52, -15) and (17, 52, -15) for the L and RvmPFC respectively. There were not significant group differences in the LvmPFC, t(46) = -0.90, *p* = .37, despite BD (*M* = .12, *SD* = .81) having lower scores than MD (*M* = .33, *SD* = -.76). The same was true for the R vmPFC, t(46) = .081, *p* = .94, where despite BD (*M* = -0.094, *SD* = 0.75) having higher scores there was not a significant difference to those of MD (*M* = -.115, *SD* = .99).

This same process was repeated for the AMYG. 5mm ROI spheres were placed at (-26, 2, -14) and (26, 2, -14) for the L and RAMYG and independent samples t-tests were run on group means. There were not significant group differences in the LAMYG, t(46) = -.52, p = .61, despite BD (M = .10, SD = 0.88) having lower scores than MD (M = .24 , SD = .97). This was not true in the RAMYG, t(46) = -.36, p = .72, despite BD (M = .082, SD = .81) having higher scores there was not a significant difference to those of MD (M = .33, SD = .76).

H3: sAA levels will be higher in BD than MD at baseline.

Averages were calculated using baseline sAA levels for both scan appointments. An independent samples t-test was run to compare means between both groups. BD (M = 102, SD = 64.7) did not have significantly greater sAA at baseline, t(46) = 1.81, p = .076, than MD (M = 71.4, SD = 44.6).



Fig, 2: Group differences between sAA averages before scanning. An independent samples t-test was run to compare means between both groups. BD (M = 102, SD = 64.7) did not have significantly greater sAA at baseline, t(46) = 1.81, p = .076, than MD (M = 71.4, SD = 44.6).

H4: BD will show less sAA response following alcohol cue observation relative to MD.

A three-factor repeated measures ANOVA was run to determine if there was an effect by time, group, and condition on sAA scores before and after alcohol cues. There was not significant group difference in sAA at time point 2 between BD and MD dependent on condition ($F(1,45) = 1.37$, $p = .25$).

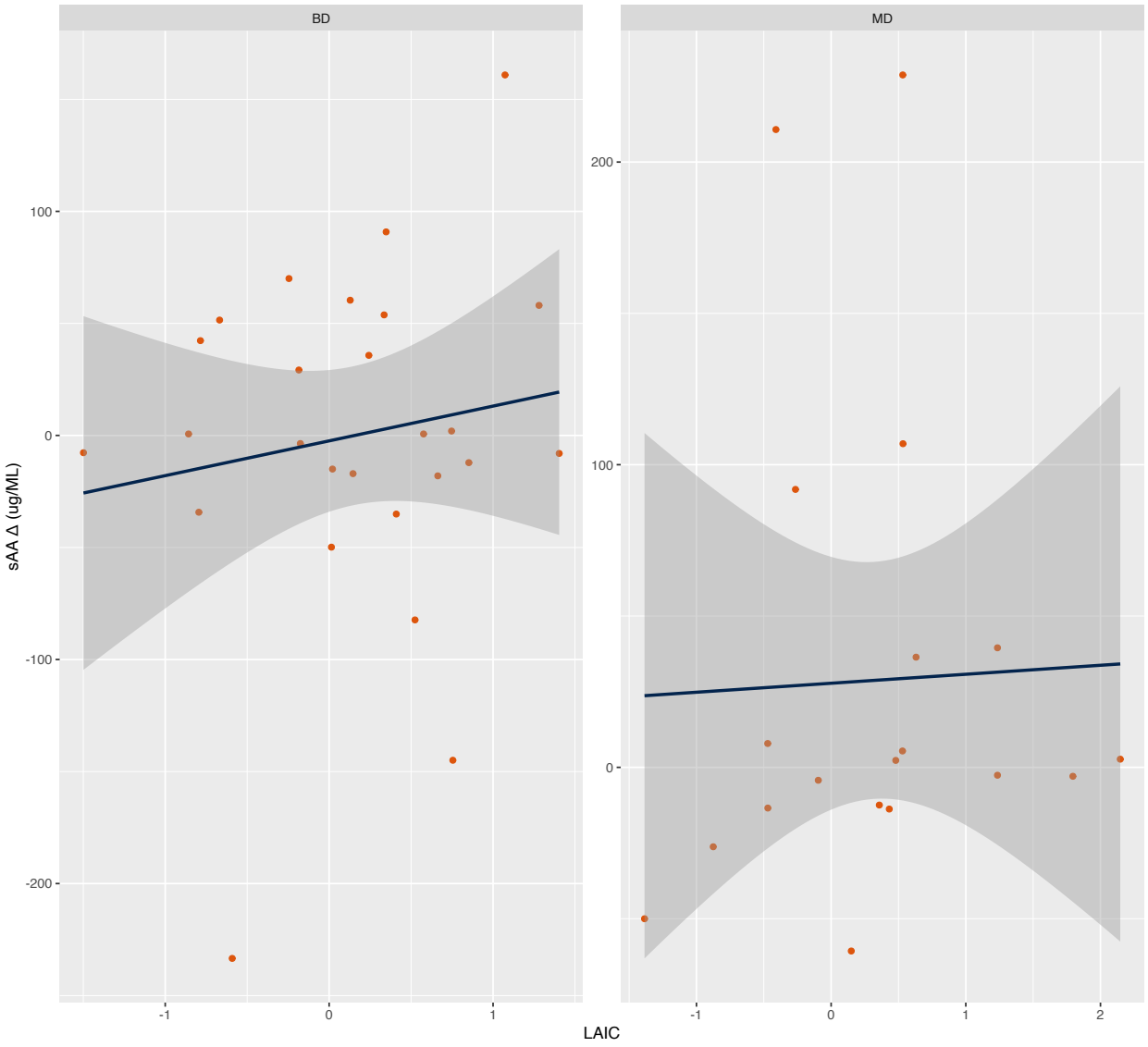
H5: AIC responsivity will be negatively correlated with sAA Δ.

sAA Δ scores were calculated for each participant. To accurately assess change during the alcohol condition, first pre-cue scores were subtracted from post-cue scores. This assessed change in response to alcohol cues. To remove baseline sAA levels, the same scores were calculated for each participants' water condition as well. Following this subtraction, water change was subtracted from alcohol change. This gave a final score for sAA change in response to alcohol. The final formula is represented below.

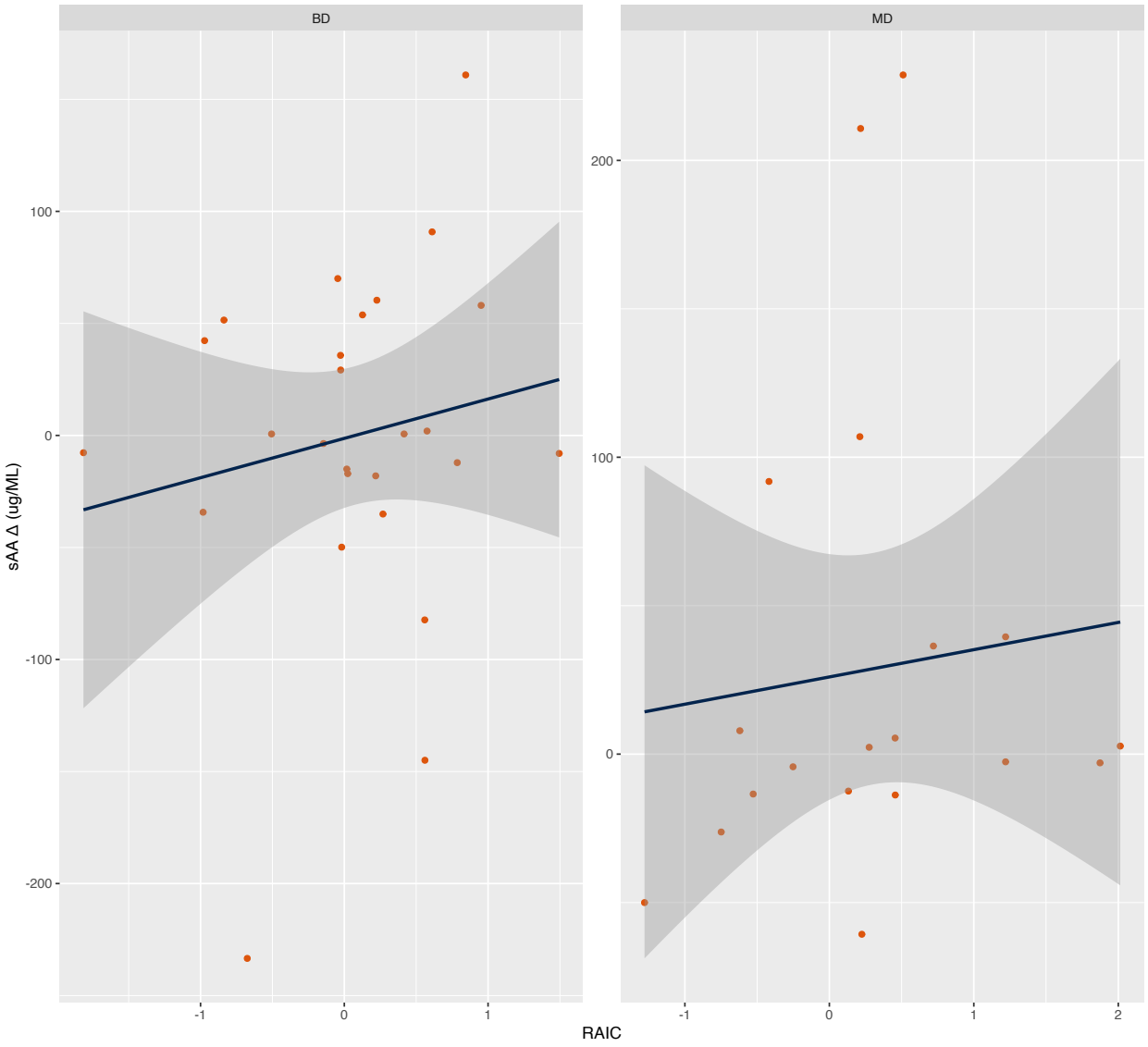
$$(sAA_{PostAlc} - sAA_{PreAlc}) - (sAA_{PostWat} - sAA_{PreWat})$$

These alcohol change scores were plotted in a regression against both sets of AIC BOLD scores.

For each 1 increase in left AIC BOLD signal, there was a 49.1 (± 167.6; 95% CI) decrease in sAA μg/MG ($p = .56$) and for each 1 increase in right AIC BOLD signal, there was a 66.9 (± 173.5; 95% CI) increase in μg/MG sAA scores ($p = .44$). There was not a significant group difference in model fit when ROI were examined individually, LAIC $p = .24$ RAIC $p = .27$, or together ($p = .34$)



Fig, 3: Group differences between models (*p* = .24) for BD and MD when fitting sAA change (ug/ML) against LAIC BOLD signal during alcohol cues.

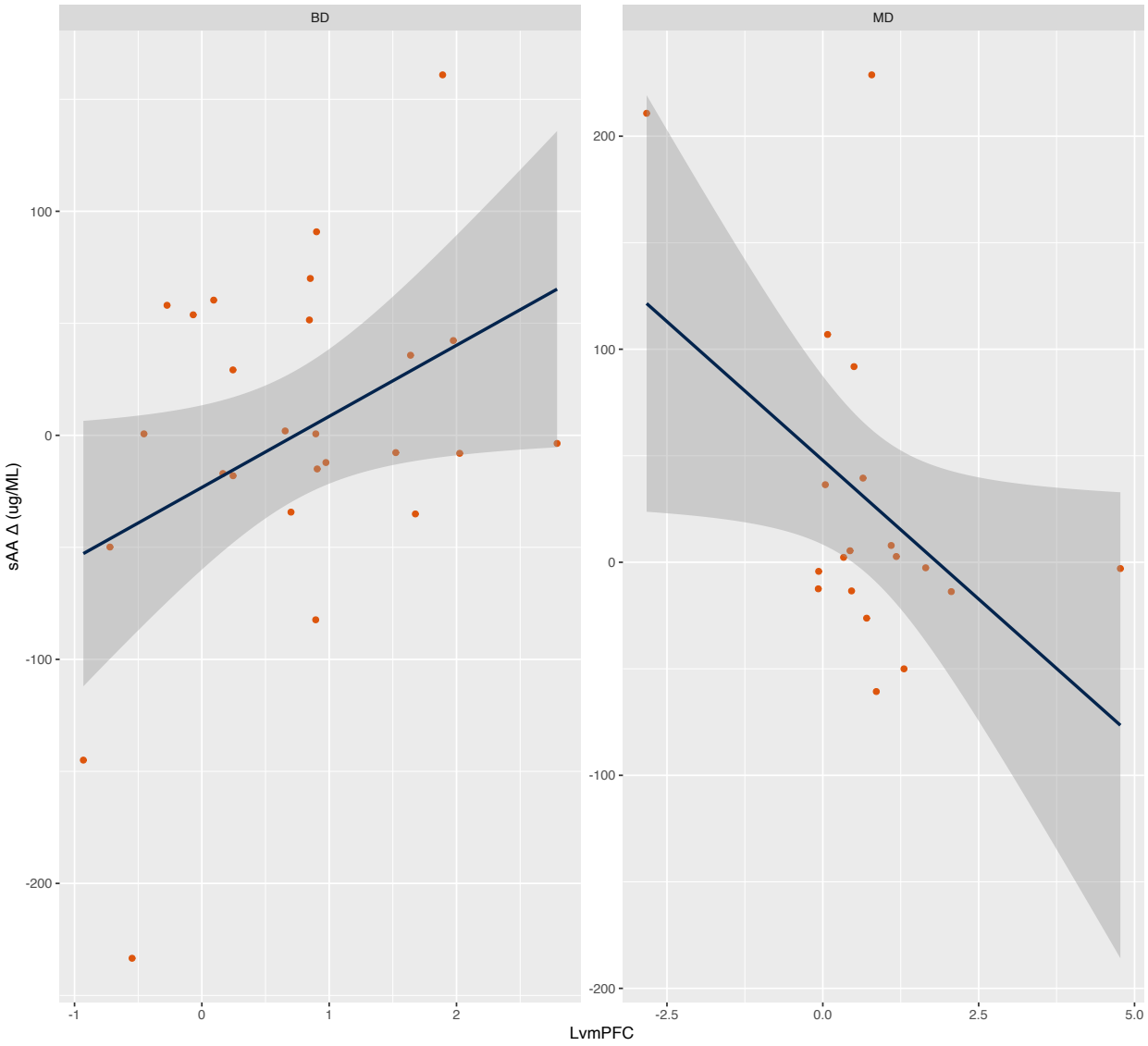


Fig, 4: Group differences (*p* = .27) between models for BD and MD when fitting sAA change (ug/ML) against RAIC BOLD signal during alcohol cues.

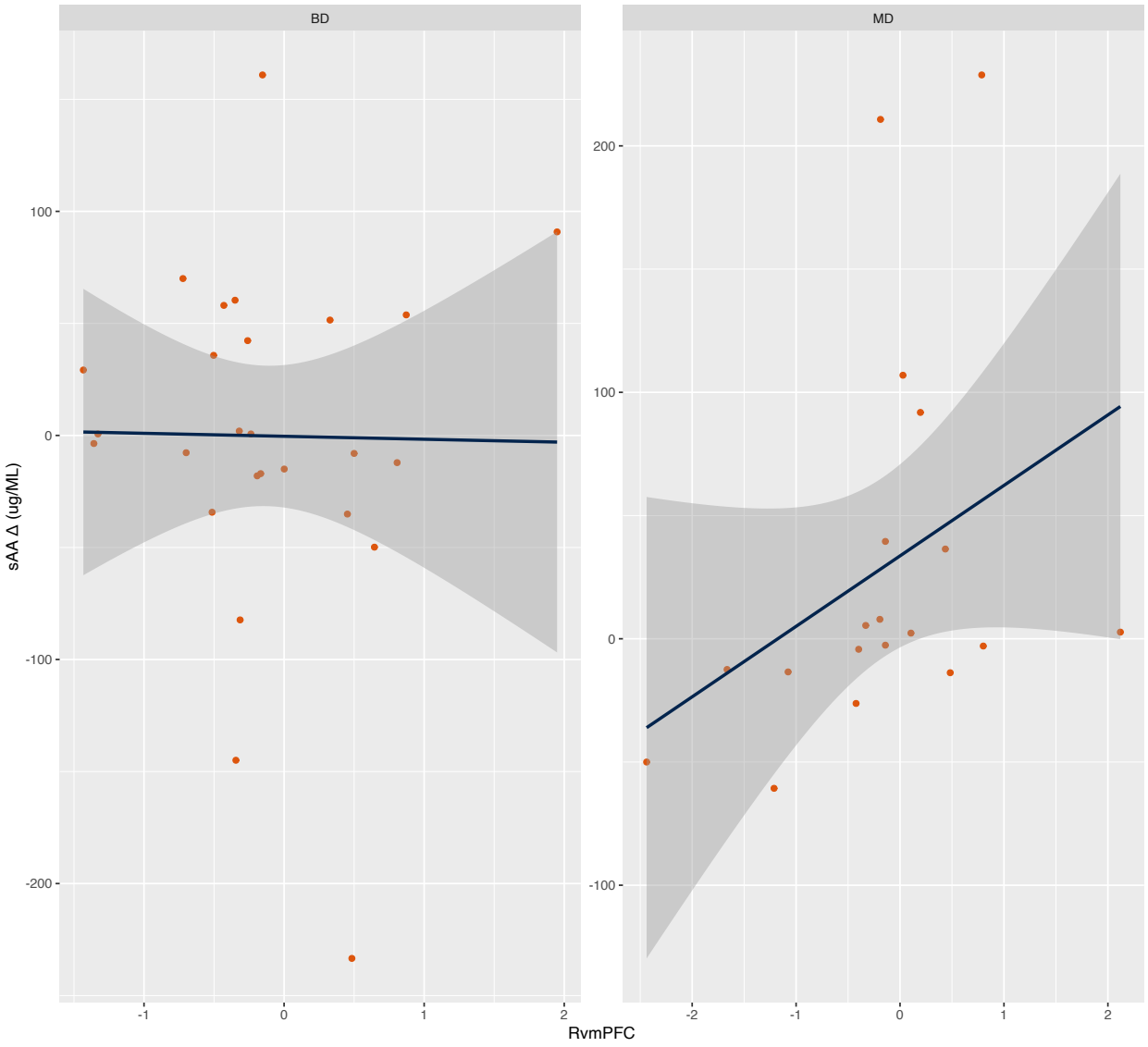
H6: vmPFC and AMYG responsivity will be positively correlated with the sAA Δ.

sAA Δ scores from H5 were utilized when calculating H6. sAA Δ was plotted onto vmPFC scores using a linear regression. For each 1 unit increase in LvmPFC BOLD signal, there was a 24.6 (± 44.3; 95% CI) decrease in sAA Δ (μg/MG) (*p* = .27) and for each 1 unit increase in RvmPFC BOLD signal, there was a 27.6 (± 173.5; 95% CI) increase in μg/MG sAA

scores (p = .18). There was not a significant group difference in model fit when ROI were examined individually, LvmPFC p = .17 RvmPFC p = .20, or together (p = .15).



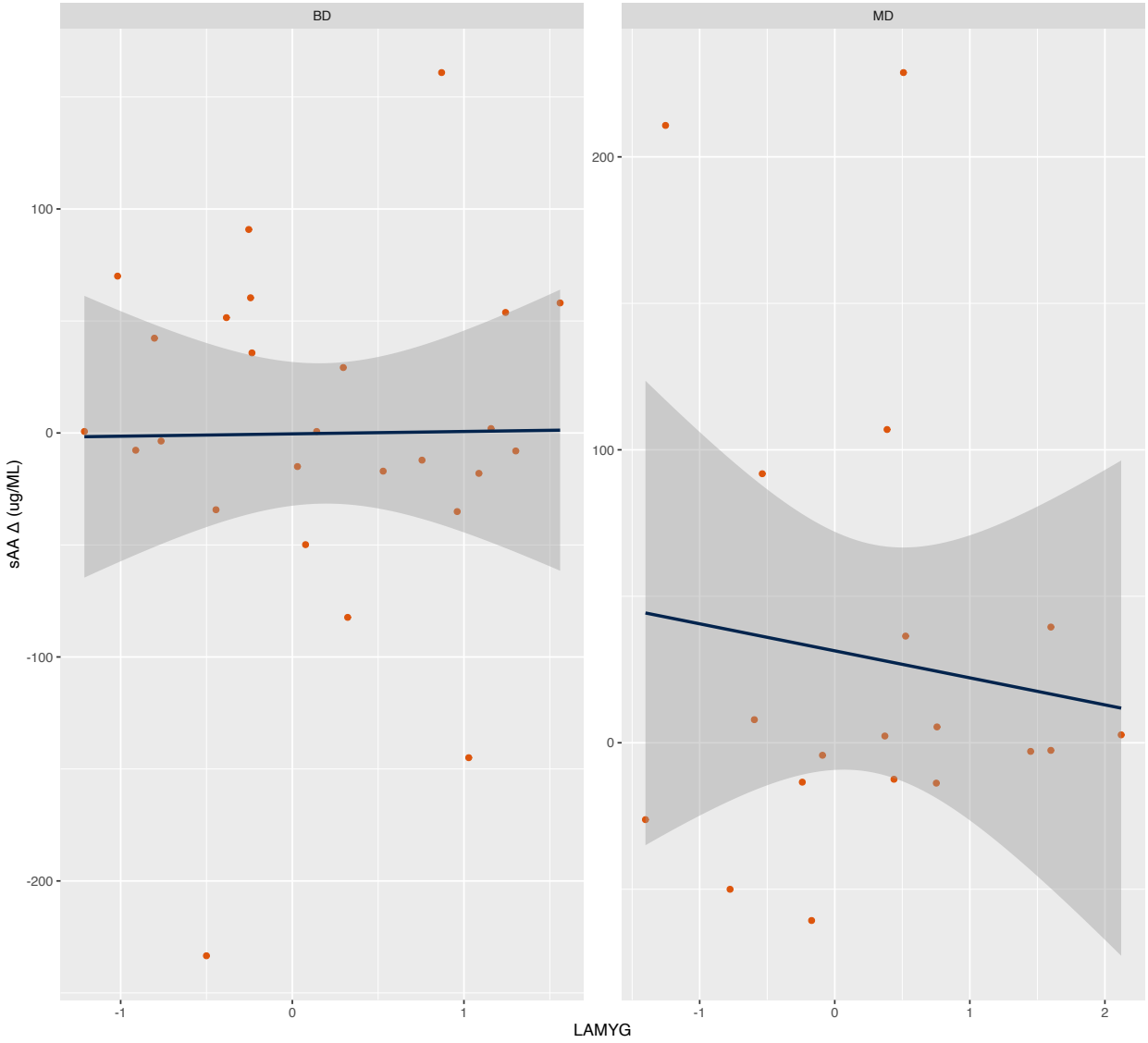
Fig, 5: Group differences (p = .17) between models for BD and MD when fitting sAA change (ug/ML) against LvmPFC BOLD signal during alcohol cues.



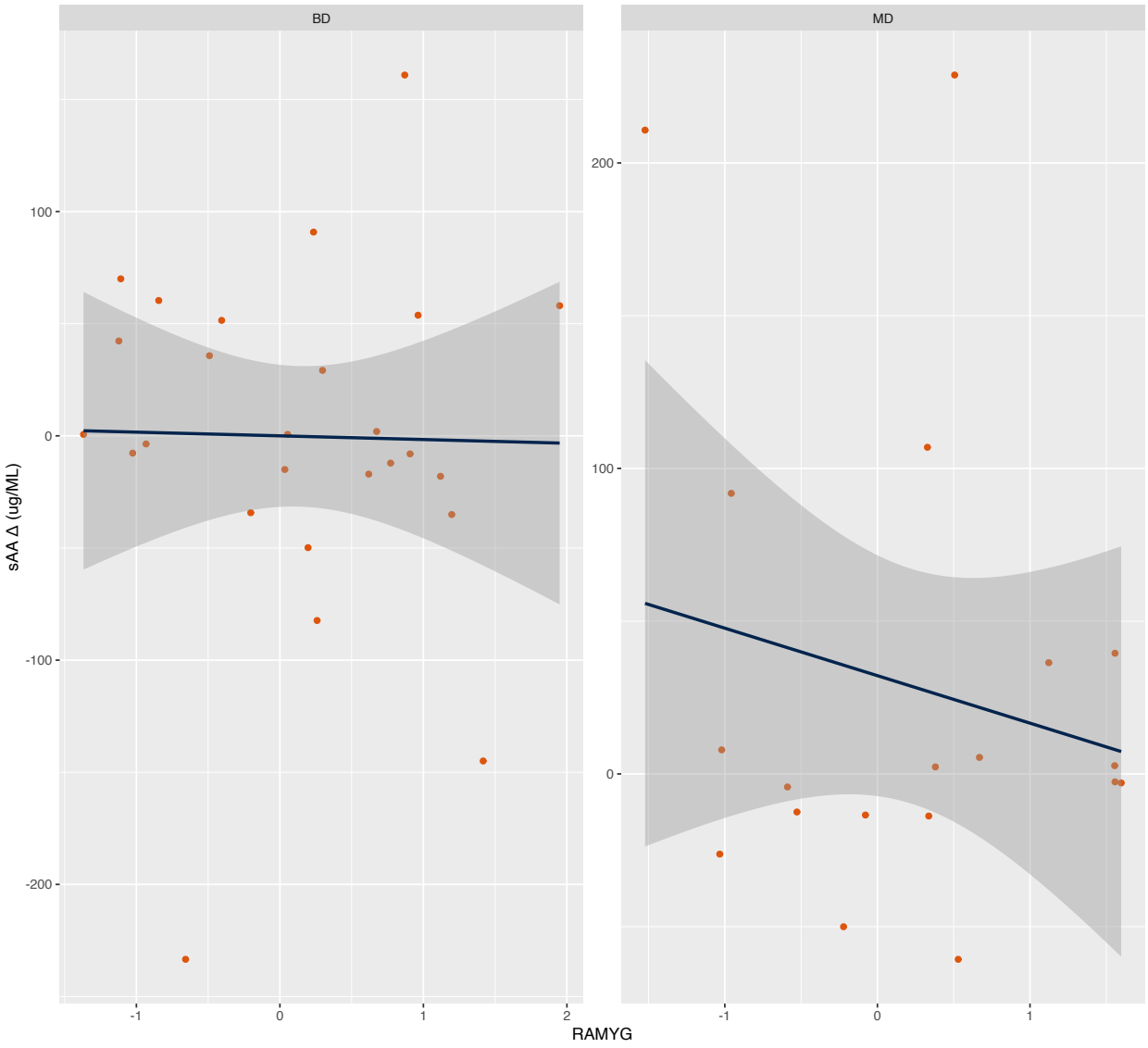
Fig, 6: Non-significant group differences (*p* = .20) between models for BD and MD when fitting sAA change (ug/ML) against RvmPFC BOLD signal during alcohol cues.

For each 1 unit increase in LAMYG BOLD signal, there was a 95.0 (± 96.2; 95% CI) decrease in sAA μg/MG (p = .053) and for each 1 unit increase in RAMYG BOLD signal, there was a 93.1 (± 93.7; 95% CI) decrease in μg/MG sAA scores (p = .051). There was not a significant group difference in when ROI were examined individually, LAMYG p = .22 RAMYG p

= .20, or together (p = .24).



Fig, 7: Non-significant group differences (p = .22) between models for BD and MD when fitting sAA change (ug/ML) against LAMYG BOLD signal during alcohol cues.



Fig, 8: Non-significant group differences (*p* = .20) between models for BD and MD when fitting sAA change (ug/ML) against RAMYG BOLD signal during alcohol cues.

Discussion

This study examined the effects of alcohol cue exposure on Binge Drinkers (BD) and moderate drinkers (MD). We sought to elucidate the correlation between the biomarker salivary alpha amylase (sAA) and neural activity in BD during alcohol cue exposure. In doing so, we sought to explore the autonomic pattern of arousal in BD and if sAA might be a biomarker indicative of sympathetic arousal in BD. 47 drinkers (26 BD; 20 MD) were recruited and underwent MRI scanning. BOLD activity was recorded during alcohol cue exposure. sAA

samples were collected before and after each scan. There are several interpretations which might contribute to the understanding of AUD.

H1 posited that the AIC in would have significant group differences during activation between BD and MD, potentially suggesting stronger bidirectional feedback between arousal facilitated in the AMYG and consolidated in the vmPFC. In group comparisons BD recorded lower BOLD scores than MD for both ROI during alcohol cues; LINS, t(46) = -0.92, p = .362 and RINS, t(46) = -1.30, p = .202 respectively. This result might be in line with prior results indicating reduced insular activity during anticipatory states of craving (Seo et al., 2013; Yang et al., 2015). This might be indicative of reduced inhibitory control during craving and a reduction of internal state monitoring (A. D. Craig, 2002; A. D. B. Craig, 2009). Through a reduction in interoception, individuals are susceptible to increased sympathetic activity by way of increased vmPFC and AMYG activity (Andrewes & Jenkins, 2019; Seeley et al., 2007; Wang et al., 2019) which might contribute to increased craving and relapse. To examine this factor and how it relates to autonomic activity, power should be increased to heighten the chances of a significant group difference in the L or R AIC. Should this result be seen, associations could be made with AMYG and vmPFC BOLD activity as well as autonomic biomarkers including, salivary or serum cortisol and salivary or serum amylase.

When examining H2, the vmPFC displayed a bidirectional relationship when comparing groups. Whereas BD LvmPFC showed reduced activity in response to alcohol cues t(46) = -0.90, p = .37, the RvmPFC showed greater activity in response to alcohol cues t(46) = .081, p = .94. Prior research suggests that the RvmPFC is more closely involved in behavioral impairment in relation to impulsivity (Boes et al., 2009). In this study, healthy boys (n=61) aged 7-17 completed a structural MRI and the Pediatric Behavior Scale (PBS). The PBS, which displays high internal reliability and consistency, predicts the development of attention deficit hyperactive disorder (ADHD), a suggested factor for developing AUD (Bozkurt et al., 2016). Boys with a high impulsivity (n = 20) score on the PBS, were compared against boys with a low impulsivity score

(n = 20). When regressing brain volume onto PBS scores, Boes et al. found RvmPFC volume but not LvmPFC was associated with the high PBS group. Our BD participants displayed greater activity in the RvmPFC and reduced activity in the LvmPFC; a bidirectional pattern which might be correspond to this volumetric difference in individuals with increased impulsivity. This could indicate that our BD have higher impulsivity correlated with their greater RvmPFC BOLD activity to go along with craving suggested by reduced L and R AIC BOLD activity. In future studies, bidirectional vmPFC volume and cue reactivity should be measured to find if there is a relation between the two.

L and RAMYG BOLD signal did not show group differences in activation, LAMYG t(46) = -.52, p = .61 and RAMYG t(46) = -.36, p = .72 respectively. This portion of H2 hypothesized that BD would have reduced BOLD AMYG signal as an indication of blunted sympathetic responsivity to alcohol cues. This could be seen as a lack of arousal from repeated conditioning to alcohol related stimuli, requiring heavier drinkers to need larger and more persistent amounts of stimulation to achieve the desired arousal. As the AMYG is a principal component of the withdrawal/negative affective stage of the addiction cycle, (Centanni et al., 2019), BD participants might not be susceptible to increased stimulation of the AMYG during alcohol cue reactivity compared with AUD individuals. This could be due to an absent compensatory response present in AUD but not BD (Wilcox et al., 2014), or BD might not have the increased autonomic activity seen in AUD individuals as a result of persistent allostatic loading (McEwen, 1998, 2000). As a result, BD SNS might not need additional stimulation to create the desired level of arousal recruitment seen in individuals with AUD. It is worth exploring the bidirectional relationship in the AMYG which was also seen in the vmPFC. BD participants had reduced activity in the left hemisphere of these two regions, but greater activity in the right hemisphere.

H3 explored baseline differences in sAA as a component of autonomic dysfunction. BD participants had higher sAA levels at baseline in comparison to MD t(46) = 1.81, p = .076. This suggests a similar pattern seen with CORT:ACTH ratio in Blaine & Sinha 2017. Directonality

suggests a biologically significant relationship; in that HPA axis and SNS are mutually elevated in BD. As with the L and R AMYG, an increased basal level of sAA might require BD to drink greater volumes in order to reach their desired level of stimulation (Blaine & Sinha, 2017). By needing greater volumes of alcohol, BD might accelerate their transition to AUD status via placing greater stress on their body i.e. allostatic load (McEwen, 1998, 2000). Our result trended towards significance, and a larger sample size would allow us to have greater power and increase our effect size and probability of significance.

For H4, we examined our three-factor repeated measures ANOVA Group * Time * Condition results ($F(1,48) = 1.37$, $p = .25$) to assess if there was a differing response between the two groups, after they were exposed to alcohol cues, controlling for condition. This result would have further replicated the results of Blaine and Sinha 2017, which saw a blunted CORT:ACTH ratio in BD but not MD in response to alcohol cues. The collection paradigm implemented might have contributed to a reduction in sAA robustness. Secondary saliva samples were collected immediately following exiting the scanner. While sAA and cortisol both follow a diurnal pattern, there is a difference in time course to peak activation. sAA has a peak volume 1 minute post-stimulation, whereas cortisol reaches peak levels 15 minutes post-stimulation (E. J. Jones et al., 2020). Therefore, collection should be considered immediately following participants exposure to alcohol cues to accurately probe Group * Time * Condition results.

H5 sought to explore sAA Δ and AIC BOLD signal between groups. It was hypothesized that in BD there would be a negative correlation between the AIC with sAA difference scores. This might have indicated that the AIC was being recruited in response to craving, which could be correlated with increased autonomic arousal. L and RAIC activity bidirectionally correlated with sAA Δ in both groups. We saw that the LAIC positively correlated with sAA Δ scores and the RAIC scores negatively correlated with sAA Δ. This is congruent with prior findings in which

increased RAIC activity occurs during the anticipation of negative affective stimuli (Simmons et al., 2008, 2013; Strigo et al., 2008, 2013). Thus, while stimuli could have been perceived as pleasurable leading to reduced activity in the RAIC, autonomic arousal was increased as seen by the sAA Δ score.

When exploring H6, BD vmPFC BOLD signal was hypothesized to have a positive correlation with sAA Δ. There was a positive correlation between sAA Δ and LvmPFC activity in BD and a negative correlation the in MD. LvmPFC activity might have a stronger correlation with craving and the autonomic response as displayed by sAA Δ. Because of this region's predictive nature in alcohol intake and relapse (Seo et al., 2013; Sinha, 2012; Thayer et al., 2009), focusing on the differential responding of the LvmPFC to alcohol cues in BD should be further researched. The RvmPFC showed the opposite pattern, in that this region was negatively correlated with sAA Δ.

Further exploring H6, both LAMYG and RAMYG BOLD activity were negatively correlated with sAA Δ. Making inferences from our results using the calculated sAA Δ should be revisited in future studies because of peak sAA concentration times. Because of this, exploratory analyses were conducted to see if sAA baseline scores were indicative of greater activity in the AMYG during alcohol cues. Indeed, sAA baseline increased significantly (p = .016) as LAMYG activity increased in both groups, suggesting that increased sAA activity was correlated with higher sympathetic activity during cue reactivity. This would follow prior literature suggesting the AMYG is associated with SNS activity (Yoshihara et al., 2016), and sAA as marker of SNS by way of the AMYG (Allendorfer et al., 2019). The features of observed lateral relationship between AMYG and vmPFC BOLD signal and sAA Δ have yet to be seen. However, prior research has shown that functional laterality can develop over a short period of time in response to stress (Ocklenburg et al., 2016). Thus, laterality and the stress response

should be explored to elucidate their contributions to the development of binge drinking and AUD.

This study has several limitations. The study design should be restructured to allow faster collection of saliva samples following alcohol cue presentation. This could be done immediately following presentation of participants with alcohol before an Alcohol Taste Test (ATT). Second, additional guidelines should be put to limit participant movement during scanning. Seven participants were excluded because motion during scanning created data which was too noisy to use during analysis. Using tape to increase participant's awareness of movement along with additional reminders before scans should decrease movement in subsequent projects.

Regardless, this study has provided information which should be considered by the scientific community. We found group sAA differences at baseline when comparing MD and BD. These baseline differences in sAA, which closely mimic that of CORT:ACTH ratio in BD, could indicate an additional facet of systemic peripheral nervous system dysregulation. Because this study examined BD, this responsivity might indicate physiological alterations prior to the development of AUD. In addition, a bidirectional relationship was found when examining BOLD signal in the vmPFC and AMYG correlated with sAA Δ. Our current design might help explore the bidirectional activation of the salience network and how it relates to the sympathetic response in BD during craving. Revisiting this paradigm could provide a novel way of exploring SNS and CNS dysregulation in BD as an etiological factor in the development of AUD.

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