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Los Angeles

Probing Inflammation and Reward

In Alcohol Use Disorder

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Neuroscience

by

Elizabeth Mar Burnette

2023

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2023

ABSTRACT OF THE DISSERTATION

Probing Inflammation and Reward

In Alcohol Use Disorder

by

Elizabeth Mar Burnette

Doctor of Philosophy in Neuroscience

University of California, Los Angeles, 2023

Professor Lara A. Ray, Chair

Alcohol use disorder (AUD) is a highly prevalent, chronic and relapsing disorder estimated to affect over 100 million people worldwide. Chronic alcohol exposure has been shown in animal models to increase both neural and systemic markers of inflammation. Alcohol-induced inflammation has been linked both to chronic alcohol-seeking and to the behavioral and neurotoxic effects of alcohol. However, the literature on inflammatory signaling and AUD is overwhelmingly preclinical, and it is unknown if this relationship can be extrapolated to clinical samples. Therefore, translation to clinical samples is necessary. In humans, addiction is often conceptualized as a reward deficit disorder, and brain activation in response to reward stimuli has been shown to be negatively associated with inflammation. However, associations between AUD, inflammation, and reward sensitivity have not yet been established. The dissertation studies presented herein combine behavioral and biological methods to elucidate this relationship. Chapter 1 consists of an investigation into the clinical and neural correlates of

individuals who self-reported their primary motivation for drinking as either reward (i.e. positive reinforcement) or relief (i.e. negative reinforcement), finding differences between the groups on clinical measures of AUD severity and neural activation to visual alcohol cues in reward-associated brain regions. Chapter 2 investigates the effects of ibudilast, a neuroimmune modulatory medication in development for AUD treatment. Ibudilast was found to reduce visual alcohol cue-elicited functional connectivity within reward-related brain circuitry, and this attenuation was correlated with reductions in alcohol consumption. Chapter 3 explores the relationship between alcohol and monocyte production of intracellular cytokines following *in vitro* stimulation with lipopolysaccharide (LPS), finding that AUD was associated with enhanced sensitivity to the cellular LPS inflammatory challenge. Finally, Chapter 4 presents a brief argument for the use of LPS as a translational tool to experimentally explore the role of inflammation in clinical samples of AUD. Taken together, these findings seek to elucidate biological mechanisms related to reward response and inflammation in AUD. These studies provide clinical and neurobiological data on the relationship between alcohol use and inflammation, and may inform precision medicine and targeted inflammatory medication development for individuals with AUD.

The dissertation of Elizabeth Mar Burnette is approved.

Naomi I. Eisenberger

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Kate Wassum

Lara A. Ray, Committee Chair

University of California, Los Angeles

2023

DEDICATION

For my A-team: Nina, Graham, and Emily Burnette and Justin Oettinger.

This dissertation is also dedicated to the individuals who participated in this research and to those whose lives are affected by substance use disorders. It is my sincere hope that this work may in some way contribute to the understanding, de-stigmatization, and treatment of addiction.

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SELECT PEER-REVIEWED PUBLICATIONS (of 21)

1. Grodin, E.N., **Burnette, E.M.,** O'Neill, J., Alger, J., & Ray, L.A. (2023). Alcohol craving and severity are associated with dorsal anterior cingulate choline levels in individuals with an alcohol use disorder. *Alcohol and Alcoholism, In Press.*
2. Grodin, E.N., Meredith, L.R., **Burnette, E.M.,** Miotto, K., Irwin, M.R., & Ray, L.A. (2022). Baseline c-reactive protein levels are predictive of treatment response to a neuroimmune modulator in individuals with alcohol use disorder: A preliminary study. *American Journal of Drug and Alcohol Dependence, In Press*.
3. **Burnette, E.M.,** Nieto, S.J., Grodin, E.N., Meredith, L.R., Hurley, B., Miotto, K., Gillis, A.J., & Ray, L.A. (2021). Novel agents for the pharmacological treatment of alcohol use disorder. *Drugs, 82*(3):251-274.
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6. **Burnette, E.M.**, Bass, L.C., Munier, J.J., Evans, C.J., & Romero-Calderón, R. (2021). Effectiveness of a virtual undergraduate student-led drug outreach program. Poster, Society for Neuroscience.
7. **Burnette, E.M.,** Ray, L.A., Irwin, M.R., & Grodin, E.N. (2021). Ibudilast attenuates alcohol cue-elicited frontostriatal functional connectivity in Alcohol Use Disorder. Poster, PsychoNeuroImmunology Research Society.
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10. **Burnette, E.**, Ocampo, G., Wander, R., Walker, Q., Zucker, N. & Kuhn, C. (2017). Adolescents show conditioned food aversion: A strategy to study disordered eating? Poster, Society for Neuroscience.

GENERAL INTRODUCTION

1. Alcohol Use Disorder

Alcohol use disorder (AUD) is the most common form of addiction and continues to present a significant disease and economic burden worldwide. AUD is a highly prevalent, chronic, relapsing condition characterized by an impaired ability to stop or control alcohol use despite clinically significant impairment, distress, or other adverse consequences (American Psychiatric Association, 2013; Grant et al., 2015). AUD is estimated to affect 100.4 million people globally (Degenhardt et al., 2018), representing a significant public health concern. The World Health Organization estimates that alcohol consumption is responsible for 5.9% of all deaths (7.6% in men, 4.0% in women) and 5.1% of global disease burden (World Health Organization, 2014). Alcohol use and misuse is thought to contribute to over two hundred related diseases and health conditions globally, including cardiovascular disease, cancer, liver cirrhosis, and injuries (World Health Organization, 2014). AUD is also often comorbid with other substance use disorders, major depressive disorder, bipolar disorder, and other psychiatric disorders (Grant et al., 2015).

In the United States (U.S.) alone, AUD is estimated to contribute to about 88,000 deaths each year (Stahre et al., 2014). Over 140 million U.S. individuals aged 12 or older reported any alcohol use within the last month in 2017, and approximately 1 in 4 people aged 12 or older reported at least one alcohol binge within the last month, defined as an instance of 4 or more drinks on one occasion for women, and 5 or more drinks for men (Substance Abuse and Mental Health Services Administration, 2018). 44.6 million U.S adults are estimated to suffer from AUD every year, and 93.4 million (approximately 33%) U.S. adults will meet or have met AUD criteria at some point in their life (Grant et al., 2015). Furthermore, the economic burden of AUD is estimated to be approximately $250 billion across the U.S. (Sacks et al., 2015). Therefore,

there is a critical need to elucidate biological mechanisms underlying AUD, which can aid in the development of novel and more efficacious treatments for this disorder.

1.1. Neural Correlates of AUD and Reward Processing

Alcohol and other substances of abuse act on the reward circuitry of the brain, specifically the mesocorticolimbic dopamine system, which is known to play an important role in habitual and goal-directed behavior (Schultz et al., 1997). Dopamine-signaling neurons connect the striatum, amygdala and hippocampus, and the prefrontal cortex (PFC), driving complex valuation of rewards and decision-making processes (Dayan, 2009; Pessiglione et al., 2006). Mesolimbic areas play a key role in in drug seeking behavior due to primary drug reinforcement, which acts as an unconditioned reward-related stimulus. Increased dopamine release within the nucleus accumbens is produced by repeated substance use, while acquisition of related stimulus-reward associations that contribute to conditioned reinforcement are enhanced by adaptations in the amygdala. These subcortical changes contribute to enhanced drug-seeking behaviors (Everitt, 2014; Jentsch & Taylor, 1999).

Alcohol cues have been shown to activate limbic and prefrontal regions, including the ventral striatum / nucleus accumbens, medial frontal gyrus, orbitofrontal cortex, PFC, and anterior cingulate cortex (ACC), among individuals with AUD (Loree et al., 2015). Furthermore, individuals with AUD also show increased neural activation in response to alcohol cues in temporoparietal areas such as the posterior cingulate cortex, precuneus, cuneus, and superior temporal gyrus, compared to healthy controls (Schacht et al., 2013). functional magnetic resonance imaging (fMRI) studies point to the interplay between mesolimbic, frontocortical, and nigrostriatal circuits as underlying cue reactivity. Alcohol cue-induced activation within these circuits is correlated with the clinical phenomenology of AUD, including alcohol addiction

severity, years of drinking, intensity of alcohol use, and self-reported craving (Jasinska et al., 2014).

In sum, chronic exposure to alcohol results in maladaptive changes to neural circuits that are involved in motivation and reward (Koob & Volkow, 2010). Traditionally, this phenomenon has been studied in animal models (Grodin & Ray, 2019); therefore, translational studies in clinical populations are critical to provide a full understanding of AUD-associated neuroadaptations.

2. The Neuroimmune Hypothesis of AUD

2.1. The Neuroimmune System in AUD

Molecular and behavioral studies suggest a central role for the innate immune system in mediating the acute and chronic effects of alcohol and support an inflammatory hypothesis of AUD (Mayfield et al., 2013). Several inflammatory pathways are heavily implicated in the development and maintenance of AUD. Toll-like receptors (TLRs), thought to play an important role implicated in AUD signaling (Crews et al., 2017), are a common family of receptors found on immune cells that can recognize pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) and catalyze an activation cascade of subsequent transcription factors including interferon (IFN) regulatory factors, cyclic adenosine monophosphate (cAMP) response elements binding protein (CREB), and nuclear factor κB (NF- κB) (Aurelian et al., 2016; Balan et al., 2018; Medzhitov, 2008). Activation of these transcription factors leads to the release of inflammatory immune protein molecules (i.e. cytokines) from immune cells. Cytokines such as interleukin (IL)-1, IL-2, IL-6, IL-8, IL-10, and tumor necrosis factor α (TNF-α) act through specific individual mechanisms to have a range of biological and behavioral effects (Dinarello, 2000; Erickson et al., 2019). In the central nervous system (CNS), neurotrophins, including glial

(GDNF) and brain derived neurotrophic factor (BDNF), are essential for basic cell signaling, including midbrain dopamine transmission (Altar et al., 1992; Lin et al., 1993) and are necessary to manage and resolve inflammatory responses.

Alcohol is thought to increase neuroinflammation and affect neuroimmune signaling both directly, via actions in the brain, where alcohol may induce neural damage and thereby initiate the release of inflammatory molecules (Crews & Vetreno, 2016; de la Monte & Kril, 2014), as well as indirectly, such as stimulating inflammation throughout the body via actions on peripheral immune receptors in the gut to produce peripheral cytokines which may then cross the blood-brain barrier (BBB) (Banks et al., 1995). Inflammatory molecules in the CNS are thought to cause adaptations in the plasticity and function of neural circuitry, and a chronic inflammatory state is suggested to contribute to increased alcohol intake (Erickson et al., 2019).

2.2. Preclinical Support for the Neuroimmune Hypothesis of AUD

In rodent models of AUD, reductions in GDNF and BDNF expression underlie dysfunctional striatal dopamine signaling, increased motivation to consume alcohol, and heightened alcohol reward (Ahmadiantehrani et al., 2014; Carnicella et al., 2009; Hensler et al., 2003). Chronic alcohol exposure produces long-lasting increases in systemic inflammation, which in turn is associated with cognitive and behavioral impairment and brain damage (Alfonso-Loeches et al., 2010). Furthermore, inflammation increases vulnerability to stress-induced drug seeking and relapse (Frank et al., 2011). Inflammation induced by LPS administration produces prolonged increases in alcohol consumption (Blednov et al., 2011), and additional preclinical studies demonstrate that voluntary ethanol consumption increases cytokines and chemokines in the CNS and periphery. In nonhuman primates, ethanol consumption was correlated with hippocampal levels of the chemokine MCP-1 (Beattie et al., 2018), and in mice, chronic ethanol consumption resulted in increased levels of cytokines (IL-1β,

IL-17, and TNF-α) and chemokines (MCP-1, MIP-1α, and CX3CL1) in the striatum and serum (Pascual et al., 2015). Preclinical evidence also supports the inverse relationship: knocking out immune-signaling genes attenuates alcohol preference and self-administration (Blednov et al., 2012). In mice, knocking out cytokines such as IL-Ra, IL-6, and IL1R and TNF1R results in reductions in ethanol consumption, indicating that these cytokines contribute to drinking behavior (Blednov et al., 2012, 2015; Chen et al., 2017; Karlsson et al., 2017). Mice with knockouts in TLR4 and TLR2 were protected from ethanol-induced cytokine upregulation, supporting the importance of TLRs in alcohol-associated neuroinflammation (Crews et al., 2017). Of note, TLR4 is particularly thought to contribute to alcohol-related neuroimmune effects, and blocking TLR4 in glial cells has also been shown to protect against ethanol-induced glial activation, proinflammatory cytokine induction, and apoptosis (Alfonso-Loeches et al., 2010). Overall, in preclinical models, alcohol consumption produces a sustained inflammatory state, and in turn, alcohol-induced neuroinflammation contributes to the behavioral and neurotoxic effects of alcohol (Cui et al., 2011).

2.3. Clinical Support for the Neuroimmune Hypothesis of AUD

Individuals with AUD are thought to have increased neuroinflammation throughout the brain (Cui et al., 2014), and elevated peripheral levels of proinflammatory cytokines have been proposed as a biomarker for AUD (Achur et al., 2010). Specifically, individuals with AUD have heightened plasma levels of proinflammatory cytokines (Heberlein et al., 2014; Leclercq et al., 2014). A recent meta-analysis of 17 studies including a range of peripheral inflammatory markers found increased concentrations of cytokines including tumor necrosis factor-α (TNF-α), as well as interleukins IL-6 and IL-8, among individuals with AUD relative to control individuals; these differences were more pronounced during active drinking and acute withdrawal states (C. Adams et al., 2020). These cytokines have been shown to cross the BBB

(Banks et al., 1995), therefore possibly contributing to CNS effects. Additionally, individuals with AUD are shown to have increased endogenous LPS levels, which may normalize after a period of abstinence (Leclercq et al., 2012; Qin et al., 2008). Nevertheless, there are contrasting findings, such that a recent imaging study reported that individuals with AUD exhibit less activated microglia in the brain and blunted peripheral proinflammatory response than controls (Hillmer et al., 2017). Clinical findings indicate that both acute and chronic alcohol intake may modulate peripheral cytokine concentrations. A recent study found that TNF-α levels were reduced and IL-6 levels elevated following acute oral alcohol administration in a population of heavy drinkers (Lee et al., 2021), Serum LPS and proinflammatory cytokine levels were elevated at baseline among treatment-seeking individuals with AUD but decreased significantly – to a level comparable with healthy controls – after approximately three weeks of detoxification (Leclercq et al., 2014). Additionally, translational studies spanning human and preclinical models has begun to enhance our understanding of the effects of alcohol-induced inflammation on neuroimmune signaling. A study transplanting fecal microbiota from humans with AUD into germ-free mice found alterations in neurotransmission and myelination, as well as increases in proinflammatory cytokines, chemokines, and microglial expression (Leclercq et al., 2020). Another recent study conducted in both humans with AUD and alcohol-preferring rats suggested a strong association between liver fibrosis, peripheral inflammation, and brain alterations (Lanquetin et al., 2021). However, the role of inflammation in AUD remains unclear and has been under-explored in clinical populations.

2.4. Inflammation, Alcohol, Reward, and Mood

Reward sensitivity is associated with AUD and neuroinflammation. Addiction has been shown to be a reward deficit disorder (Joyner et al., 2016), with reward threshold heightening after the initiation of substance use acting as a major reinforcing effect of continued use (Koob,

2013). Reward sensitivity is a marker of initial risky drinking, such that individuals with baseline higher reward sensitivity are at increased risk of problematic alcohol use (Jonker et al., 2014; Lyvers et al., 2010; Nees et al., 2012). However, in the transition from early problematic use to AUD, continued alcohol use eventually impairs neuronal circuits that are involved in reward sensitivity, thereby shifting alcohol use from an innately rewarding activity into drinking to relieve withdrawal and negative symptoms (Volkow et al., 2010).

Reward responsiveness is also associated with neuroinflammation. Preclinical studies show that LPS-induced inflammation alters reward sensitivity in mice (Lasselin et al., 2017; Vichaya et al., 2014). Furthermore, chronic binge-like alcohol consumption in alcohol-preferring rats resulted in an anhedonia phenotype (i.e. reduced ability to experience reward, shown in the model as significant decreases in hedonic response to sucrose) (Briones et al., 2013). In humans, neuroinflammation has been implicated in reward processing impairments seen in Major Depressive Disorder (Felger et al., 2016). Additionally, previous studies in human control samples indicate that brain activation in response to reward stimuli is attenuated after experimentally-induced increases in inflammation (Eisenberger et al., 2010).

Similar to the relationships seen with reward processing, negative mood is also associated with both AUD and neuroinflammation. There is a well-established relationship between AUD and negative mood (Raimo & Schuckit, 1998). While negative mood can induce alcohol seeking due to its effects on craving (Dvorak et al., 2014; Hogarth et al., 2018), alcohol use also inhibits negative emotion regulation, i.e. the ability to alleviate negative mood states through one’s own efforts (Lyvers et al., 2010). Negative emotionality, a comprehensive set of emotional states related to unpleasant feelings or a lack of feelings (e.g. sadness, anxiety, malaise, anhedonia) (Kwako et al., 2018) are well-associated with alcohol addiction, such that individuals with AUD demonstrate higher levels of overall low mood (Kwako et al., 2017; Sinha et al., 2009).

Neuroinflammation has been associated with the emergence of negative emotionality (Brites & Fernandes, 2015), such that cytokines have been shown to play a causal role in the onset of negative mood (Harrison et al., 2009; Miller et al., 2009; Reichenberg et al., 2001; Wright et al., 2005). In preclinical research, artificially-induced inflammation via LPS or cytokine administration was dose-dependently associated with alcohol withdrawal-induced anxiety (Breese et al., 2008). Chronic binge drinking induced a microglia-driven neuroinflammatory response in the PFC, leading to synapse loss and increases in anxiety-like behavior (Whitman et al., 2013), whereas microglia depletion both decreased anxious behaviors and prevented increases in voluntary ethanol consumption in mice (Warden et al., 2020). Taken together, reward sensitivity and negative mood have both been associated with AUD and with inflammation, but separately. The link between AUD, inflammation, and these behavioral outcomes has not yet been explored. This dissertation aims to translate preclinical findings regarding the relationship between inflammation, reward processing, and AUD into clinical samples using functional neuroimaging and psychoneuroimmunology techniques.

3. Clinical and Biological Models and Methods Used

3.1. Reward and Relief-Motivated Drinking

AUD is a highly heterogeneous disorder, and one way to address this heterogeneity is by identifying subpopulations within the larger field of individuals with AUD (Jellinek, 1960; Leggio et al., 2009). This approach can help in tailoring treatment to certain common clinical features, in a step toward precision medicine.

One effort to parse discrete subgroups of individuals with AUD is by separating those who drink primarily for positive (rewarding) effects from those who drink primarily for the relief of negative effects. This theory is strengthened by the allostatic model of addiction, which

characterizes the transition from early-phase “liking” to later-phase “wanting.” In some individuals with AUD, repeated intoxication – withdrawal cycles shift motivation for alcohol from positive reinforcement towards negative reinforcement, wherein individuals drink alcohol to alleviate negative emotional states (Koob & Le Moal, 2005).

Two recent studies (Z. W. Adams et al., 2016; Grodin et al., 2019) developed measures with the aim of differentiating the positive reinforcement / reward and negative reinforcement / relief drinking phenotypes. Adams and colleagues developed the Reasons for Heavy Drinking Questionnaire (RHDQ) and identified a two-factor solution, interpreting these two subscales as Reinforcement (i.e., positive reinforcement / reward) and Normalizing (i.e., negative reinforcement or restoration of allostatic balance / relief). While both subscales were associated with AUD severity, the normalizing subscale score was more strongly associated with severity than the reinforcement subscale score. Grodin et al.’s brief UCLA Reward, Relief, Habit Drinking Scale (RRHDS) categorizes subjects into reward, relief, or habit drinking subgroups. The RRHDS was shown to successfully differentiate reward drinkers from relief drinkers based on clinical characteristics including alcohol craving measures, withdrawal symptoms, and anxiety and depression symptomology. While habit drinking was also assessed, it was determined that habit and relief drinkers were not dissociable on clinical measures, suggesting that the two constructs may overlap phenotypically. Taken together, these studies demonstrate that individuals whose drinking is primarily motivated by positive reinforcement / reward and those motivated by negative reinforcement relief are dissociable from each other, and that these differences may inform treatment matching.

3.2. Neuroimaging & Alcohol Cue-Reactivity Paradigm

As mentioned above, translational studies are a critical step toward fully understanding the pathology of AUD. To that end, neuroimaging represents an important and widely-used

noninvasive clinical tool to conduct such translational studies corroborating preclinical findings in human populations with AUD (Grodin & Ray, 2019). Neuroimaging studies can be used to identify mechanisms underlying clinical AUD phenotypes, as well as aiding in the development and assessment of pharmacotherapies.

fMRI is a widely-used modality to study addiction due to its relative accessibility and high spatial resolution (Fowler et al., 2007). Briefly, increased neuronal activity within a brain region is associated with increased cerebral blood flow and changes in oxygen consumption (Hoge et al., 1999), resulting in an increased concentration of diamagnetic oxyhemoglobin and a decreased amount of deoxyhemoglobin (Buxton et al., 2004). fMRI detects changes in this oxygenated to deoxygenated hemoglobin ratio in blood vessels within the brain as a proxy measurement of neuronal activity (Heeger & Ress, 2002). These changes, termed blood oxygen level-dependent (BOLD) signals have been shown to correlate highly with neural activity (Logothetis, 2003; Mukamel et al., 2005) and are illustrated by color variations in different regions of the brain (Parvaz et al., 2011).

A common method to investigate brain circuits thought to be involved in AUD is by presenting alcohol-related stimuli to induce alcohol craving, termed cue-reactivity (Monti et al., 1987). fMRI cue-reactivity studies show cue-induced activation in learning, memory, and reward regions including the prefrontal cortex, striatum, insula, cingulate, and precuneus (Courtney et al., 2016; Schacht et al., 2013). The fMRI studies included in this dissertation use a visual alcohol cue-reactivity task (Schacht et al., 2011). During the scan, participants are shown interspersed visual stimuli that include images of alcoholic and non-alcoholic ("neutral") beverages, blurred versions of both image types that lack object recognition, and a fixation cross to serve as visual controls. These stimuli are presented in six 120-second epochs to total a 12-minute-long task. Each epoch consists of four, 24-second blocks (one block each of alcohol,

neutral beverage, blurred images, and fixation cross). Each of these 24-second blocks has five individual images, which are specific to a beverage type within a block, (e.g. five images of beers, or five images of wines). Each block is followed by a 6-second washout period, to allow the hemodynamic response from the previous stimulus block to decline before the next block is presented. During these washout periods, participants are presented with a 1-4 Likert scale asking them to assess their "urge to consume alcohol" (Grodin & Ray, 2019). All analyses utilize the contrast of activation during alcoholic beverage images compared to non-alcoholic beverage images (ALC vs. BEV).

3.3. Neuroimaging Methods

For all fMRI studies discussed in this dissertation, neuroimaging took place at the UCLA Center for Cognitive Neuroscience (CCN) on a 3.0T Siemens Prisma Scanner (Siemens Medical Solutions USA, Inc., Malvern, PA). A T2-weighted, high-resolution matched-bandwidth (MBW) anatomical scan (time to repetition (TR) = 5,000 ms, time to echo (TE) = 34 ms, flip angle = 90°, voxel size: 1.5 mm × 1.5 × 4 mm, field of view (FOV) = 192 mm2, 34 slices, ~1.5 minutes) and a T1-weighted magnetization-prepared rapid gradient-echo (MPRAGE) sequence (TR = 2,530 ms, TE = 1.74 ms, time to inversion = 1,260 ms, flip angle = 7°, voxel size: 1 mm3, FOV = 256 mm2, ~6.2 minutes) were acquired for co-registration to the functional data. A T2*-weighted echo planar imaging (EPI) scan (TR = 2,200 ms, TE = 35ms, flip angle = 90°, FOV = 192 mm, slices = 36, 3.0 mm, ~12 minutes) was acquired to examine the blood oxygen-level dependent (BOLD) signal during the visual alcohol cue reactivity task detailed above.

Preprocessing of neuroimaging data followed conventional procedures as implemented in FMRIB Software (FSL v6.0.1 http://www.fmrib.ox.ac.uk/fsl), including motion correction (Jenkinson et al., 2002), high-pass temporal filtering (100-second cut-off), and smoothing with a 5-mm full-width, half-maximum Gaussian kernel. Functional and structural data were skull-

stripped to remove non-brain tissue. Each subject's functional images were registered to their MBW, followed by their MPRAGE using affine linear transformations, and then were normalized to the Montreal Neurological Institute (MNI) 152-brain-average template through non-linear registration (Andersson et al., 2007). All fMRI data was examined for quality control issues, including excessive motion, and were excluded under the following criteria: >2mm translational displacement, >1.5˚ rotation.

3.4. Novel Neuroimmune Modulator Ibudilast

Ibudilast is an inhibitor of phosphodiesterases (PDE)-3, -4, -10, and -11 (Gibson et al., 2006) and of macrophage migration inhibitory factor (MIF) (Y. Cho et al., 2010). Ibudilast has been shown to dose-dependently suppress pro-inflammatory cytokines, such as interleukins IL-1β, IL-6, and TNF-α, and to increase the anti-inflammatory cytokine IL-10 and neurotrophic factors (Mizuno et al., 2004). As increases in inflammation are found in AUD (Mayfield & Harris, 2017), ibudilast's effects in treating AUD are thought to be driven by its anti-inflammatory and pro-neurotrophic qualities (Johnson et al., 2014).

Preclinically, ibudilast was demonstrated to reduce alcohol intake in two rat models, and decreased drinking selectively in alcohol-dependent mice in comparison to non-dependent mice (Bell et al., 2015). These preclinical findings align with prior rodent studies in which pharmacological inhibition of PDE also reduced alcohol intake (Blednov et al., 2014; Logrip, 2015; Wen et al., 2012).

Ibudilast is well-tolerated and has few side effects (Rolan et al., 2008; Grodin et al., 2021). The most common adverse side effects include gastrointestinal symptoms (nausea, vomiting, abdominal pain, and diarrhea), headaches, and depression (Fox et al., 2018; Ray et al., 2017). In a seven-day human laboratory crossover trial (N=24), ibudilast was well-tolerated and decreased tonic craving in comparison to placebo. Additionally, ibudilast improved mood during

exposure to alcohol and stress cues and reduced the mood-altering and stimulant effects of alcohol among participants with more severe depressive symptoms (Ray et al., 2017).

3.5. Lipopolysaccharide Stimulation of Monocyte Intracellular Cytokine Production

As mentioned in **section 2.1**, preclinical and clinical evidence indicate that the immune and neuroimmune system is related to AUD symptomatology, but specific mechanisms remain unclear. Innate and adaptive immune mechanisms serve as the human body's primary defense against pathogens (Bonilla & Oettgen, 2010; Slavich & Irwin, 2014). When the innate immune system is activated, inflammatory responses are provoked by the detection of pathogen-associated molecular patterns (PAMPs), such as the bacterial ligand lipopolysaccharide (LPS), by toll-like receptors (TLRs), which are widely implicated in neuroimmune signaling processes related to alcohol use (Meredith et al., 2021). LPS can also serve as a biomarker of AUD, such that individuals with AUD are shown to have elevated LPS levels but may re-normalize after abstinence (Leclercq et al., 2012; Qin et al., 2008).

A novel method to characterize mechanisms of AUD-related immune signaling is to probe monocyte production of intracellular cytokines (ICCs) following *in vitro* ligation of the TLR4 receptor with LPS, which allows for the direct detection of ICC levels without extracellular background (J. H.-J. Cho et al., 2019). Monocytes comprise approximately 5% of circulating leukocytes and are a major contributor to proinflammatory cytokine production in peripheral blood (O'Connor et al., 2007). The acute inflammatory state induced by LPS stimulation is thought to be reflective of stress, as physiological and psychological stressors both activate inflammatory processes (Black, 2002), and LPS-induced ICC expression reflects the inflammatory responsivity of cells to these stressors (Bale, 2006). In the depression literature, ICC levels following LPS stimulation have shown positive associations with depressive symptom severity (Suarez et al., 2003, 2004). However, despite the known relationship between

AUD and inflammation, to our knowledge, no studies have yet used this method to investigate the associations between alcohol use and ICC response to LPS challenge.

3.6. Clinical Assessments

3.6.1. Alcohol Dependency Scale (ADS)

The Alcohol Dependency Scale (Skinner & Allen, 1982) is a 25-item scale that measures alcohol dependence symptoms over the past 12-months. The ADS is a self-report measure that assesses problems that are relevant for alcohol dependent drinkers.

3.6.2. Alcohol Use Disorder Identification Test (AUDIT)

The Alcohol Use Disorders Identification Test (Saunders et al., 1993) is a self-report measure used to identify persons with hazardous and harmful patterns of alcohol consumption. The AUDIT was developed by the World Health Organization (WHO) as a simple method of screening for excessive drinking.

3.6.3. Beck Depression Inventory (BDI-II)

The BDI-II (Beck et al., 1996) is the most widely-used instrument for detecting depression symptomology. It is a brief, self-report inventory designed to measure symptom severity, with clinical categories including minimal (score ≤ 13), mild (score 14-19), moderate (score 20-28), and severe (score ≥ 29).

3.6.4. Clinical Institute Withdrawal Assessment-Alcohol Revised (CIWA-Ar)

The CIWA-Ar (Sullivan et al., 1989) is a brief 10-item measure used to provide a quantitative index of the severity of the alcohol withdrawal syndrome. The CIWA-AR has been used both in clinical and research applications and has demonstrated both reliability and validity.

3.6.5. Daily Diary Assessment

The electronic daily diary assessment asks participants to report on their mood, alcohol and cigarette craving, and drinking behavior from the previous day (Grodin et al., 2021). Participants received daily text message reminders with a link to the assessment.

3.6.6. Fagerström Test for Nicotine Dependence (FTND)

The Fagerström Test for Nicotine Dependence (Heatherton et al., 1991) is used to assess smoking status and motivation to change smoking behavior. As nicotine and alcohol co-use is known to be highly prevalent (Dawson, 2000), current smoking status as determined by the FTND is used as a covariate in models throughout this dissertation.

3.6.7. Hamilton Depression Rating Scale

The Hamilton Depression Rating Scale (Hamilton, 1960) is a clinician-administered depression assessment used to assess severity of and change in depressive symptoms experienced over the past week.

3.6.8. Obsessive Compulsive Drinking Scale (OCDS)

The OCDS (Anton, 2000) is a 14-item self-report measure used to assess drinking patterns and a participant's attempts to control their drinking over the past week. The OCDS includes three subscales: Resistance / Control Impairment (RCI), Obsession, and Interference (Roberts et al., 1999).

3.6.9. Penn Alcohol Craving Scale (PACS)

The PACS (Flannery et al., 1999) is a five-item, self-report measure that includes questions about the frequency, intensity, and duration of craving, the ability to resist drinking, and asks for an overall rating of craving for alcohol for the previous week.

3.6.10. Reasons for Heavy Drinking Questionnaire (RHDQ)

The RHDQ (Z. W. Adams et al., 2016) is a 6-item self-report measure that measures an individual's motivation for drinking. This measure is split into two subscales: positive reinforcement and negative reinforcement.

3.6.11. UCLA Reward, Relief, Habit Drinking Scale (RRHDS)

The Reward-Relief Drinking Scale (Grodin et al., 2019) is a 4-item scale that measures an individual's reward- and relief-motivated drinking tendencies. This measure was adapted from the Inventory of Drinking Situations.

3.6.12. Structured Clinical Interview for DSM-5 (SCID)

The SCID (First et al., 1995) is a semi-structured interview for making the major DSM-5 diagnoses. For all studies included in this dissertation, the SCID was performed by a master's level clinician. The SCID-5 is used to assess current (past 12-month) AUD diagnosis (moderate or severe) as well as exclusionary diagnoses (e.g., lifetime psychosis).

3.6.13. Thirty-Day Timeline Follow-Back Interview (TLFB)

The TLFB (Sobell & Sobell, 1992) is an interview-format assessment administered to assess quantity and frequency of alcohol, cigarette and marijuana use over the past month. For all studies included in this dissertation, the TLFB was administered by a trained interviewer.

4. Overview of Dissertation Chapters

4.1. Chapter 1: Clinical and Neural Correlates of Reward and Relief Drinking

Reviewed above, one way to parse the heterogeneity inherent in AUD is by phenotyping individuals by their underlying motivation to drink, specifically drinking for reward (i.e., positive reinforcement) or for relief (i.e., negative reinforcement/normalizing). Reward- versus relief-motivated behavior is thought to be associated with a shift from ventral (VS) to dorsal (DS) striatal neural activation. This study examined whether reward and relief drinking were

differentially associated with other clinical characteristics and with alcohol cue-elicited activation of the ventral and dorsal striatum.

Non–treatment-seeking heavy drinkers (N = 184) completed assessments of reward- and relief-motivated drinking and other measures of AUD-associated behaviors; a subset of these (N = 45) also completed a functional neuroimaging alcohol cue-reactivity task. Relief drinkers demonstrated greater AUD severity than reward drinkers on a host of clinical measures and displayed higher cue-elicited DS activation compared with reward drinkers ($p < 0.05$). Overall, findings supported and extended the differentiation of reward- from relief- motivated drinking and suggested that differences in DS response to conditioned alcohol cues may underlie this distinction.

4.2. Chapter 2: Neuroimmune Modulator Ibudilast Attenuates Alcohol Cue-Elicited Frontostriatal Functional Connectivity

This study probed the effects of ibudilast, a novel neuroimmune modulator being studied to treat AUD, on alcohol cue-elicited functional connectivity (i.e., temporally correlated activation) in reward processing brain circuitry from a ventral striatum (VS) seed. The study also tested the association between functional connectivity and alcohol use during the trial. Non–treatment-seeking participants (N = 45) with AUD received twice-daily dosing with either ibudilast (50 mg; N = 20) or placebo (N = 25) for two weeks. Ibudilast reduced alcohol cue–elicited functional connectivity between the VS seed and reward-processing regions, including the orbitofrontal and anterior cingulate cortices, compared to placebo ($p < 0.05$). Cue-elicited functional connectivity was correlated with alcohol consumption (drinks per drinking day) ($R^2 = 0.5351$, $p < 0.001$), and ibudilast reduced this association in similar reward-processing regions. Overall, findings indicated that ibudilast's effects on drinking outcomes may be related to the attenuation of functional connectivity in reward processing circuitry.

4.3. Chapter 3: Alcohol Use Disorder is Associated with Enhanced Sensitivity to Cellular Lipopolysaccharide Challenge

This study evaluated associations between AUD and monocyte ICC production following cellular LPS challenge. Blood samples from 36 participants (AUD N=14; Controls N=22), collected across five timepoints, were assessed for monocyte ICC expression at baseline and after LPS challenge (10 repeated measures/participant). Biomarkers of interest included tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), co-expressing TNF-α and IL-6 monocytes, and interferon (IFN). For each biomarker, linear mixed models were constructed with AUD status and LPS stimulation status as fixed effects (BMI and time course as covariates), allowing for random slope and intercept. AUD × stimulation was included as an interaction term.

For TLR4 stimulated monocyte production of TNF-α, there were effects for AUD ($p < 0.01$), LPS ($p < 0.001$), and AUD × LPS interaction ($p < 0.05$), indicating that individuals with AUD showed greater resting levels of monocyte expression of TNF-α and also greater TLR4 stimulated monocyte production of TNF-α. Similarly, for TLR4 stimulated monocyte co-expression of TNF-α and IL-6, there were effects for AUD ($p < 0.01$), LPS ($p < 0.001$), and interaction ($p < 0.05$). No AUD or LPS effects were found for TLR4 stimulated production of IL-6. Time point effects were also observed on IL-6 and TNF-α / IL-6 co-expression ($p < 0.001$). Finally, for TLR4 stimulated monocyte production of IFN, AUD ($p < 0.05$), LPS ($p < 0.001$), and AUD × LPS ($p < 0.001$) effects were found. This study extends previous preclinical and clinical findings on the roles of proinflammatory cytokines in AUD and serves as a critical proof-of-concept for the use of a novel method in probing the neuroimmune mechanisms underlying AUD.

4.4. Chapter 4: Endotoxin for Alcohol Research: A Call for Experimental Medicine Using Lipopolysaccharide Challenge

After exploring the relationship between alcohol and inflammatory signaling using a cellular LPS challenge, this commentary article highlights the potential for a systemic, *in vivo* LPS inflammatory challenge in clinical research. A growing body of literature implicates inflammation in psychiatric disorders, including AUD. However, studies of inflammation in AUD are overwhelmingly preclinical, and experimental approaches to establish a causal link between inflammation and clinical phenotypes of AUD in human populations are both currently lacking and necessary for the next step in the translation of the neuroimmune hypothesis of AUD. This brief commentary article discusses the use of LPS endotoxin, which has been used in previous human challenge studies within the field of affective disorders. Such studies offer proof of not only safety and reliability in human subjects, but also strengthen the case for the use of endotoxin inflammatory challenge as a method for studying AUD based on the well-established relationship between AUD and emotion regulation. LPS challenge presents a method through which the complex relationship between AUD and inflammatory signaling may be elucidated, and can aid in the development of neuroimmune treatments for AUD.

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CHAPTER 1

CLINICAL AND NEURAL CORRELATES OF REWARD AND RELIEF DRINKING

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Abstract

Background: Alcohol use disorder (AUD) is heterogenous. One approach to parsing this heterogeneity is to phenotype individuals by their underlying motivation to drink, specifically drinking for reward (i.e. positive reinforcement) or for relief (i.e. negative reinforcement/normalizing). Reward- vs. relief-motivated behavior is thought to be associated with a shift from ventral to dorsal striatal signaling. The present study examined whether reward and relief drinking were differentially associated with other clinical characteristics and with alcohol cue-elicited activation of the ventral and dorsal striatum.

Methods: Non-treatment-seeking heavy drinkers (N=184; 61 female, 123 male) completed the UCLA Reward, Relief, Habit Drinking Scale (RRHDS) and the Reasons for Heavy Drinking Questionnaire (RHDQ), to categorize drinking motivation. Measures of alcohol use, alcohol problems, mood, and craving were also collected. A subset of participants (N=45; 17 female, 28 male) also completed a functional neuroimaging alcohol cue reactivity task.

Results: RRHDS-designated relief/habit drinkers scored lower than reward drinkers on the RHDQ Reinforcement subscale (p=0.04) and higher on the RHDQ Normalizing subscale (p=0.004). Relief/habit drinkers also demonstrated greater AUD severity on a host of clinical measures. Relief/habit drinkers displayed higher cue-elicited dorsal striatal activation compared to reward drinkers (p=0.04), while ventral striatal cue-elicited activation did not significantly differ between groups.

Conclusions: Our findings support and extend the differentiation of reward from relief/habit-motivated drinking and suggest that differences in dorsal striatal response to conditioned alcohol

cues may underlie this distinction. Elucidating neurobiological and clinical differences between these subtypes may facilitate treatment matching and precision medicine for AUD.

Keywords: RRHDS, motivation, AUD, fMRI, RHDQ

Summary: This study examined whether reward- and relief-motivated drinking were differentially associated with clinical characteristics and alcohol cue-elicited striatal activation in a sample of non-treatment-seeking heavy drinkers. Relief/habit-motivated drinkers demonstrated greater Alcohol Use Disorder severity than reward-motivated drinkers on clinical measures. Relief/habit drinkers also displayed higher dorsal striatal cue-reactivity compared to reward drinkers. These findings support the differentiation of reward from relief drinking and suggest that differences in dorsal striatal response to conditioned alcohol cues may underlie this distinction.

Introduction

Alcohol use disorder (AUD) is a heterogenous disorder, subtypes of which present distinct characteristics and may require distinct treatment strategies. As such, there have been extensive efforts to parse this heterogeneity into typologies and other clinical phenotypes with the goal of matching each AUD subtype with the most effective treatment (Leggio et al., 2009). One of the latest developments in categorizing AUD subgroups has focused on the underlying motivation for drinking, namely drinking for reward (i.e., feeling good) or drinking for relief (i.e., alleviating unpleasant feelings). This approach may have clinical implications, as recent studies demonstrated that individuals whose drinking is driven by positive reinforcement (i.e., reward drinkers) benefit more from naltrexone, a medication known to blunt the rewarding effects of alcohol, than from other medications (Mann et al., 2018; Witkiewitz et al., 2019).

The contrast between reward- and relief-based alcohol use is generally consistent with the allostatic and incentive salience models of addiction, which propose a transition from positive to negative reinforcement (Koob and Schulkin, 2019), or from initial “liking” to later “craving” (Robinson and Berridge, 1993), respectively. This shift from positive to negative reinforcement may be accompanied by a transition from ventral striatal (VS) to dorsal striatal (DS) activation to alcohol cues. In a neuroimaging study, heavy drinkers showed higher DS activation to alcohol cues than lighter drinkers, whereas lighter drinkers showed higher VS activation (Vollstädt-Klein et al., 2010). The VS is implicated in reward-motivated decision-making, while the DS is thought to be involved in more compulsive or “habit-like” behavior (Burton et al., 2015). The notion that compulsive drug seeking may depend on the formation of long-lasting stimulus-response associations mediated by the DS is supported by animal research. It is thought that once drug use becomes compulsive, it is linked to cue-elicited DS dopamine release (Ito et al., 2002), and drug-seeking behavior can be limited by dopamine receptor blockade only in the DS, not in the VS

(Vanderschuren et al., 2005). In human neuroimaging studies, alcohol cue-induced activation in the VS and DS has been shown in participants with AUD (Heinz et al., 2004; Schacht et al., 2011), and cue-reactivity throughout the striatum is associated with risk of relapse in abstinent subjects (Courtney et al., 2016; Grüsser et al., 2004).

Our group (Grodin et al., 2019) and others (Adams et al., 2016) have recently developed and validated brief scales with the goal of identifying reward and relief drinkers. Adams and colleagues (Adams et al., 2016) developed the Reasons for Heavy Drinking Questionnaire (RHDQ) and identified a two-factor solution, interpreting these two subscales as Reinforcement (i.e., positive reinforcement / reward) and Normalizing (i.e., negative reinforcement or restoration of allostatic balance / relief). While both subscales were associated with AUD severity, the normalizing subscale score was more strongly associated with severity than the reinforcement subscale score. Our group's brief UCLA Reward, Relief, Habit Drinking Scale (RRHDS) (Grodin et al., 2019) categorizes subjects into reward, relief, or habit drinking subgroups. The RRHDS was shown to successfully differentiate reward drinkers from relief drinkers based on clinical characteristics including alcohol craving measures, withdrawal symptoms, and anxiety and depression symptomology. While habit drinking was also assessed, it was determined that habit and relief drinkers were not dissociable on clinical measures, suggesting that the two constructs may overlap phenotypically. Therefore, we proposed combining the relief and habit groups, supported by previous research in the domain which characterizes only reward and relief subtypes (Glöckner-Rist et al., 2013; Mann et al., 2018; Roos et al., 2017).

While these recent efforts are promising and may carry important clinical implications, much work remains to be done. One gap in the literature is understanding how the RHDQ and RRHDS self-report scales relate to one another. Admittedly, for clinical application, shorter

scales are likely to have the most acceptability; however, clinical sensitivity should not be compromised. Another critical area is the biological validation of these phenotypes using neural markers, as reward and relief phenotypes are thought to be subserved by VS and DS neural circuitry, respectively. These systems are related, yet distinct and represent targets for precision pharmacotherapy. No studies to date have examined the association between self-report scales of reward and relief with measures of VS and DS activation to alcohol or other alcohol-related biomarkers.

With the overarching goal of identifying clinically meaningful drinking phenotypes, this study compares two scales of reward and relief drinking in a sample of non-treatment seeking heavy drinkers. We hypothesized that the two scales would largely align, such that participants categorized as reward drinkers on the RRHDS would have higher Reinforcement scores on the RHDQ, while relief/habit drinkers would have higher Normalizing scores. Furthermore, we examined the relationship between self-reported reward and relief drinking and neural activation to visual alcohol cues during a functional magnetic resonance imaging (fMRI) task in a subset of participants. Our analyses of neural activation are informed by the literature on reward and relief/habit drinking, which posits a dissociation between ventral and dorsal striatal activation in heavy drinkers (Vollstädt-Klein et al., 2010). As such, we hypothesized that reward drinkers would show higher cue-induced VS activation while relief/habit drinkers would show higher DS response to alcohol cues.

Materials and Methods

This study was performed as part of a two-week randomized controlled clinical trial (ClinicalTrials.gov NCT03489850) of ibudilast for drinking reduction. The trial was approved by the Institutional Review Board of the University of California, Los Angeles. All study participants provided written informed consent for screening, medication, and neuroimaging procedures. The current study used data from the initial in-person screening visit for all participants, and fMRI data from a subset of these individuals who completed the neuroimaging visit.

Participants

Participants included 184 non-treatment-seeking heavy drinkers [61 female, 123 male, mean±SD age 31.98±8.69], 45 of whom completed the fMRI neuroimaging paradigm [17 female, 28 male, mean±SD age 32.51±8.59] after being randomly assigned to take either ibudilast or placebo. Ibudilast was titrated as follows: 20 mg b.i.d. on days 1-2 and 50 mg b.i.d. on days 3-14. The neuroimaging session occurred after participants had been taking medication for seven days. Participants were recruited through social media and mass transit advertisements. Initial screening was conducted through telephone interviews. Eligible participants were invited for the in-person assessment visit. Data from all individuals who completed the full in-person screening visit and individual differences battery were included in the aim of comparing drinker subtypes on clinical measures.

Eligibility criteria for the fMRI scan included an age range between 21 and 50 years; meeting DSM-5 criteria for current AUD; and drinking more than 14 drinks per week for men (more than 7 for women) in the 30 days prior to screening. Exclusion criteria included currently receiving or seeking treatment for AUD; past year DSM-5 diagnosis of any other substance use

disorder (excluding nicotine); lifetime diagnosis of schizophrenia, bipolar disorder, or any psychotic disorder; non-removable ferromagnetic objects in body; claustrophobia; serious head injury or prolonged period of unconsciousness (>30 minutes); medical conditions thought to interfere with safe participation (unstable cardiac, renal or liver disease, uncontrolled hypertension, diabetes, or elevated liver enzymes); and pregnancy, nursing, or refusal to use reliable birth control (women).

Assessments

Participants completed a battery of assessments at the in-person screening visit. These measures included the Structured Clinical Interview for DSM-5 (SCID) (First et al., 1995), Clinical Institute Withdrawal Assessment – Alcohol Revised (CIWA-Ar) (Sullivan et al., 1989), and the 30-day Timeline Followback interview (TLFB) (Sobell and Sobell, 1992) for alcohol, cigarette, and marijuana use. Participants completed assessments regarding alcohol use and related problems, including the Alcohol Dependency Scale (ADS) (Skinner and Allen, 1982) and Alcohol Use Disorders Identification Test (AUDIT) (Saunders et al., 1993); and measures of alcohol craving, including the Penn Alcohol Craving Scale (PACS) (Flannery et al., 1999) and the Obsessive Compulsive Drinking Scale (OCDS) (Anton, 2000). The three subscales of the OCDS (Resistance/Control Impairment (RCI), Obsession, and Interference) (Roberts et al., 1999) were analyzed separately. Participants completed the Fägerstrom Test for Nicotine Dependence (Heatherton et al., 1991), a measure of smoking severity. Measures of interest for the current study were the UCLA Reward, Relief, Habit Drinking Scale (RRHDS) (Grodin et al., 2019) and the Reasons for Heavy Drinking Questionnaire (RHDQ) (Adams et al., 2016), which assess motivations for drinking.

Derivation of Reward and Relief Groups

Reward and relief groups were derived by self-categorization on the RRHDS. Continuous scales (questions 2-4) ask subjects to rate on a 1-7 Likert scale how often they drank alcohol for its rewarding effects (e.g. to feel good, excited, or confident), relief effects (e.g. to feel less bad, sad, or nervous), or habit (e.g. without thinking about the effects), respectively. The highest rating on these Likert scales is used to categorize subjects into reward, relief, and habit groups. In the event of a participant rating more than one dimension equally highly, the first question, which asks participants to select their primary drinking motivation, is used as a tie-breaker (Grodin et al., 2019).

fMRI Data Acquisition

Neuroimaging took place at the UCLA Center for Cognitive Neuroscience (CCN) on a 3.0T Siemens Prisma Scanner (Siemens Medical Solutions USA, Inc., Malvern, PA). A T2-weighted, high-resolution matched-bandwidth (MBW) anatomical scan (time to repetition (TR) = 5,000 ms, time to echo (TE) = 34 ms, flip angle = 90°, voxel size: 1.5 mm × 1.5 × 4 mm, field of view (FOV) = 192 mm2, 34 slices, ~1.5 minutes) and a T1-weighted magnetization-prepared rapid gradient-echo (MPRAGE) sequence (TR = 2,530 ms, TE = 1.74 ms, time to inversion = 1,260 ms, flip angle = 7°, voxel size: 1 mm3, FOV = 256 mm2, ~6.2 minutes) were acquired for co-registration to the functional data. A T2*-weighted echo planar imaging (EPI) scan (TR = 2,200 ms, TE = 35ms, flip angle = 90°, FOV = 192 mm, slices = 36, 3.0 mm, ~12 minutes) was acquired to examine the blood oxygen-level dependent (BOLD) signal during the visual alcohol cue reactivity task.

Participants completed a 720s-long visual alcohol cue-reactivity task (Schacht et al., 2013), in which they were presented with 24 pseudo-randomly interspersed blocks of alcoholic

beverage images (ALC), non-alcoholic beverage images (BEV), blurred images to serve as visual controls, and a fixation cross. Each block was composed of 5 individual pictures of the same type, each presented for 4.8 seconds, for a total of 24-seconds. Each block was followed by a 6-second washout period during which participants reported on the urge to drink. Alcoholic beverage blocks were distributed between images of beer, wine, and liquor (2 of each).

Data analysis

Preprocessing of neuroimaging data followed conventional procedures as implemented in FMRIB Software (FSL v6.0.1 http://www.fmrib.ox.ac.uk/fsl), including motion correction (Jenkinson et al., 2002), high-pass temporal filtering (100-second cut-off), and smoothing with a 5-mm full-width, half-maximum Gaussian kernel. Functional and structural data were skull-stripped to remove non-brain tissue. Each subject's functional images were registered to their MBW, followed by their MPRAGE using affine linear transformations, and then were normalized to the Montreal Neurological Institute (MNI) 152-brain-average template through non-linear registration (Andersson et al., 2007).

All data analysis was conducted in R (RStudio 1.2.5001). Two-sample T-tests, Chi-Squared tests, and univariate analyses of variance (ANOVAs) were used to compare continuous and categorical behavioral variables across participants divided into two (i.e. reward vs. relief+habit drinkers) or three (i.e. reward vs. relief vs. habit drinkers) groups, respectively. T, X^2, and F- statistics, along with corresponding p-values, are reported in **Table 1.1 (entire sample)** and **Table 1.2 (fMRI subset)**. A correlation matrix of all assessments con- ducted is reported in **Table 1.3.**

For the fMRI task, the mean percent signal change between alcohol and non-alcoholic beverage blocks of the task was extracted from *a priori* striatal regions of interest (ROIs). The

first ROI, bilateral ventral striatum (VS), was defined anatomically as the nucleus accumbens from the Harvard-Oxford subcortical structure probability atlas, binarized at a 0.5 probability threshold (Kaag et al., 2019; Ray et al., 2014). The bilateral dorsal striatum (DS) ROI was defined anatomically as the caudate and putamen from the Harvard-Oxford atlas, also binarized at a 0.5 probability threshold. Overlap between the VS and DS regions were subtracted from the DS mask to distinguish between dorsal and ventral striatal areas (Kaag et al., 2019; Liu et al., 2017). Analyses of group differences in DS and VS cue-reactivity, as well as associations between cue-reactivity and behavioral measures, were conducted in R as general linear models. Since the relief group scored higher than the reward group on the ADS, a measure of AUD severity, all neuroimaging analyses controlled for ADS score (as well as medication assignment and interaction effects) in order to probe neural activation differences between groups over and beyond what could be explained by AUD severity or medication. As the current study was conducted within the framework of a medication trial, medication effects and interaction effects were tested within these models, and exploratory analyses of DS and VS cue-reactivity within the placebo and medication groups were conducted separately as well.

Results

In the initial sample of 184 subjects, 122 were categorized as reward drinkers, 28 as relief drinkers, and 34 as habit drinkers (i.e. 62 relief+habit drinkers). Mean RHDQ scores for the full sample were 7.48±1.72 for Reinforcement and 2.88±2.63 for Normalizing. Cronbach's α was 0.507 for Reinforcement and 0.813 for Normalizing, which are consistent with previous studies using these reward and relief scales. Of the 45 participants who underwent the fMRI paradigm, 27 were categorized as reward drinkers and 18 as relief+habit drinkers. The proportion of participants randomized to ibudilast vs. placebo did not differ between the reward and relief+habit participants who completed the fMRI session.

RRHDS-defined relief/habit drinkers reported higher craving on the OCDS than reward drinkers [t=-3.60; p<0.0001]. The same pattern emerged for the PACS [t=-3.06; p=0.003], ADS [t=-2.60; p=0.011], and all subscales of the OCDS [Resistance/Control Impairment (RCI) t=-3.42; p<0.001; Obsession t=-2.918; p=0.004; Interference t=-3.478; p<0.001.]. The groups did not differ significantly on the CIWA-Ar measure of withdrawal. See **Table 1.1** for complete results for clinical variables.

Among the 45 participants who completed the fMRI task, reward and relief/habit groups differed on the OCDS RCI subscale [t=-2.05; p=0.04]. Unlike the broader sample, the two groups did not differ significantly on the PACS, ADS, total OCDS score, or the other OCDS subscales. See **Table 1.2** for complete results.

Comparison of Reward and Relief Drinking Assessments

RRHDS-defined reward and relief+habit groups differed significantly from each other on both the Reinforcement [t=2.06; p=0.04] and Normalizing [t=-2.94; p=0.004] subscales of the RHDQ

(Figure 1.1a). When relief and habit drinkers were separated, the three groups differed significantly only on the Normalizing subscale [$F = 4.49$; p=0.01] **(Figure 1.1b)**.

Neuroimaging Results

Reward and relief+habit groups differed in activation within the DS only, such that the relief+habit group showed higher cue-elicited DS activation [reward mean = -0.0001±0.14; relief+habit mean = 0.09±0.13; p=0.04] **(Figure 1.2a)**. As all fMRI participants were in a medication trial, medication and interaction effects were examined to evaluate if medication influenced this result. A main effect of medication was not seen (p>0.34), but a medication × group interaction effect was present ($p<0.05$). An exploratory analysis estimated the models separately within each medication group. This analysis indicated that the difference in DS cue-elicited activation was driven by the placebo group [placebo reward mean = -0.02±0.13; placebo relief+habit mean= 0.15±0.13; p=0.002] **(Figure 1.2b),** as there was no significant difference in the group receiving active medication [ibudilast reward mean = 0.03±0.16; ibudilast relief+habit mean= 0.04±0.12; p=0.45] **(Figure 1.2c).** No significant differences were found in VS activation between groups.

Cue-elicited VS activation was significantly negatively correlated with a continuous measure of relief on the RRHDS (Question 3: "How often do you drink alcohol because it reduces negative feelings (e.g. makes you feel bad, sad, or nervous)?", rated on a 1-7 Likert scale) [R^2=0.32; p=0.03] **(Figure 1.3).** Due to responses on the continuous scale being moderately right-skewed (skewness=0.592), the continuous data was transformed on a log scale. As with the previous result, medication and interaction effects were examined to evaluate if medication influenced this result. Neither a main effect of medication ($p>0.12$) or a medication × group interaction effect ($p>0.15$) were present. The other two continuous RRHDS items (Questions 2 or 4) were not

significantly correlated with VS or DS activation (p>0.09); nor were there any correlations between VS or DS cue-reactivity and RHDQ scores (p>0.41).

Discussion

This study sought to elucidate clinical and neural correlates of reward and relief drinking, captured through recently developed self-report instruments. Results replicated original findings regarding the score distributions and internal consistency of the RHDQ (Adams et al., 2016). Group differences between reward and relief/habit drinkers on alcohol measures including the PACS, ADS, and OCDS were also replicated in this independent sample (Grodin et al., 2019), such that relief/habit drinkers reported significantly higher scores than reward drinkers on these measures, with relief drinkers scoring highest when separated into three groups.

As hypothesized, characterizations based on the RRHDS and RHDQ largely aligned, such that RRHDS-designated reward drinkers scored significantly higher than relief/habit drinkers on the Reinforcement subscale of the RHDQ, whereas relief/habit drinkers scored significantly higher than reward drinkers on the Normalizing subscale. Further analysis separating relief from habit drinkers found that the three groups all significantly differed from each other only on the Normalizing subscale, suggesting that the difference seen in Reinforcement when the relief and habit groups were combined may be driven by habit drinkers' lower reinforcement scores.

Neuroimaging results indicated that, as hypothesized, relief/habit drinkers showed greater DS activation to visual alcohol cues than reward drinkers. However, contrary to our hypotheses, cue-elicited VS activation did not differ significantly between groups. A possible interpretation of these results may be that among relief/habit drinkers, reward is not lost; rather, a dimension of drinking for relief is gained or amplified. This finding aligns with previous studies showing that

positive, stimulatory response to alcohol, but not the negative, sedative response, predicts the development of escalated drinking and AUD (King et al., 2019, 2014, 2011). This hedonic response to alcohol, or "liking" as it is termed in the incentive salience model (Robinson and Berridge, 1993), is thought to be associated with reward, and serves as positive reinforcement under the allostatic model of addiction (Koob and Schulkin, 2019). The interpretation that reward is not lost while relief is amplified is also supported by a previous finding that alcohol-dependent participants did not show a blunted stimulation response compared to heavy-drinking controls, but did show a higher sedation response as alcohol administration began (Bujarski and Ray, 2014) – a result that is distinct from what would be hypothesized simply due to tolerance syndrome, in which all domains of subjective response are expected to be blunted (Morean and Corbin, 2008). It should be noted that while previous studies (Bujarski and Ray, 2014; Schacht et al., 2013) administered alcohol, the present study did not. Nevertheless, we reference those studies as they capture drinking responses and motives in "real-time" through controlled alcohol administration models.

This finding generally aligns with the allostatic and incentive salience models, but may suggest that the current participants are still somewhat early in the process of transitioning from positive reinforcement to negative reinforcement, or from liking to wanting/craving. This may be a result of the current sample being a relatively high-functioning, outpatient group that did not reach the most severe levels of AUD, with the vast majority of participants categorized as reward drinkers. Future studies should examine ventral and dorsal striatal cue-reactivity in participants with more severe AUD, whom might be farther along in the transition to negative reinforcement and as such, more likely to be categorized as relief drinkers. It is also important to note that while the RHDQ measure was developed in treatment-seeking samples (Adams et al., 2016), the current study is comprised of non-treatment seeking heavy drinkers. While the results of the

current study largely replicated the findings seen in treatment seekers, the two populations differ, with treatment-seekers reporting a greater number of AUD symptoms, consuming more drinks per drinking day, and having higher ADS and OCDS scores than non-treatment-seeking participants (Ray et al., 2017). Thus, the findings identified in the current study, particularly with regards to neural activation, should be tested in treatment seekers, who are more likely to reach more severe levels of AUD. In the between-groups finding presented herein, exploratory follow-up analyses found that the observed effects were largely driven by the placebo group, which calls for further neuroimaging studies that do not involve a medication component.

Further neuroimaging results examining correlations between continuous measures of reward, relief, and habit and neural alcohol cue-reactivity found that cue-elicited VS activation was significantly negatively correlated with the continuous measure of relief. This finding was unexpected, as we would have hypothesized that there would be a positive relationship between relief and DS cue-reactivity rather than the observed negative correlation with VS cue-reactivity. Again, this may be a function of our outpatient sample not reaching the most severe levels of AUD. Changes in reward and relief scores may be best studied within-person and longitudinally.

The current study creates subtypes based on reward (reinforcement) and relief (normalizing); however, previous work focusing on precision medicine (Mann et al., 2018) differentiates high reward/low relief individuals from those who are high relief/low reward, high in both, or low in both. While the RRHDS was developed with the aim of characterizing participants' primary motivations for drinking, the continuous 1-7 Likert scales (questions 2-4) can be dichotomized (i.e., "high" reward or relief = 4 or higher; "low" = 3 or lower). Using these designations, in the current sample of 184 participants, 83 (45%) were characterized as high reward/high relief, 86 (47%) as high reward/low relief, 7 (4%) as low reward/high relief, and 8 (4%) as low reward/low relief. As previously discussed, our high-functioning outpatient sample

did not reach the most severe levels of AUD, so these categorizations are unsurprising. Additionally, few individuals were low on both reward and relief, despite higher prevalence in this category within treatment-seeking samples. It may be a limitation of the RRHDS measure that does not provide an opportunity for individuals to be low in both dimensions, as it emphasizes participants' highest dimension. Future studies should examine these further subtypes in a sample with more severe AUD, which may yield more participants with low reward scores.

These results should be considered in light of the study's strengths and limitations. Strengths include the integration of two novel measures of drinking motivation, as well as the combination of clinical phenotyping and neuroimaging (fMRI) methodologies. A notable limitation is that participants in the fMRI analysis were originally from a larger medication study and were scanned after one week on medication. Medication effects were not the focus of the present study and was controlled for in all analyses presented; however, exploratory analyses of interaction effects indicated that group differences in striatal cue-reactivity were driven by the placebo group. In light of this limitation, it is critical that these results be replicated in independent samples, including studies that do not have a medication component. An additional limitation was the moderate sample size within the neuroimaging group, which limited our ability to probe the neural differences in differences between reward and relief/habit drinkers (e.g., through whole-brain analyses). On balance, these results represent a first step towards characterizing the underlying neural correlates of neuroscience-informed drinking phenotypes in a clinical sample. The study is also limited by the reliability of assessments used, as the RRHDS has been shown to have strong test-retest reliability for reward drinkers, but to be less reliable for relief drinkers (Grodin et al., 2019) and the RHDQ had low reliability on the reinforcement subscale both in its original development (Adams et al., 2016) and in our sample. Additionally,

the current sample is relatively young, with average age at least ten years younger than in comparable studies (Mann et al., 2018; Ooteman et al., 2006; Witkiewitz et al., 2019). While age did not distinguish between reward and relief within the current sample, in samples with a higher representation of older adults, age may differentiate these two groups. Specifically, relief drinkers may be older than reward drinkers, which is also associated with longer drinking history and potential for higher severity. Furthermore, the small neuroimaging sample size limited our ability to further probe additional factors such as sex differences. While the current sample did not show sex differences in reward/relief categorization, future studies may explore sex differences within a sample with a wider range of AUD severity, as men and women have been shown to drink for different reasons (Peltier et al., 2019) and sex differences have also been implicated in differential cue-reactivity signatures in smoking studies (Cosgrove et al., 2014). Finally, the disproportionate ratios of reward drinkers to relief and habit drinkers in both the fMRI sample and the larger behavioral sample necessitated the combining of relief and habit drinkers with regards to neuroimaging analyses. Nonetheless, the decision to combine these groups was informed both by previous research (Grodin et al., 2019; Mann et al., 2018; Roos et al., 2017) and by the lack of significant differences between groups on clinical measures within the fMRI sample.

In conclusion, this study reports on the neural and clinical characterization of heterogenous AUD subtypes based on motivations for drinking, as assessed by the RHDQ and the UCLA RRHDS. The overall agreement between these two measures suggests a consistent differentiation between reward-driven and relief-driven alcohol use, providing indirect support for the allostatic and incentive salience models of addiction (Koob and Schulkin, 2019; Robinson and Berridge, 1993). The present study also elucidates neural mechanisms (i.e. cue-elicited striatal activation) underlying these AUD subgroups. The clinical and neural correlates of reward

and relief/habit drinking found in this study may present a path towards the refinement of these neuroscience-informed phenotypes with the ultimate goal of informing personalized treatments for AUD.

Table 1.1. *Clinical characteristics of reward, relief, habit, and relief+habit groupings within the entire sample. 2-group comparison refers to reward group vs. relief+habit combined group; 3-group comparison refers to reward vs. relief vs. habit groups. ± indicates standard deviation.*

Measure	Sex F/M	Age	Smoker	THC+	Total Drinking	Drinks / Day	Drinks/ Week	Drinks/ Drinking
Total (n=184)	61/123 33/67%	31.98±8.69	87 47%	55 30%	19.05±7.53	3.57±2.98	25.06±20.91	5.51±3.26
Reward (n=122)	43/79 35/65%	32.34±9.16	55 45%	33 27%	17.69±7.40	3.25±2.75	22.77±19.27	5.43±3.22
Relief (n=28)	10/18 36/64%	32.11±8.53	14 50%	7 25%	19.75 ±7.00	3.48±2.33	24.42±16.31	5.24±2.65
Habit (n=34)	8/26 24/76%	30.59±6.98	18 53%	15 44%	23.39±6.84	4.84±3.92	33.89±27.45	6.00±3.87
Relief +Habit	18/44 29/71%	31.27±7.70	32 52%	22 35%	21.72±7.10	4.22±3.33	29.55±23.33	5.65±3.36
2-group comparison	X^2=0.46 p=0.496	t=0.835 p=0.405	X^2=0.47 p=0.495	X^2=1.02 p=0.312	t=-3.559 **p<0.001**	t=-1.954 p=0.054	t=-1.954 p=0.054	t=-0.420 p=0.675
3-group comparison	X^2=1.75 p=0.417	F=0.544 p=0.581	X^2=0.76 p=0.685	X^2=4.07 p=0.131	F=8.175 **p<0.001**	F=3.793 **p=0.024**	F=3.793 **p=0.024**	F=0.508 p=0.603

ADS (α=0.853)	PACS (α=0.904)	OCDS Interference	OCDS Obsession	OCDS RCI	OCDS	AUDIT	CIWA-Ar
12.53±7.09	12.41±6.86	1.67±2.17	5.33±3.85	10.83±4.78	17.83±9.81	15.62±7.27	0.98±2.18
11.53±6.58	11.30±6.47	1.23±1.71	4.72±3.53	10.00±4.67	15.95±8.95	14.52±6.89	0.95±2.07
15.29±7.04	16.54±6.25	2.96±2.86	8.07±3.83	13.643±3.73	24.68±8.99	17.86±5.61	0.89±1.89
13.85±8.19	13.00±7.49	2.18±2.52	5.26±4.09	11.50±5.05	18.94±10.92	17.71±8.95	1.15±2.79
14.50±7.67	14.60±7.12	2.53±2.68	6.53±4.19	12.468±4.59	21.53±10.42	17.77±7.57	1.03±2.41
t=-2.599 **p=0.011**	*t*=-3.064 **p=0.003**	*t*=-3.478 **p<0.001**	*t*=-2.918 **p=0.004**	*t*=-3.422 **p<0.001**	*t*=-3.597 **p<0.001**	*t*=-2.836 **p=0.005**	*t*=-0.228 p=0.819
F=4.049 **p=0.019**	*F*=7.723 **p<0.001**	*F*=9.136 **p<0.001**	*F*=9.429 **p<0.001**	*F*=7.513 **p<0.001**	*F*=10.22 **p<0.001**	*F*=4.256 **p=0.016**	*F*=0.132 p=0.877

Table 1.2. *Clinical characteristics of reward and relief+habit groupings within the subset of individuals who participated in neuroimaging. ± indicates standard deviation.*

Measure	fMRI Total (n=45)	fMRI Reward (n=27)	fMRI Relief+Habit (n=18)	2-group comparison
Sex F/M	17/28 38/62%	10/17 37/63%	7/11 39/61%	$X^2<0.001$ $p=1$
Age	32.5±8.59	32.48±8.81	32.56±8.51	$t=-0.02$ $p=0.978$
Smoker	24 53%	15 56%	9 50%	$X^2=0.004$ $p=0.951$
THC+	13 29%	6 22%	7 39%	$X^2=0.76$ $p=0.383$
Total Drinking Days	20.53±6.58	17.78±5.73	24.67±5.63	$t=-3.996$ $\mathbf{p<0.001}$
Drinks / Day	3.92±3.14	3.46±3.04	4.62±3.25	$t=-1.201$ $p=0.238$
Drinks/ Week	27.51±22.00	24.27±21.29	32.38±22.77	$t=-1.201$ $p=0.238$
Drinks/ Drinking Day	5.65±3.37	5.73±3.56	5.52±3.14	$t=0.205$ $p=0.839$
CIWA-Ar	0.58±1.41	0.89±1.71	0.11±0.47	$t=2.231$ $\mathbf{p=0.033}$
AUDIT	16.53±6.21	15.00±5.36	18.83±6.81	$t=-2.008$ $p=0.053$
OCDS	18.6±9.02	16.56±8.21	21.67±9.53	$t=-1.861$

				p=0.072
OCDS RCI Factor	11.30±4.60	10.15±4.36	12.49±4.57	t=-2.049 **p=0.048**
OCDS Obsession Factor	5.69±3.65	5.15±3.60	6.50±3.67	t=-1.220 p=0.230
OCDS Interference Factor	1.64±1.79	1.26±1.53	2.22±2.02	t=-1.721 p=0.096
PACS	12.00±6.31	11.07±6.31	13.39±6.23	t=-1.215 p=0.232
ADS	12.20±6.56	11.81±7.22	12.78±5.58	t=-0.503 p=0.618
IBUD	20 44%	11 40.7%	9 50%	X^2=0.09 p=0.759

Table 1.3. *Correlation matrix of all assessments conducted. Reported values are R^2. * =p<0.05, **=p<0.01, ***= p<0.001.*

Assessments numbered in Table 1.3:

1. RHDQ – Reinforcing

2. RHDQ – Normalizing

3. RRHDS – Reward

4. RRHDS – Relief

5. RRHDS – Habit

6. CIWA-Ar

7. AUDIT

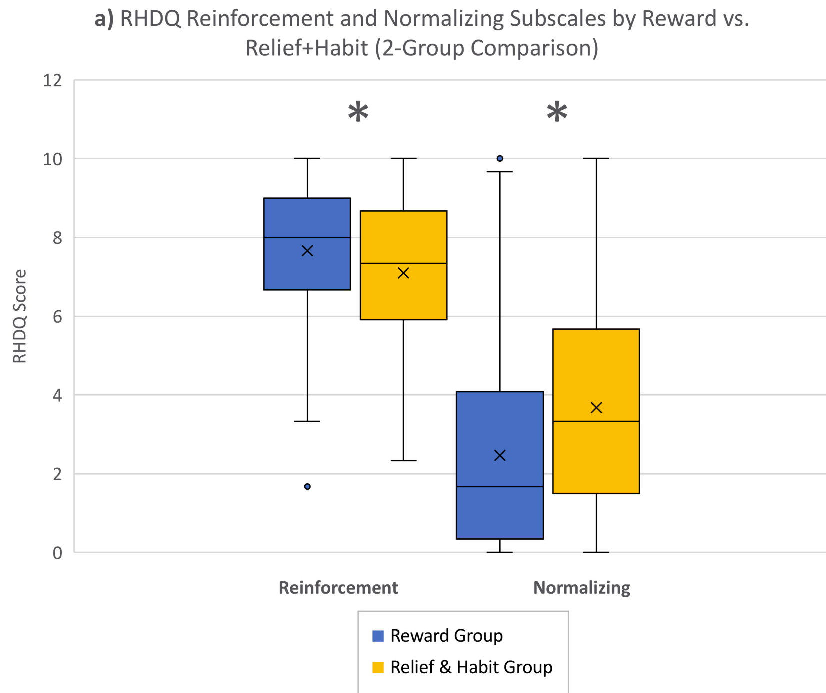
8. OCDS

9. PACS

10. ADS

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
1	-								
2	0.119***	-							
3	0.171***	0.003	-						
4	0.142***	0.258***	0.003	-					
5	0.010	0.244***	0.002	0.063***	-				
6	0.015	0.234***	0.004	0.051**	0.045**	-			
7	0.095***	0.457***	0.004	0.176***	0.143***	0.190***	-		
8	0.061***	0.418***	0.000	0.221***	0.167***	0.251***	0.554***	-	
9	0.098***	0.402***	0.016	0.286***	0.179***	0.219***	0.464***	0.686***	-
10	0.073***	0.313***	0.002	0.179***	0.117***	0.145***	0.608***	0.462***	0.410***

Figure 1.1. *RHDQ Reinforcement and Normalizing scores by reward / relief drinkers.* ***a)*** *Two-group comparison between reward (blue) and relief+habit (yellow) drinking groups;* ***b)*** *Three-group comparison between reward (blue), relief (yellow), and habit (green) drinking groups.*



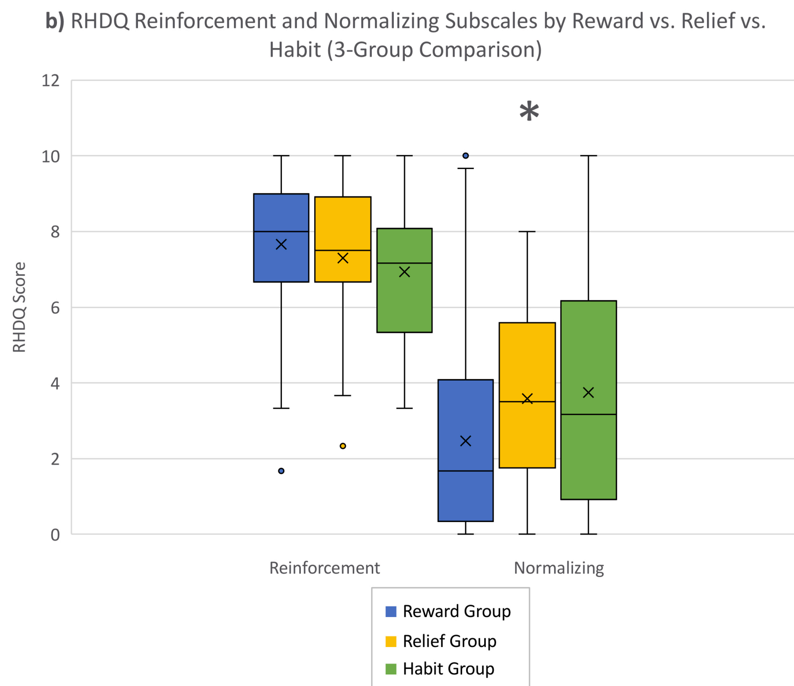


Figure 1.2. *Dorsal and ventral striatal cue-reactivity in reward (blue) vs. relief+habit (yellow) drinkers.* ***a)*** *all fMRI participants;* ***b)*** *placebo group only;* ***c)*** *ibudilast group only.*

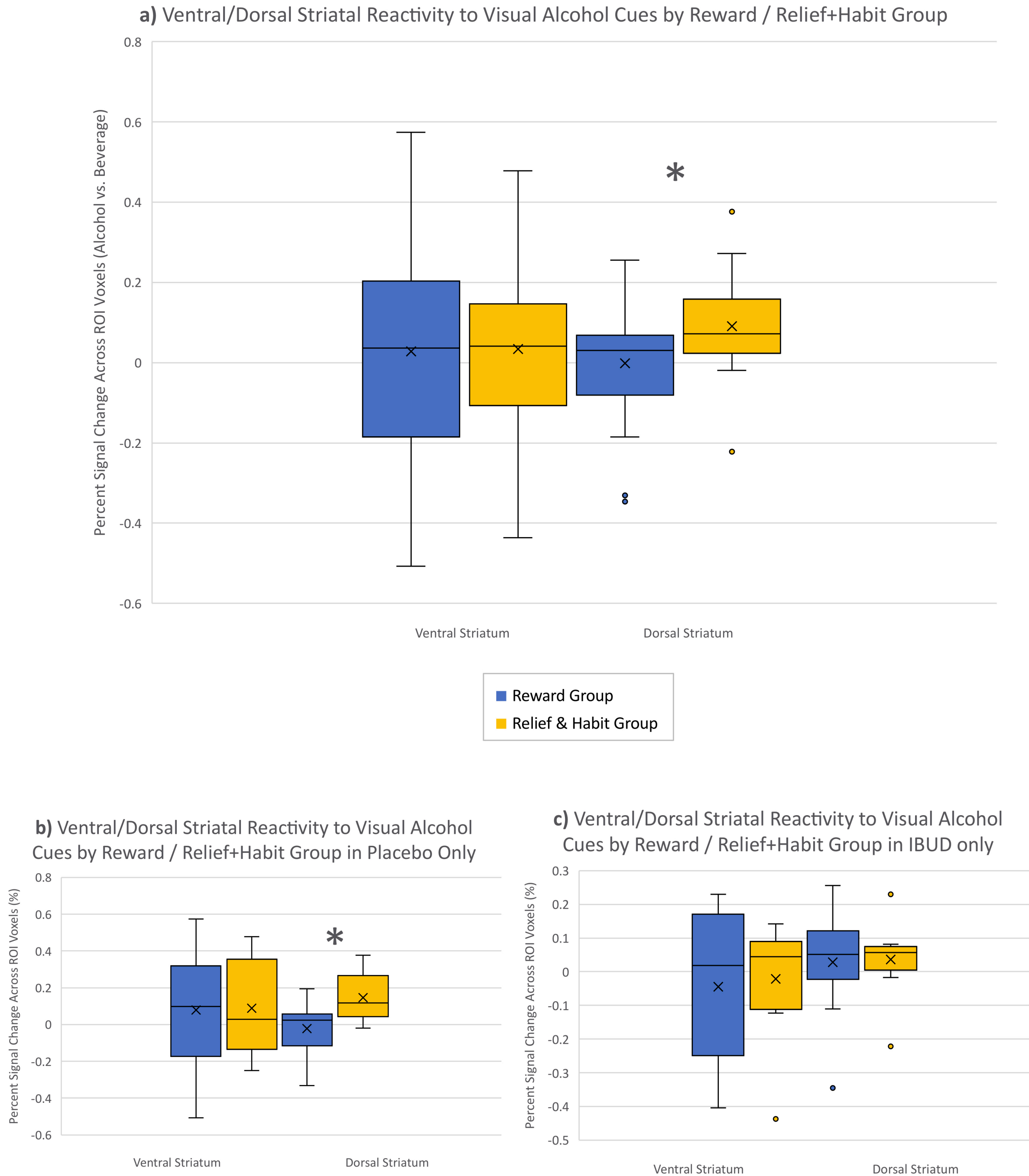
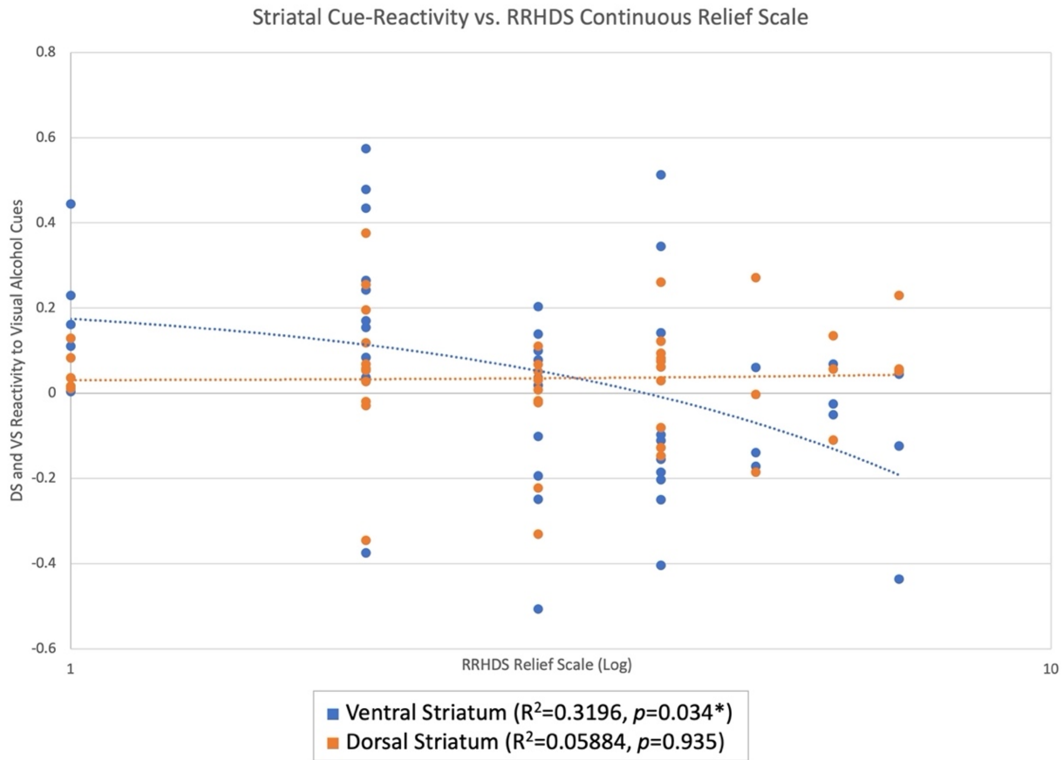


Figure 1.3. *Ventral (blue) and dorsal (orange) striatal cue-reactivity vs. RRHDS continuous relief scale.*



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CHAPTER 2

IBUDILAST ATTENUATES ALCOHOL CUE-ELICITED FRONTROSTRIATAL FUNCTIONAL CONNECTIVITY IN ALCOHOL USE DISORDER

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Abstract

Background: Ibudilast, a novel neuroimmune modulator to treat alcohol use disorder (AUD), was shown in a randomized controlled trial (NCT03489850) to reduce ventral striatum (VS) activation in response to visual alcohol cues. The present study extended this finding by probing the effects of ibudilast on alcohol cue-elicited functional connectivity (i.e., temporally-correlated activation) with the VS seed. The study also tests the association between functional connectivity and alcohol use during the trial.

Methods: Non-treatment-seeking participants (n=45) with current alcohol use disorder were randomized to receive either ibudilast (50 mg/BID; n=20) or placebo (n=25). Upon reaching the target dose on the medication, or placebo, participants completed a functional neuroimaging alcohol cue-reactivity paradigm. Drinks per drinking day were assessed at baseline and daily during the two-week trial.

Results: Ibudilast reduced alcohol cue-elicited functional connectivity between the VS seed and reward processing regions including the orbitofrontal and anterior cingulate cortices compared to placebo ($p<0.05$). Cue-elicited functional connectivity was correlated with drinks per drinking day ($R^2=0.5351$, $p<0.001$), and ibudilast reduced this association in similar reward processing regions compared to placebo.

Conclusions: Ibudilast's effects on drinking outcomes may be related to attenuation of functional connectivity in frontostriatal circuits related to reward processing. These results provide an important proof-of-concept for this novel pharmacotherapy and support the clinical utility of incorporating neuroimaging – and especially functional connectivity – analyses into medications development.

Keywords: AUD, fMRI, Ibudilast, functional connectivity

Summary: This study examines the effects of Ibudilast, neuroimmune modulator, on cue-elicited functional connectivity in alcohol use disorder (AUD). Ibudilast (compared to placebo) significantly attenuated functional connectivity between the ventral striatum and regions related to reward processing, including the orbitofrontal and anterior cingulate cortices, and reduced the association between functional connectivity and drinks per drinking day compared to placebo. Findings indicate that ibudilast's effects on drinking outcomes may be related to its attenuation of functional connectivity in frontostriatal reward-processing circuitry.

Introduction

Alcohol use disorder (AUD) is a highly prevalent chronic relapsing disorder (Grant et al., 2015); however, it is among the most undertreated health conditions (Carvalho et al., 2019), with only 7% of adults with AUD receiving treatment (Hasin et al., 2007). The Food and Drug Administration has approved only four pharmacotherapies for the treatment of AUD to date, and these medications are limited in efficacy (Ray et al., 2019). There is a great need to develop new and more effective treatments for AUD, with a specific focus on novel molecular targets (Litten et al., 2016, 2012).

One such novel pharmacotherapy is ibudilast (IBUD; also known as MN-166, previously AV411 and available as Ketas in Japan for the treatment of bronchial asthma and for cerebrovascular disorders). Ibudilast is a selective phosphodiesterase (PDE) inhibitor (inhibiting PDE3, 4, 10, and 11) (Gibson et al., 2006) and an allosteric macrophage migration inhibitory factor (MIF) inhibitor (Cho et al., 2010), which has shown promising preclinical and clinical outcomes in the treatment of alcohol use disorder. IBUD has been shown to reduce drinking and relapse in preclinical rodent models of AUD, including preferentially reducing drinking in dependent compared to non-dependent mice (Bell et al., 2015). In a previous human laboratory study by our group, IBUD was shown to reduce craving and improve mood following stress and alcohol cue exposure (Ray et al., 2017a), but the neurobiological processes related to these clinical outcomes remain unclear.

A useful tool for identifying neural mechanisms of novel pharmacotherapies is the use of functional magnetic resonance imaging (fmri) to examine the modulation of brain activation and connectivity in regions associated with AUD (Grodin and Ray, 2019). In order to explore the mechanisms of action of IBUD in the human brain, a recent clinical trial from our group (Grodin et al., in press) employed a functional magnetic resonance imaging (fMRI) paradigm of visual

alcohol cue exposure to investigate the effect of IBUD on cue-elicited neural activity in the ventral striatum (VS). This region is commonly associated with reward and has been shown to have a high expression of PDE4A, B, and D (Pérez-Torres et al., 2000) and to be highly relevant for alcohol cue-reactivity tasks (Schacht et al., 2013). The study found that IBUD significantly reduced VS activity in response to alcohol cues relative to placebo. Further, reductions in VS activity due to ibudilast were associated with reductions in drinking during the 2-week trial, as compared to placebo (Grodin et al., in press). Cue-reactivity has been shown to be predictive of treatment response (Schacht et al., 2017), demonstrating the clinical utility of functional neuroimaging in providing mechanistic data for pharmacotherapy development.

The current study is a secondary analysis of the aforementioned trial (ClinicalTrials.gov identifier: NCT03489850). While the registered aim of the main trial examined the effects of IBUD on VS cue-reactivity using an *a priori* defined region of interest (ROI), the current study further probes this aim and evaluates regions in which neural activation in response to alcohol cues is temporally correlated with VS cue-reactivity. This strategy, often referred to as functional connectivity (O'Reilly et al., 2012), offers a more complex, holistic picture of the circuits involved, in comparison to a single ROI (Courtney et al., 2016; Lim et al., 2019). A functional connectivity approach also builds on the main study by using the same VS region as an *a priori* designated seed, since its activity in response to alcohol cues was shown to be affected by IBUD in this sample (Grodin et al., in press). Previous medication studies from our group have successfully used this cue-reactivity task-based functional connectivity approach. For example, in previous studies we reported that naltrexone enhanced cue-elicited functional connectivity (relative to placebo) from a VS seed in heavy drinkers (Lim et al., 2019) and from caudate and precuneus seeds in methamphetamine users (Courtney et al., 2016).

Based on the premise that novel compounds, such as ibudilast, require proof-of-mechanism via a host of brain-based biomarkers, the primary aim of the present study was to test whether IBUD altered functional connectivity. Given that we previously found that VS cue-reactivity was significantly attenuated under IBUD, we hypothesized that VS functional connectivity would similarly be reduced in the IBUD condition compared to placebo. To connect brain-to-behavior, the current study also tested whether IBUD's effects on functional connectivity were associated with one of the primary registered drinking outcomes of the main trial: drinks per drinking day in the week following the fMRI scan. The previous report (Grodin et al., in press) found that VS cue-reactivity predicted the drinks per drinking day outcome, such that individuals in the IBUD group who had attenuated VS activation had the fewest number of drinks per drinking day. Therefore, we hypothesized that individuals in the IBUD group who had reduced functional connectivity from the VS seed would also have the fewest drinks per drinking day. While the VS is our *a priori* seed of interest, we considered the broader literature on neural reactivity to alcohol cues and explored a host of additional seeds. The dorsal striatum (DS), anterior cingulate cortex (ACC), posterior cingulate cortex (PCC), and precuneus were selected as alternative exploratory seeds, as these regions have shown a strong cue-reactivity signal and modulation by pharmacological and behavioral treatments (Schacht et al., 2013).

Materials and Methods

This study was performed as part of a two-week randomized controlled trial (ClinicalTrials.gov NCT03489850) of IBUD for drinking reduction. The trial was approved by the Institutional Review Board of the University of California, Los Angeles. All study participants provided written informed consent for screening, medication, and neuroimaging procedures. The current study used fMRI data from individuals who completed the neuroimaging visit.

Participants

Participants were recruited between July 2018 and March 2020 from the greater Los Angeles metropolitan area via mass transit and social media advertisements. Detailed description of the screening and experimental procedures has been published elsewhere (Grodin et al., in press). Briefly, participants included 45 non-treatment-seeking individuals with an AUD [20 IBUD, 25 Placebo; 17 female, 28 male; mean±SD age 32.51±8.59], who completed the fMRI neuroimaging paradigm after being randomly assigned to take either IBUD or placebo.

Eligibility was initially assessed through a telephone interview, after which eligible participants underwent in-person screening in the laboratory. Eligibility criteria included an age range between 21 and 50 years; meeting criteria for current AUD as assessed with the Structured Clinical Interview for DSM-5 (American Psychiatric Association, 2013) and drinking more than 14 drinks per week for men (more than 7 for women) in the 30 days prior to screening. Exclusion criteria included currently receiving or seeking treatment for AUD; past year DSM-5 diagnosis of any other substance use disorder (excluding nicotine); lifetime diagnosis of schizophrenia, bipolar disorder, or any psychotic disorder; non-removable ferromagnetic objects in body; claustrophobia; serious head injury or prolonged period of unconsciousness (>30 minutes); medical conditions thought to interfere with safe participation (unstable cardiac, renal or liver

disease, uncontrolled hypertension, diabetes, or elevated liver enzymes); and pregnancy, nursing, or refusal to use reliable birth control (women). Participants were also excluded if taking medications that could interact with ibudilast or alter their alcohol use.

Participants were also assessed for a broader scope of alcohol use measures, including: a daily diary assessment, from which the study's drinks per drinking day outcome was derived; 30-day Timeline Follow-Back (Sobell and Sobell, 1992), from which the baseline drinks per drinking day variable was derived; Structured Clinical Interview for DSM-5 (SCID) (First et al., 1995); Alcohol Use Disorder Identification Test (AUDIT) (Saunders et al., 1993); Clinical Institute Withdrawal Assessment – Alcohol Revised (CIWA-Ar) (Sullivan et al., 1989); Alcohol Dependency Scale (ADS) (Skinner and Allen, 1982); Penn Alcohol Craving Scale (PACS) (Flannery et al., 1999); and Obsessive Compulsive Drinking Scale (OCDS) (Anton, 2000). Clinical and demographic characteristics of the participants are detailed in **Table 2.1.**

At each in-person visit, participants were required to have a breath alcohol concentration (BrAC) of 0.00 g/dl and to test negative on a urine toxicology screen for all drugs of abuse (except cannabis) and urine pregnancy test (if female). IBUD was titrated as follows: 20 mg b.i.d. on days 1-2 and 50 mg b.i.d. on days 3-14. The neuroimaging session occurred at the midpoint visit, after participants had been taking medication for seven days.

Procedures

fMRI Data Acquisition

Neuroimaging took place at the UCLA Center for Cognitive Neuroscience (CCN) on a 3.0T Siemens Prisma Scanner (Siemens Medical Solutions USA, Inc., Malvern, PA). A T2-weighted, high-resolution matched-bandwidth (MBW) anatomical scan (time to repetition (TR) = 5,000 ms, time to echo (TE) = 34 ms, flip angle = 90°, voxel size: 1.5 mm × 1.5 × 4 mm, field

of view (FOV) = 192 mm2, 34 slices, ~1.5 minutes) and a T1-weighted magnetization-prepared rapid gradient-echo (MPRAGE) sequence (TR = 2,530 ms, TE = 1.74 ms, time to inversion = 1,260 ms, flip angle = 7°, voxel size: 1 mm3, FOV = 256 mm2, ~6.2 minutes) were acquired for co-registration to the functional data. A T2*-weighted echo planar imaging (EPI) scan (TR = 2,200 ms, TE = 35ms, flip angle = 90°, FOV = 192 mm, slices = 36, 3.0 mm, ~12 minutes) was acquired to examine the blood oxygen-level dependent (BOLD) signal during the visual alcohol cue reactivity task.

Participants completed a well-validated 720s-long visual alcohol cue-reactivity task (Schacht et al., 2013), in which they were presented with 24 pseudo-randomly interspersed blocks of alcoholic beverage images (ALC), non-alcoholic beverage images (BEV), blurred images to serve as visual controls, and a fixation cross. Each block was composed of 5 individual pictures of the same type, each presented for 4.8 seconds, for a total of 24 seconds. Each block was followed by a 6-second washout period during which participants reported on the urge to drink. Alcoholic beverage blocks were distributed between images of beer, wine, and liquor (2 of each).

Data Analysis

Preprocessing of neuroimaging data followed conventional procedures as implemented in FMRIB Software (FSL v6.0.1 http://www.fmrib.ox.ac.uk/fsl), including motion correction (Jenkinson et al., 2002), high-pass temporal filtering (100-second cut-off), and smoothing with a 5-mm full-width, half-maximum Gaussian kernel. Functional and structural data were skull-stripped to remove non-brain tissue. Each subject's functional images were registered to their MBW, followed by their MPRAGE using affine linear transformations, and then were normalized to the Montreal Neurological Institute (MNI) 152-brain-average template through

non-linear registration (Andersson et al., 2007). All fMRI data had been used in previous studies (Burnette et al., 2021; Grodin et al., in press), and, as such, met criteria for quality control (exclusion criteria: >2mm translational displacement, >1.5˚ rotation). Therefore, no participants or images were excluded for quality control issues, including motion, as part of this study. The time series of activation was extracted from an a priori defined region of interest: bilateral ventral striatum (VS), a 6 mm-radius sphere centered at MNI coordinates x=12, y=6, z=9 (Schacht et al., 2017), which was then reverse-registered from standard space to each participant's anatomical image.

Functional connectivity analyses were conducted in FSL using psychophysiological interaction (PPI) to examine the interaction of task conditions and functional connectivity between the time course of activation for specific seed regions with the rest of the brain (O'Reilly et al., 2012). PPI analyses were conducted to examine the interaction of the ALC>BEV contrast and the VS seed region for the comparisons: IBUD > PLAC and PLAC > IBUD. The first-level PPI models included four regressors: the main 'psychological' regressor to model the difference in task conditions (ALC-BEV), a second 'psychological' regressor to account for the shared variance between task conditions (ALC+BEV), a 'physiological' regressor to model the seed time course, and a 'psychophysiological interaction' regressor which is the product of the main 'psychological' and 'physiological' regressors. Age, sex, and cigarette smoking status were entered as neuroimaging-relevant covariates often associated with differential brain activation in fMRI studies. Whole-brain contrast images were generated with cluster-forming thresholds of $Z>2.3$ and cluster-probability thresholds of $p<0.05$ (Worsley, 2001). Average drinks per drinking day in the last week of the study was added as a covariate of interest in separate higher-level analyses paralleling those described above. In these analyses, baseline drinks per drinking day was also included as a covariate.

Clusters revealed by the PPI to significantly correlate with drinks per drinking day in both groups were selected as ROIs. The activation profile (percent signal change) was then extracted for these PPI ROIs using the Featquery tool in FSL for all subjects in the model, regardless of whether or not a subject had significant task-related activation in the cluster (Bradley et al., 2016). General linear model (GLM) analyses probing medication effects on the relationship between the PPI ROI activation profiles and drinks per drinking day were conducted in R (RStudio 1.2.5001), controlling for baseline drinks per drinking day. Associations between PPI ROI percent signal change and drinks per drinking day were assessed across groups, as well as separately in the IBUD and placebo groups.

Exploratory PPI analyses were also conducted to examine functional connectivity from additional seeds. The dorsal striatum (DS), anterior cingulate cortex (ACC), posterior cingulate cortex (PCC), and precuneus were selected as alternative seeds (anatomical ROIs derived from the Harvard-Oxford atlas), as these regions have shown a strong cue-reactivity signal and modulation by pharmacological and behavioral treatments (Schacht et al., 2013). Age, sex, and cigarette smoking were included as neuroimaging-relevant covariates in these exploratory analyses as well.

Results

IBUD Effects on Functional Connectivity

As compared to placebo, treatment with IBUD resulted in reduced alcohol cue-elicited functional connectivity from the VS seed, as indicated by PPI analysis. Specifically, IBUD, compared to placebo, resulted in reduced functional connectivity from the VS to multiple regions including the left orbitofrontal cortex, right medial frontal cortex, and bilateral anterior cingulate (see **Figure 2.1** and **Table 2.2**). Whole-brain results were thresholded using cluster-corrected statistics with a height-threshold of $Z > 2.3$ and cluster-forming threshold of $p < 0.05$.

Functional Connectivity and Drinks per Drinking Day

Further PPI analysis examined the association between alcohol cue-elicited functional connectivity with the VS seed and drinks per drinking day. Whole-brain results showed that, overall (i.e., across medication groups) functional connectivity with the VS seed was correlated with drinks per drinking day and that this correlation was stronger in the placebo group than in the IBUD group. Regions in which functional connectivity showed a stronger correlation with drinks per drinking day in the placebo group compared to the IBUD group included the left caudate, temporal pole, and orbitofrontal cortex, bilateral anterior cingulate, and right lateral occipital cortex within the ALC>BEV contrast (see **Figure 2.2** and **Table 2.3**). Whole-brain results were thresholded using cluster-corrected statistics with a height-threshold of $Z > 2.3$ and cluster-forming threshold of $p < 0.05$.

Across both groups, functional connectivity with the VS seed correlated positively with activation in regions including the left parahippocampal gyrus and postcentral gyrus, right frontal pole, and right inferior temporal gyrus (see **Table 2.4**). Further GLM analysis of the correlation

between drinks per drinking day and connectivity between the VS seed and these regions (controlling for baseline drinks per drinking day) revealed this association to be significant across groups (R^2=0.5351, p<0.001) and in the placebo group (R^2=0.7363, p<0.001), but not in the IBUD group (R^2=0.09506, p>0.05) (see **Figure 2.3**). These associations in the placebo and ibudilast groups were significantly different from each other (p<0.005).

Exploratory Analyses

PPI analyses from alternative seeds – DS, ACC, PCC, and precuneus – were conducted. Of these, only functional connectivity from the DS seed showed an effect of IBUD. Specifically, in comparison to placebo, IBUD reduced functional connectivity from the DS to the right temporal pole and middle temporal gyrus (see **Figure 2.4** and **Table 2.5**). Whole-brain results were thresholded using cluster-corrected statistics with a height-threshold of $Z > 2.3$ and cluster-forming threshold of $p < 0.05$. This attenuation of functional connectivity from the DS seed did not show a significant correlation with drinks per drinking day.

Discussion

This study investigated the effects of ibudilast on temporally correlated activation to visual alcohol cues (i.e. cue-elicited functional connectivity) from a VS seed in a sample of individuals with current AUD. Functional connectivity analyses were employed to further our understanding of the effects of ibudilast on neural responses to alcohol cues.

Consistent with results from the main study, which found that IBUD diminished alcohol cue-reactivity in the VS ROI, IBUD was also found to reduce correlation in activity between the VS seed and frontal regions including the orbitofrontal cortex (OFC) and anterior cingulate cortex (ACC). These brain regions are heavily implicated in reward processing, decision-making, and selective attention (Volkow et al., 2011). Disrupted function in the OFC is a characteristic of addiction broadly and of AUD in particular (Moorman, 2018). Given the OFC's primary role in controlling flexible, goal-directed behavior and its association with reward identification and acquisition, this region is implicated in regulating alcohol seeking in AUD. Of interest within the context of the current study, preclinical and clinical studies have shown cellular correlates of neuroinflammation in the OFC in both humans with AUD (Vetreno et al., 2013) and animals with chronic alcohol exposure (Qin and Crews, 2012), making this region a salient target for neuroimmune modulators like ibudilast. The ACC is strongly implicated in the experience of craving (Goldstein and Volkow, 2002), with human neuroimaging studies indicating that BOLD signal in the ACC increases in response to alcohol cues (Grüsser et al., 2004; Heinz et al., 2007). Individuals with AUD also have greater glutamate levels within the ACC than healthy controls, and ACC glutamate levels were shown to be significantly reduced by acamprosate (Frye et al., 2016; Umhau et al., 2010), another medication for AUD that may also act through neuroimmune mechanisms (Germany et al., 2018). Studies suggest that ibudilast may work similarly to protect against the hyper-glutamatergic state and maintain glutamate homeostasis in the brain (Bachtell

et al., 2017; Tominaga et al., 1996). Animal (Johansson et al., 2012) and human (Pérez-Torres et al., 2000) studies show that PDE4A, B, and D are well-expressed in the cingulate and frontal cortices, indicating that IBUD may inhibit PDE throughout this reward-processing circuit. Additionally, research shows that subjective craving is correlated with alcohol cue-induced functional connectivity between the VS and regions including the OFC and ACC (Strosche et al., 2021); therefore, diminishing these connections through ibudilast may facilitate the inhibition of reward processing and craving, and ultimately a reduction in alcohol use.

To further examine this connection between brain and behavior, we conducted an exploratory analysis of associations between cue-elicited functional connectivity and drinks per drinking day in the week following the fMRI scan. Across medication groups, drinks per drinking day was positively correlated with alcohol cue-elicited functional connectivity from the VS seed. Further probing of this association revealed that brain areas in which this correlation was stronger in the placebo group than in the IBUD group included similar reward-processing regions. These results indicate that IBUD's effects on reducing functional connectivity were indeed beneficial, as it also reduced drinks per drinking day during the two-week trial. Additionally, recency of drinking was considered as a variable. The IBUD and placebo groups did not differ significantly on recency of drinking ($p>0.05$). When included as a covariate in the models, recency of drinking did not significantly impact the results, and therefore was not included in the final model.

In order to probe the specificity of the effects seen from the VS seed, we conducted exploratory PPI analyses from alternative seeds, including the DS, ACC, PCC, and precuneus. Of these exploratory analyses, only the DS seed showed an IBUD-associated reduction in functional connectivity, and did not predict drinks per drinking day. These results indicate that the findings from the VS seed were relatively specific, especially those that correlated with

drinks per drinking day. However, it is worth noting that IBUD's effects on alcohol-induced functional connectivity from the VS may not be the only mechanism underlying the effects of IBUD on drinking outcomes.

This study has several strengths and limitations that should be considered in evaluating its findings. It is strengthened by the combination of neurobiological and self-reported behavioral (real-world drinking) outcomes. Another strength lies in the utilization of psychophysiological interaction (PPI) analyses to explore functional connectivity, allowing us to visualize effects of IBUD on broader reward processing circuitry beyond the ventral striatum itself. However, as mentioned in the main paper, the study is limited by its modest neuroimaging sample size, as well as its recruitment of a non-treatment-seeking sample, meaning that these results may not generalize to a sample of treatment-seeking participants (Ray et al., 2017b). An ongoing randomized controlled trial of IBUD (NCT03594435) aims to expand these results to a larger sample of treatment-seeking individuals with AUD. Additionally, IBUD's actions on PDE have the potential to result in vascular effects. In order to probe these effects, blood pressure was collected at every in-person visit. However, the IBUD and placebo groups did not differ significantly on systolic or diastolic blood pressure at either baseline or at the scan visit, nor did either group's blood pressure at the scan visit differ significantly from their baseline blood pressure ($p>0.05$). Finally, this study was conducted in a relatively high-functioning outpatient sample with mild-to-severe AUD, which may have limited our ability to detect effects of IBUD on pathologies associated with greater AUD severity levels. The ongoing trial in treatment-seekers may serve to address this outstanding question as well, as treatment-seeking populations tend to report a greater number of AUD symptoms and consume more drinks per drinking day (Ray et al., 2017b). Severity of AUD and overall brain pathology may be particularly relevant

given that ibudilast has been studied for a host of brain-based biomarkers in clinical trials for multiple sclerosis (Fox et al., 2018; Naismith et al., 2021).

Ibudilast's effects are hypothesized to be mediated by its effects on neuroinflammation and brain volume and structural integrity (Mizuno et al., 2004). Therefore, while beyond the scope of the current paper, future studies associating neural effects of ibudilast with inflammatory markers, as well as work probing possible long-term effects of ibudilast on brain morphometry, are warranted.

The use of neuroimaging in medications development continues to evolve (Grodin and Ray, 2019). Understanding the neurobiological mechanisms of action of novel pharmacotherapies represents an important aspect of medications development, especially when these biological findings are paired with disorder-related behavioral outcomes (i.e. drinking outcomes as in the case of the current study), representing a window into the clinical utility of neuroimaging in the development of pharmacological treatments. This study's combination of a functional connectivity analysis based on an original *a priori* ROI analysis extends previous research to explore the actions of IBUD beyond a single region, showing its attenuation of functional connectivity throughout a reward-processing circuit including the ventral striatum, orbitofrontal cortex, and anterior cingulate cortex. Furthermore, the current study supports the primary neuroimaging finding from the main clinical trial – i.e. that IBUD diminishes VS reactivity to visual alcohol cues and that this effect is associated with drinking outcomes – and expands on these findings to give an early proof of mechanism that IBUD's effects on drinking outcomes may be specifically related to effects in broader frontostriatal neural circuitry related to reward processing.

Table 2.1. Demographic and Clinical characteristics (collected at baseline screening visit) of ibudilast and placebo medication groups within the sample and comparisons between the groups.

Variable	**Ibudilast (n=20)**	**Placebo (N=25)**	**Comparison**
Age	34.40 ± 9.67	31.00 ± 7.49	$T = -1.293$ $p = 0.205$
Gender, No. (%)			
Male	13 (65%)	16 (64%)	$X^2 = 0.001$ $p = 0.973$
Female	7 (35%)	9 (36%)	
Race/Ethnicity, No. (%)			
White	14 (70%)	11 (44%)	$X^2 = 8.577$ $p = 0.127$
African-American	4 (20%)	2 (8%)	
Asian	0 (0%)	4 (16%)	
Pacific Islander	0 (0%)	1 (4%)	
Mixed Race	1 (5%)	5 (20%)	
Another Race	1 (5%)	2 (8%)	
Hispanic or Latino	5 (25%)	6 (24%)	
Years of Education	15.30 ± 2.79	15.28 ± 1.72	$T = -0.075$ $p = 0.940$
Cigarette Smokers, No. (%)	10 (50%)	15 (60%)	$X^2 = 0.492$ $p = 0.483$
Baseline THC+, No. (%)	7 (35%)	6 (24%)	$X^2 = 0.228$ $p = 0.633$
AUD Severity	0/4/9/7	1/6/13/5	$X^2 = 1.929$ $p = 0.587$

PACS Total Score	12.50 ± 5.52	11.60 ± 6.96	$T = 0.484$ $p = 0.631$
OCDS Total Score	19.35 ± 8.00	18.00 ± 9.88	$T = 0.506$ $p = 0.615$
ADS Total Score	13.20 ± 6.59	11.40 ± 6.57	$T = 0.911$ $p = 0.368$
CIWA-Ar Total Score	0.75 ± 1.74	0.44 ± 1.08	$T = 0.695$ $p = 0.492$
AUDIT Total Score	16.70 ± 6.30	16.40 ± 6.26	$T = 0.159$ $p = 0.874$
Drinking Days (30-day baseline)	21.25 ± 7.00	19.96 ± 6.30	$T = 0.642$ $p = 0.525$
Drinks per Day (30-day baseline)	4.02 ± 2.21	3.85 ± 3.77	$T = 0.196$ $p = 0.846$
Drinks per Week (30-day baseline)	28.19 ± 15.49	26.97 ± 26.41	$T = 0.196$ $p = 0.846$
Drinks per Drinking Day (30-day baseline)	5.91 ± 2.72	5.45 ± 3.85	$T = 0.461$ $p = 0.647$

Figure 2.1. Whole-brain analysis clusters, IBUD<PLAC, Ventral Striatum Seed. PPI analyses indicating functional connectivity from ventral striatum seed during ALC>BEV contrast in regions where functional connectivity was lower in the IBUD group than the placebo group (see **Table 2.2** for list of clusters). Color bar represents z-values. Whole-brain results are thresholded at z > 2.3, cluster-forming threshold of $p<0.05$. Brain maps are displayed in radiological convention (right = left).

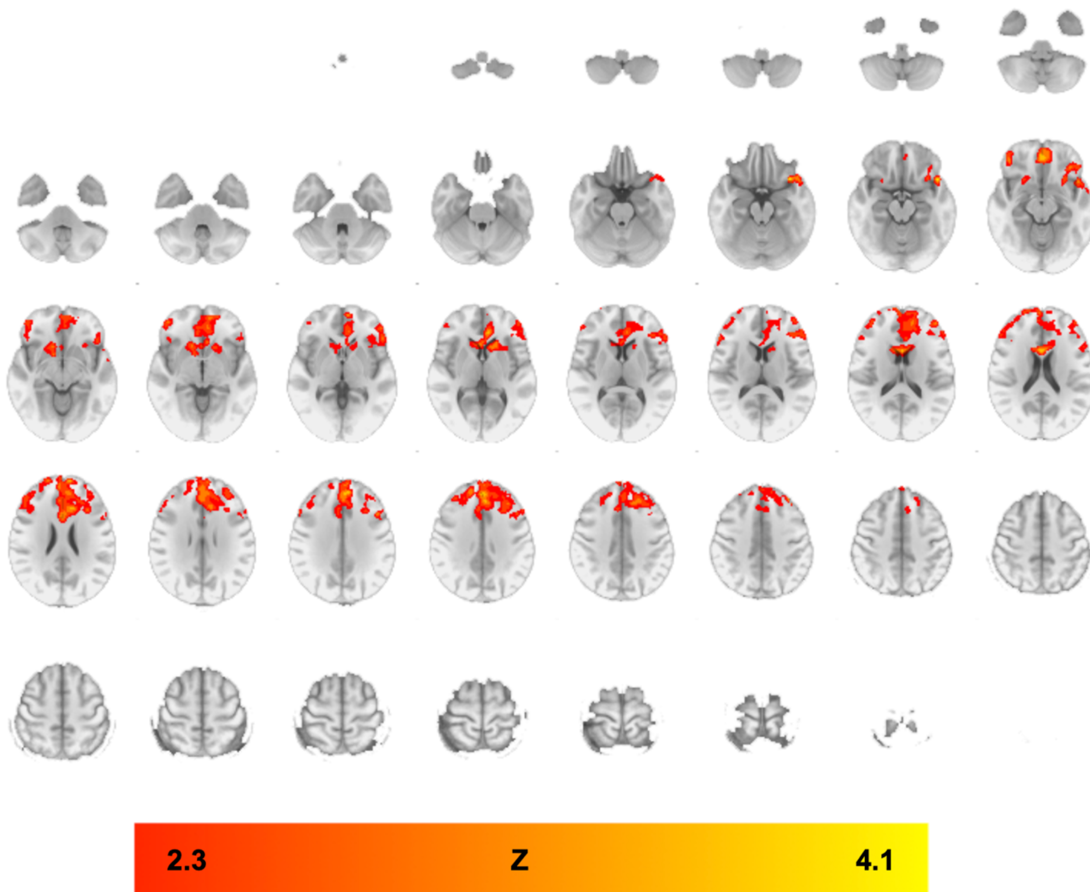

Table 2.2. Significant clusters for psychophysiological interaction analyses using the Alcohol > Beverage contrast, IBUD<PLAC, Ventral Striatum seed. Z-statistic maps were thresholded using cluster-corrected statistics with a height-threshold of Z > 2.3 and cluster-forming threshold of p < 0.05. All coordinates are in MNI space.

Brain Region	**Cluster Voxels**	**Max Z-statistic**	**x**	**y**	**z**
Placebo > Ibudilast – PPI during Alc>Bev; VS Seed					
L Orbitofrontal Cortex	9599	4.13	-46	18	-16
Bilateral Anterior Cingulate		3.8	0	14	18
R Medial Frontal Cortex		3.68	2	46	-12
L Superior Frontal Gyrus		3.65	-6	46	32

Figure 2.2. Drinks per Drinking Day whole-brain analysis clusters, IBUD<PLAC, Ventral Striatum Seed. PPI analyses indicating functional connectivity from ventral striatum seed during ALC>BEV contrast with drinks per drinking day as a covariate, in regions where functional connectivity was lower in the IBUD group than the placebo group (see **Table 2.3** for list of clusters). Color bar represents z-values. Whole-brain results are thresholded at $z > 2.3$, cluster-forming threshold of $p<0.05$. Brain maps are displayed in radiological convention (right = left).

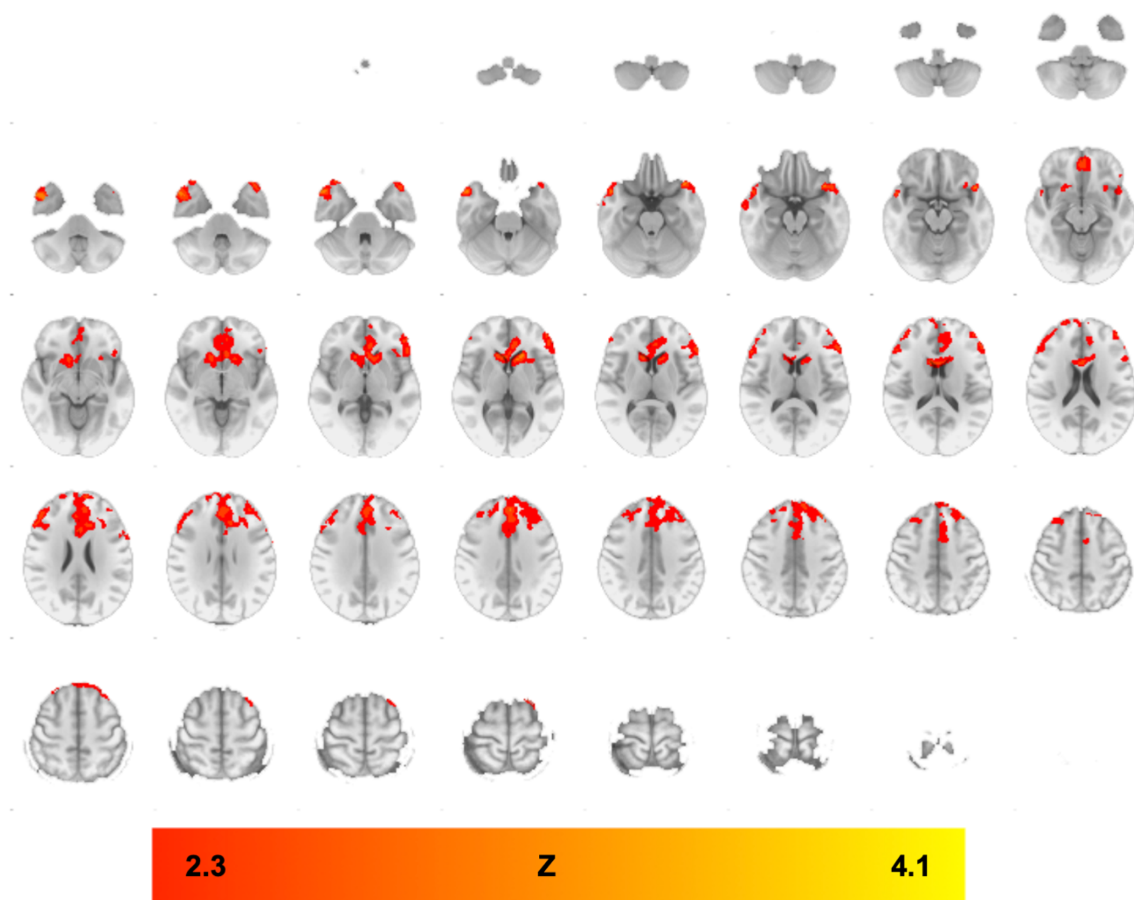

Table 2.3. Significant clusters for psychophysiological interaction analyses with drinks per drinking day as a covariate using the Alcohol > Beverage contrast, IBUD<PLAC, Ventral Striatum Seed. Z-statistic maps were thresholded using cluster-corrected statistics with a height-threshold of $Z > 2.3$ and cluster-forming threshold of $p < 0.05$. All coordinates are in MNI space.

Brain Region	Cluster Voxels	Max Z-statistic	x	y	z
Placebo > Ibudilast – PPI during Alc>Bev x DPDD; VS Seed					
L Orbitofrontal Cortex	8477	4.1	-38	20	-20
L Caudate		4.01	-18	24	4
L Temporal Pole		3.91	-44	24	-24
Bilateral Superior Frontal Gyrus		3.7	0	44	36
Bilateral Anterior Cingulate		3.63	-6	36	6
R Caudate		3.62	12	22	6
R Temporal Pole	828	4.07	46	8	-38

Table 2.4. Significant clusters for psychophysiological interaction analyses with drinks per drinking day as a covariate using the Alcohol > Beverage contrast, across groups. Z-statistic maps were thresholded using cluster-corrected statistics with a height-threshold of $Z > 2.3$ and cluster-forming threshold of $p < 0.05$. All coordinates are in MNI space.

Brain Region	**Cluster Voxels**	**Max Z-statistic**	**x**	**y**	**z**
Mean functional connectivity across groups – PPI during Alc>Bev x DPDD; VS Seed					
L Parahippocampal Gyrus	2368	5.12	22	-16	-26
L Postcentral Gyrus	2050	4.63	-6	-40	72
R Frontal Pole	1162	4.52	24	48	-20
R Inferior Temporal Gyrus	952	4.74	48	-32	-26

Figure 2.3. Correlation between functional connectivity from Ventral Striatum seed and Drinks per Drinking Day. Activation profile (percent signal change) within clusters showing correlated activation from VS seed vs. Drinks per Drinking Day in the last week of the trial (controlling for baseline drinks per drinking day. Across groups (green): R^2=0.5351, p<0.001; IBUD (blue): R^2=0.09506, p>0.05: Placebo (yellow): R^2=0.7363, p<0.001.

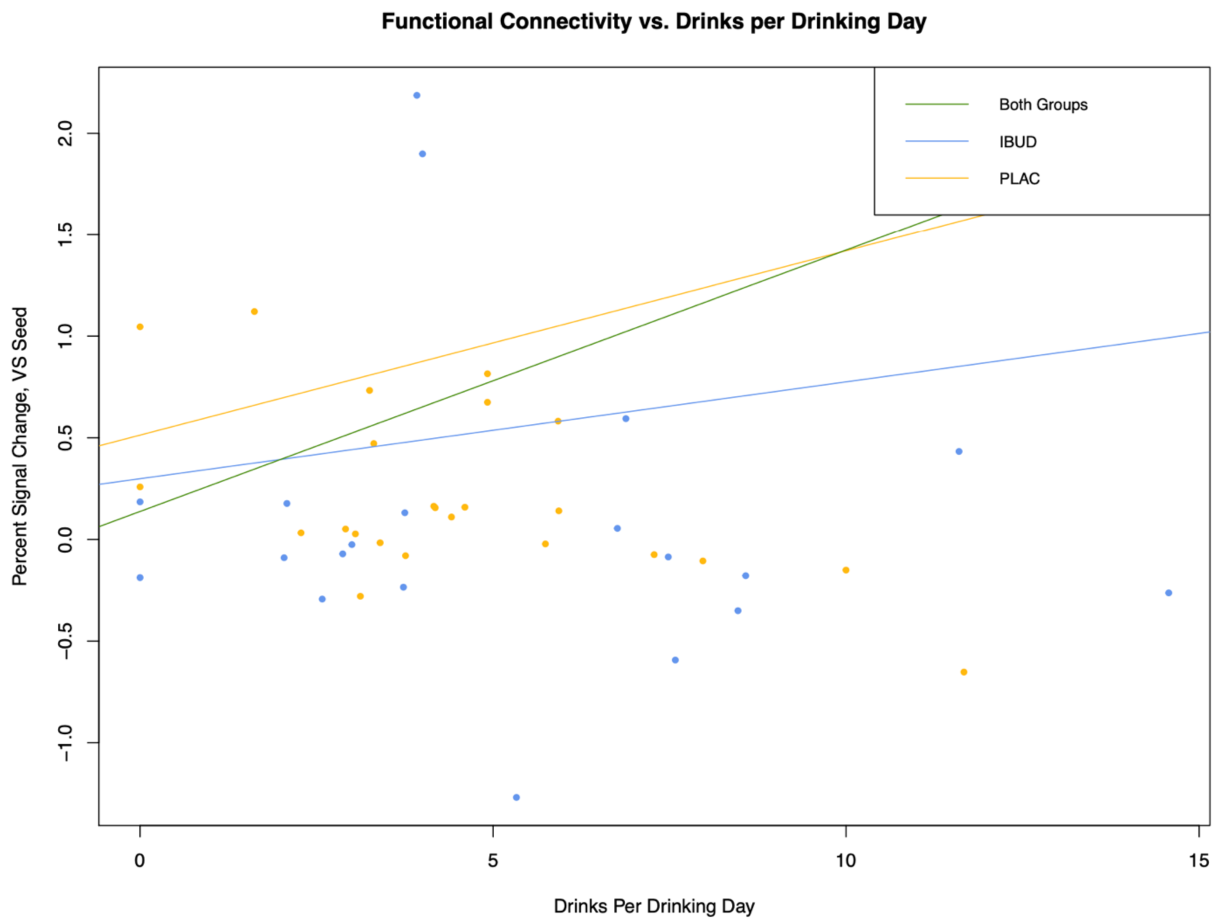

Figure 2.4. Whole-brain analysis clusters, IBUD<PLAC, Dorsal Striatum Seed. PPI analyses indicating functional connectivity from dorsal striatum seed during ALC>BEV contrast in regions where functional connectivity was lower in the IBUD group than the placebo group (see **Table 2.5** for list of clusters). Color bar represents z-values. Whole-brain results are thresholded at z > 2.3, cluster-forming threshold of p<0.05. Brain maps are displayed in radiological convention (right = left).

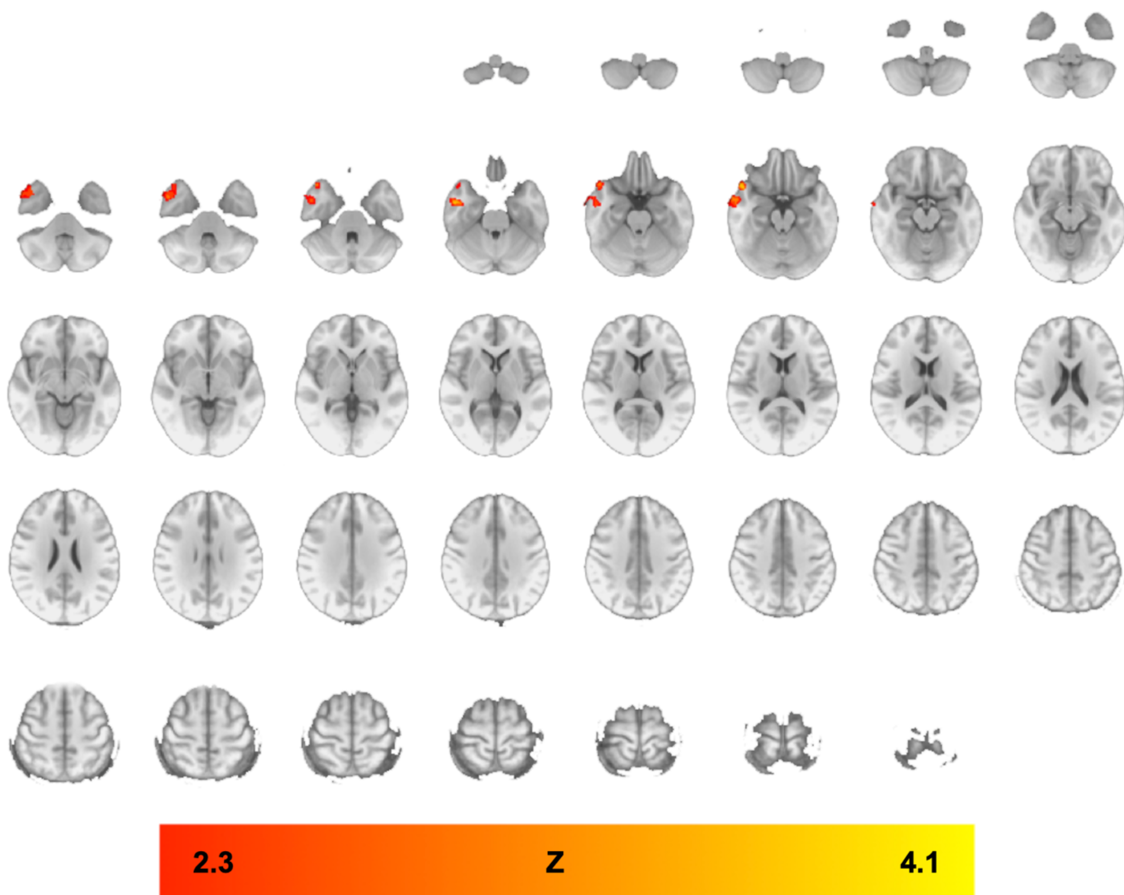

Table 2.5. Significant clusters for psychophysiological interaction analyses using the Alcohol > Beverage contrast, IBUD<PLAC, Dorsal Striatum seed. Z-statistic maps were thresholded using cluster-corrected statistics with a height-threshold of Z > 2.3 and cluster-forming threshold of p < 0.05. All coordinates are in MNI space.

Brain Region	**Cluster Voxels**	**Max Z-statistic**	**x**	**y**	**z**
Placebo > Ibudilast – PPI during Alc>Bev; DS Seed					
R Temporal Pole	781	3.68	58	12	-42
Middle Temporal Gyrus		3.24	66	6	-34

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CHAPTER 3

ALCOHOL USE DISORDER IS ASSOCIATED WITH ENHANCED SENSITIVITY TO CELLULAR LIPOPOLYSACCHARIDE CHALLENGE

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Abstract

Background: Inflammation is implicated in alcohol use disorder (AUD). A novel method to characterize AUD-related immune signaling is by evaluating Toll-like receptor (TLR)-4 stimulated monocyte production of intracellular cytokines (ICCs) in response to lipopolysaccharide (LPS). This study evaluated relationships between AUD and levels of monocyte ICC production at rest and in response to LPS.

Methods: This secondary analysis used blood samples from 36 participants (AUD N=14; Controls N=22), collected across five timepoints, with assessment of monocyte expression of ICC at rest and after LPS stimulation (10 repeated measures/participant). Proinflammatory markers of interest included tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), co-expressing TNF-α and IL-6 monocytes, and interferon (IFN). For each marker, linear mixed models were constructed with AUD status, LPS status, and time point as fixed effects (BMI as a covariate), allowing for random slope and intercept. AUD × LPS was included as an interaction term.

Results: For TLR4 stimulated monocyte production of TNF-α, there were effects for AUD ($p < 0.01$), LPS ($p < 0.001$), and AUD × LPS interaction ($p < 0.05$), indicating that individuals with AUD showed greater resting levels of monocyte expression of TNF-α and also greater TLR4 stimulated monocyte production of TNF-α. Similarly, for TLR4 stimulated monocyte co-expression of TNF-α and IL-6, there were effects for AUD ($p < 0.01$), LPS ($p < 0.001$), and interaction ($p < 0.05$). No AUD or LPS effects were found for TLR4 stimulated production of IL-6. Time point effects were also observed on IL-6 and TNF-α / IL-6 co-expression ($p < 0.001$). Finally, for TLR4 stimulated monocyte production of IFN, AUD ($p < 0.05$), LPS ($p < 0.001$), and AUD × LPS ($p < 0.001$) effects were found.

Conclusions: In individuals with AUD, resting levels of intracellular monocyte expression of TNF-α and co-expression of IL-6 and TNF-α were elevated as compared to controls. Additionally, AUD was associated with increased TLR4 stimulated monocyte production of TNF-α, and co-production of IL-6 and TNF-α. This is, to our knowledge, the first study using this method to investigate the relationships between AUD and cellular production of proinflammatory cytokines, at rest and in response to TLR4 stimulation. This study extends previous preclinical and clinical findings on the roles of proinflammatory cytokines in AUD and serves as a critical proof-of-concept for the use of a novel method in probing the neuroimmune mechanisms underlying AUD.

Keywords: Alcohol Use Disorder, Inflammation, Cytokine, Lipopolysaccharide

Summary: This study evaluated relationships between AUD and toll-like receptor (TLR)-4 stimulated monocyte production of intracellular cytokines (ICCs) in response to lipopolysaccharide (LPS). Resting levels of intracellular monocyte expression of TNF-α and co-expression of IL-6 and TNF-α were elevated in individuals with AUD compared to controls. Additionally, individuals with AUD showed increased TLR4 stimulated monocyte production of TNF-α, and co-production of IL-6 and TNF-α. Findings indicate that AUD is associated with enhanced sensitivity to inflammatory challenge.

Introduction

Inflammation has been implicated in the development and maintenance of alcohol use disorder (AUD), termed the neuroimmune hypothesis of AUD (Cui et al., 2011; Mayfield and Harris, 2017). In preclinical models, chronic alcohol exposure has been shown to increase both central and peripheral markers of inflammation (Mayfield et al., 2013, Crews et al., 2015). Preclinical research also indicates that inflammation heightens motivation for alcohol consumption, enhances alcohol-related reward, and contributes to substance use-related cognitive impairment and depression-like behavior (Alfonso-Loeches et al., 2010, Briones and Woods, 2013). In humans, post-mortem brain tissue of individuals with AUD shows increased levels of proinflammatory gene expression (He and Crews, 2008, Liu et al., 2006), and individuals with AUD have heightened levels of peripheral proinflammatory biomarkers relative to healthy controls (Achur et al., 2010, Adams et al., 2020). A prolonged or excessive proinflammatory response can have detrimental effects on health and, in populations with AUD, is suggested to contribute to compulsive alcohol intake and other AUD symptomatology (Cui et al., 2011; Leclercq et al., 2014; Lee et al., 2021).

Essential for survival, innate and adaptive immune mechanisms serve as the human body's primary defense against pathogens (Bonilla and Oettgen, 2010; Slavich and Irwin, 2014). When the innate immune system is activated, inflammatory responses are provoked by the detection of pathogen-associated molecular patterns (PAMPs) such as the bacterial ligand lipopolysaccharide (LPS). *In vitro* LPS stimulation has been shown to induce microglial expansion and increase microglial TSPO binding, a clinical marker of neuroinflammation used in positron emission tomography (PET) (Tournier et al., 2020). LPS also serves as a biomarker of AUD such that individuals with AUD are shown to have elevated LPS levels but may re-normalize after abstinence (Leclercq et al., 2012; Qin et al., 2008).

Toll-like receptors (TLRs) are widely implicated in neuroimmune signaling processes related to alcohol use (Meredith et al., 2021). Commonly found on immune cells, TLRs recognize PAMPs. When TLR4 is bound by LPS, activation of transcription factors, such as interferon (IFN) regulatory factors, nuclear factor-κB (NF-κB), and cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) (Aurelian et al., 2016; Balan et al., 2018). These activated transcription factors drive the expression of proinflammatory cytokines, immune protein molecules released by immune cells. Cytokines coordinate inflammatory cellular functions and, with the ability to cross the blood-brain barrier (Banks et al., 1995), have been shown to affect physiological and behavioral responses (Dinarello, 2000).

Chronic ethanol consumption has been shown to result in increased levels of plasma cytokines such as tumor necrosis factor α (TNF-α) and interleukins IL-1β, IL-17 in wild-type mice (Pascual et al., 2015). Mice with TLR system (i.e., TLR4, TLR2) knockouts were protected from these effects, however, providing evidence in support of the TLR system's importance in alcohol-related neuroinflammation (Crews et al., 2017). In humans, a recent meta-analysis of 17 clinical studies (Adams et al., 2020) found increased cytokine concentrations (e.g., IL-6, TNF-α, IL-8) among individuals with AUD compared to healthy controls; these abnormalities were more prominent during active drinking and acute withdrawal periods compared to periods of early or prolonged abstinence. In sum, preclinical and clinical evidence indicate that the immune and neuroimmune system is related to AUD symptomatology, but specific mechanisms remain unclear.

A novel method to characterize mechanisms of AUD-related immune signaling is to probe monocyte production of intracellular cytokines (ICCs) at rest and following *in vitro* TLR4 stimulation with LPS. This method provides insight into the source of systemic inflammation, independent of extracellular levels (Cho et al., 2019). Monocytes comprise approximately 5% of

circulating leukocytes and are a major contributor to proinflammatory cytokine production in peripheral blood (O'Connor et al., 2007). The acute inflammatory state induced by LPS stimulation is thought to be reflective of stress, as physiological and psychological stressors both activate inflammatory processes (Black, 2002), and TLR4-induced ICC expression reflects the inflammatory responsivity of cells to these stressors (Bale, 2006). Whereas higher levels of TLR4 stimulated production of TNF-α has been found to correlate with depression symptom severity, (Suarez et al., 2004, 2003), no study to our knowledge yet used this method to examine the associations between alcohol use and ICC response to LPS challenge, even though systemic inflammation is reported to occur in AUD.

The current study evaluated relationships between alcohol use disorder and monocyte intracellular cytokine production following ligation of the TLR4 receptor with LPS. Proinflammatory cytokines of interest included tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interferon (IFN). Participants with AUD were hypothesized to show higher resting levels of monocyte intracellular expression of the inflammation, and also to show greater response to TLR4 stimulation with LPS, consistent with an interaction between AUD and LPS on monocyte production of inflammatory markers.

Materials and Methods

The present study was a secondary analysis of data and blood samples from a study designed to assess associations between sleep deprivation and monocyte ICC expression. Detailed methodology of the experimental procedures has been published previously (Irwin et al., 2006).

Participants

Participants included 36 volunteers (AUD N=14; Controls N=22). Inclusion criteria required that participants be healthy by medical screening interview and physical examination; none had a history of inflammatory disorder, cancer, or chronic / active infections. Subjects were 70% male, between the ages of 25 and 55, and had an average body mass index (BMI) of 25.35 $kg/m^2 \pm 4.46\ kg/m^2$. Sample demographics broken down by AUD status can be found in **Table 3.1.** Subjects in the AUD group had a DSM-IV diagnosis of current, primary alcohol dependence with or without secondary depression and no other primary affective or other psychiatric disorders; controls were diagnosed as having no history of any major psychiatric disorder via structured clinical interview for DSM-IV (American Psychiatric Association, 1994). Participants in the AUD group were required to have been abstinent for between 14 and 21 days. Exclusion criteria included suicide risk, immunosuppression from neoplastic disease, corticosteroids, or other immunosuppressive therapy, and use of psychotropic or anti-hypertensive medications.

Participants were assessed for alcohol and nicotine use via the clinician-administered Semi-Structured Assessment for Genetics of Alcoholism (SSAGA) (Bucholz et al., 1994). Depression symptomatology was also assessed via the Beck Depression Inventory (BDI-II) (Beck et al., 1996), Hamilton Depression Rating Scale (HRS-D) (Hamilton, 1960), and the

depression subscale of the Profile of Mood States (POMS) (McNair et al., 1971). Alcohol use and depression symptomatology statistics can be found in **Table 3.2.**

Procedures

Sample Collection

Blood samples were collected from participants across five timepoints (0800, 1200, 1600, 2000, and 2300) over the span of a 15-hour period via an indwelling venous forearm catheter. Samples were assessed for expression of intracellular proinflammatory cytokine production in peripheral blood mononuclear cell (PBMC) populations at rest and after stimulation with LPS for a total of 10 repeated measures per participant (5 unstimulated timepoints, 5 stimulated timepoints), or approximately 360 total observations.

Intracellular monocyte assay

Monocyte intracellular cytokine production in response to whole-blood LPS stimulation was assessed by flow cytometry using peridinin chlorophyll protein (PerCP)-labeled CD14 mAb and phycoerythrin (PE)-labeled anti-IL-6 Ab, as previously described (Collado-Hidalgo et al., 2006; Irwin et al., 2006; Prussin and Metcalfe, 1995). In brief, heparin-treated blood (1mL) was mixed with 100 pg/mL of LPS (Sigma, St. Louis, MO) and 10 μg/mL brefeldin A (Sigma, St. Louis, MO) and incubated for 4 h at 37°C in a platform mixer followed by an overnight incubation at 4°C. Red blood cells were lysed in FACS lysing solution (BD Biosciences, San Jose, CA), remaining cells were permeabilized in FACS permeabilizing buffer (BD Biosciences, San Jose, CA), and fluorescence-conjugated antibodies were added for 30 min at room temperature in the dark. Cells were then washed and resuspended in 1% paraformaldehyde for flow cytometry. Three-color flow cytometric analysis was conducted on a Coulter Elite flow

cytometer using Coulter Elite software. Forward and side scatter were used to gate on the target population (on the population consisting of monocytes and granulocytes). For the monocyte population, the percentage of cytokine-secreting (PE positive) cells among CD14-PerCP-positive population was determined by counting about 12,000 CD14+ events. Proinflammatory cytokines of interest included tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), TNF-α / IL-6 co-expressing monocytes, and interferon (IFN). Results for cytokine-positive monocytes were expressed as percentages of the total CD14+ cells. For the purposes of this study, "unstimulated" refers to this percentage within resting whole blood; "stimulated" refers to the total level of percentage cytokines expressed after stimulation with LPS.

Statistical Analysis

Data were analyzed using R statistical software (RStudio 1.2.5001). For each marker of interest, a linear mixed model was constructed with AUD status (i.e. AUD vs. control), LPS stimulation status (i.e. unstimulated vs. stimulated), and time point (i.e. hour 08, 12, 16, 20, or 23) as fixed effects, along with BMI as a covariate, allowing for random slope and intercept based on participant effects. AUD × LPS Stimulation status was included as an interaction term. Four individual models were run, one for each biomarker of interest. We did not correct for multiple comparisons, due to the exploratory nature of the study.

Results

Participant Demographics and Clinical Variables

AUD and control groups did not significantly differ on demographic variables (see **Table 3.1**), other than current smoking status [$X^2 = 17.039$; $p < 0.001$]. Participants in the AUD group reported consuming a total of 556.38 (SD: 601.63) drinks over the last 3 months, or an average of 11.67 (SD: 9.18) drinks per day; participants in the control group reported consuming a total of 16.18 (SD: 33.68) drinks in the last 3 months [t=-3.234; $p < 0.01$], or an average of 0.70 (SD: 0.98) drinks per day [t=-4.124; $p < 0.01$] (see **Table 3.2**).

Participants with AUD had significantly greater levels of depressive symptomatology than controls, as assessed by the BDI-II [t=-4.439; $p < 0.001$], HRS-D [t=-3.189; $p < 0.01$], and POMS [t=-2.188; $p < 0.05$]; both groups remained, on average, under clinical thresholds for depressive symptoms (see **Table 3.2**). Due to collinearity between AUD diagnosis and depression symptomatology, as well as the lack of clinical significance, these measures were not included as factors in the mixed model analysis.

Linear Mixed Models

Results from the linear mixed models for each inflammatory marker of interest are presented in **Table 3.3**. For monocyte expression of TNF-α (**Figure 3.1**), effects of AUD ($p < 0.01$), LPS ($p < 0.001$), and an interaction effect between alcohol and LPS stimulation ($p < 0.05$) were seen, such that individuals with an AUD had greater resting monocyte expression of TNF-α levels and also greater TLR4 stimulated monocyte production of TNF-α. While no AUD or AUD × LPS effects were seen for IL-6 alone (**Figure 3.2**), there was an effect of AUD ($p < 0.01$), LPS ($p < 0.001$), and AUD x LPS interaction ($p < 0.05$) on the levels of TNF-α and IL-6 co-expressing monocytes (**Figure 3.3**), driven by the effects seen in the TNF-α model alone. Of

note, a diurnal pattern of expression was seen for both IL-6 and TNF-α / IL-6 co-expressing monocytes (driven by the effects seen in the IL-6 model), with a significant effect of time point ($p < 0.001$) on both models. Finally, similar AUD ($p < 0.05$), LPS ($p < 0.001$), and AUD × LPS ($p < 0.001$) effects were found on IFN levels (**Figure 3.4**).

Discussion

This study investigated the relationship between alcohol use disorder and monocyte intracellular cytokine production following a cellular inflammatory challenge with lipopolysaccharide. As hypothesized, alcohol use disorder status (i.e., AUD versus controls) and LPS challenge were both associated with higher levels of proinflammatory markers. Importantly, interaction effects between these two factors were found, such that LPS ligation of TLR4 yielded greater monocyte expression of TNF-α and IFN in blood samples from participants with AUD compared to controls, indicating that AUD was associated with enhanced sensitivity to cellular LPS challenge.

Chronic alcohol consumption is associated with increased TNF-α levels in rodents and humans (Heberlein et al., 2014; Leclercq et al., 2014); elevated TNF-α levels are also correlated with liver dysfunction and can be used as an early indicator of alcohol-associated hepatitis (Gonzalez-Quintela et al., 2008). Alcohol intake has also been shown to promote a systemic proinflammatory IFN response in mice resulting from chronic ethanol exposure and subsequent alcohol dependence (Frank et al., 2020). Previous studies on alcohol's effects on IFN provide inconsistent conclusions, with some studies showing alcohol consumption leading to increased IFN levels and others showing the opposite (Laso et al., 1999; Song et al., 2002; Starkenburg et al., 2001; Zhang et al., 2015). In the present study, stimulated levels of IFN across AUD groups

were much lower than that of TNF-α and IL-6 results. Although there was no overlap between unstimulated and stimulated ICC levels, it was also apparent that only a small percentage of stimulated monocytes expressed IFN. While monocytes can express IFN, this cytokine mainly comes from T-cells (Pang et al., 2011; Parmar and Platanias, 2003), and as such, LPS may not be the best stimulus to affect IFN expression, especially in monocytes.

IL-6 is well-studied as an inflammatory marker in psychiatric disorders. Previous studies, especially in the depression literature, have demonstrated effects of LPS stimulation on IL-6 (Cho et al., 2019; Irwin et al., 2006). In the alcohol field, IL-6 has been emphasized as an important marker associated with chronic alcohol exposure (Moura et al., 2022) and withdrawal (Gruol et al., 2018; Roberts et al., 2019). Therefore, it was counter to our original hypothesis that there was neither a main effect of AUD nor an AUD × LPS stimulation effect on IL-6 observed in our study. Our results indicate that IL-6 was more sensitive to time course than other markers of interest, showing a distinct diurnal pattern of expression (Vgontzas et al., 2005). We hypothesize that due to the limited sample size, possible IL-6 effects may have been affected by the marker's sensitivity to time course. Although timepoint × alcohol interactions may be interesting to probe, especially considering the observed diurnal pattern of IL-6 expression, our statistical model did not converge due to lack of power for the inclusion of these additional multiple comparisons. We suggest that a larger sample size be used to study potential interactions between timepoint, alcohol, and LPS stimulation. Importantly, previous work has also shown that serum IL-6 levels decrease steeply in early withdrawal (over the course of 14 days of abstinence from alcohol), declining to a level non-significantly differentiable from controls by day 14. In comparison, TNF-α levels were shown to remain at an elevated state throughout withdrawal (Heberlein et al., 2014). As our participants were required to have abstained from alcohol for at least 14 days, it is possible that they had experienced a similar level

of recovery in IL-6 effects. In particular, these findings speak to the effects of protracted withdrawal on inflammatory markers, as compared to the acute alcohol withdrawal phase.

This study has several strengths and limitations that should be considered when interpreting its results. Study strengths include the probing of monocyte production of ICCS following LPS stimulation, which captures an acute cellular immune response and reduces extracellular background. Strengths also include demographically comparable AUD and control groups, and multiple repeated measures within subjects. We considered including depression symptomology in our analysis; however, such analyses were limited both by depression symptoms being highly collinear with alcohol use status in our sample and by our sample on average not reaching clinical significance on depressive symptom metrics. In future studies, we recommend investigating the relationships between alcohol use, inflammation, and depression with the inclusion of participants who may have AUD but not clinically significant depressive symptoms as well as participants who may have clinical depression but not AUD. As mentioned above, participants in this sample were required to have abstained from alcohol for 14-21 days. Therefore, these participants may have already experienced some recovery of baseline inflammatory markers and were likely in a state of protracted withdrawal (Heberlein et al., 2014). It is likely that greater baseline effects may be seen in participants who were actively drinking and/or undergoing acute withdrawal, as opposed to protracted withdrawal, and we suggest that future studies include such a population. Finally, previous work has shown sex differences in LPS challenge-induced monocyte cytokine production (O'Connor et al., 2007). However, the current study was underpowered to examine sex effects. Future studies with a greater sample size should explore these effects.

In conclusion, this is, to our knowledge, the first study to investigate the relationships between alcohol use disorder and monocyte ICC production in response to cellular LPS

challenge. This study extends previous preclinical and clinical findings on the roles of proinflammatory cytokines in AUD. Main effects of AUD and LPS stimulation, as well as AUD × LPS stimulation interaction effects, were observed on monocyte intracellular expression of TNF-α, IFN, and TNF-α / IL-6 co-expression indicating elevated levels of cellular inflammation at rest and response to TLR4 activation in AUD. In other words, monocytes from individuals with AUD are more sensitive to inflammatory challenge than those of controls. Insofar as the cellular LPS challenge mimics a stress response, these findings suggest that individuals with AUD may mount a more robust inflammatory response to systemic stress than healthy controls without AUD or heavy drinking. This differential response, in turn, may render individuals with AUD more vulnerable to chronic alcohol use. These analyses serve as a critical proof-of-concept for the use of this novel method in probing the neuroimmune mechanisms underlying AUD.

Table 3.1. *Sample demographics, as separated by AUD vs. Control groups. ± indicates standard deviation. N.S. indicates p > 0.05.*

	AUD (N=14)	**Control (N=22)**	**Statistic**	**P-Value**
Gender (%M)	71%	68%	$X^2 = 0.028$	*N.S.*
Age (Mean ± SD)	37.31 ± 5.42	37.05 ± 9.36	t=-0.105	*N.S.*
Ethnicity - Asian	0%	18.2%	$X^2=6.592$	*N.S.*
Pacific Islander	0%	4.5%		
Black/AA, Non-Hispanic	28.5%	22.7%		
Black/AA, Hispanic	14.3%	4.5%		
White, Non-Hispanic	35.7%	36.4%		
White, Hispanic	14.3%	4.5%		
Other	7.1%	9.1%		
% Current Smoker	64.3%	0%	$X^2 = 17.039$	$\boldsymbol{p < 0.001}$
BMI	26.09 ± 3.03	24.92 ± 5.14	t= -0.854	*N.S.*

Table 3.2. *Alcohol use and depression clinical characteristics, as separated by AUD vs. Control groups. ± indicates standard deviation. N.S. indicates p > 0.05.*

	Alcohol (N=14)	**Control (N=22)**	**Statistic**	**P-Value**
Average Days / Month	22.57 ± 10.34	5.45 ± 14.06	t=-4.198	$p < 0.001$
Average Drinks / Day	11.67 ± 9.18	0.70 ± 0.98	t=-4.124	$p < 0.005$
Maximum Drinks / Day (last 3 months)	21.25 ± 13.40	1.50 ± 1.44	t=-5.269	$p < 0.001$
Total Drinks (last 3 months)	556.38 ± 601.63	16.18 ± 33.68	t=-3.234	$p < 0.01$
BDI-II Total	7.46 ± 4.47	1.65 ± 1.89	t=-4.439	$p < 0.001$
Hamilton Total	6.85 ± 6.20	1.19 ± 1.97	t=-3.189	$p < 0.01$
POMS - Depression Subscale	5.92 ± 8.10	0.90 ± 2.07	t=-2.188	$p < 0.05$

Table 3.3. *Mixed model results: alcohol use, LPS stimulation status, time point, BMI, alcohol × stimulation effects on inflammatory marker outcomes. N.S. indicates p > 0.05.*

Outcome	**TNF-α**	**IL-6**	**TNF/IL-6 Co-Expressing**	**IFN**
Alcohol	t=2.604 $\boldsymbol{p < 0.01}$	t=0.042 *N.S.*	t=3.005 $\boldsymbol{p < 0.01}$	t=2.607 $\boldsymbol{p < 0.05}$
LPS Stimulation	t=34.505 $\boldsymbol{p < 0.001}$	t=21.496 $\boldsymbol{p < 0.001}$	t=26.676 $\boldsymbol{p < 0.001}$	t=15.599 $\boldsymbol{p < 0.001}$
Time point	t=0.293 *N.S.*	t=7.111 $\boldsymbol{p < 0.001}$	t=5.493 $\boldsymbol{p < 0.001}$	t=0.563 *N.S.*
BMI	t=0.404 *N.S.*	t=0.813 *N.S.*	t=0.070 *N.S.*	t=0.952 *N.S.*
Alcohol × LPS Stimulation	t=1.978 $\boldsymbol{p < 0.05}$	t=1.219 *N.S.*	t=2.418 $\boldsymbol{p < 0.05}$	t=2.879 $\boldsymbol{p < 0.001}$

Figure 3.1. *Mixed model results predicting monocyte intracellular TNF-α expression. Yellow = control group; blue = AUD group. Circle points = unstimulated; triangle = stimulated.*

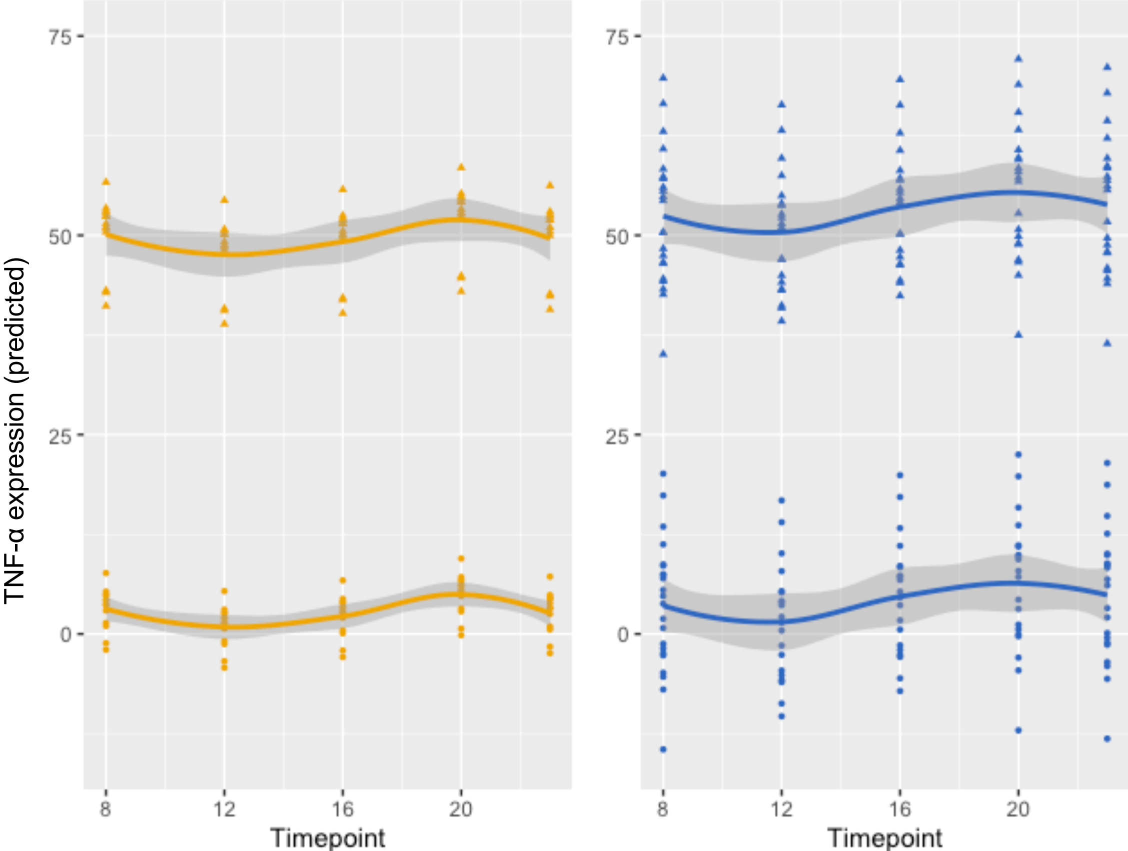


Figure 3.2. *Mixed model results predicting monocyte intracellular IL-6 expression. Yellow = control group; blue = AUD group. Circle points = unstimulated; triangle = stimulated.*

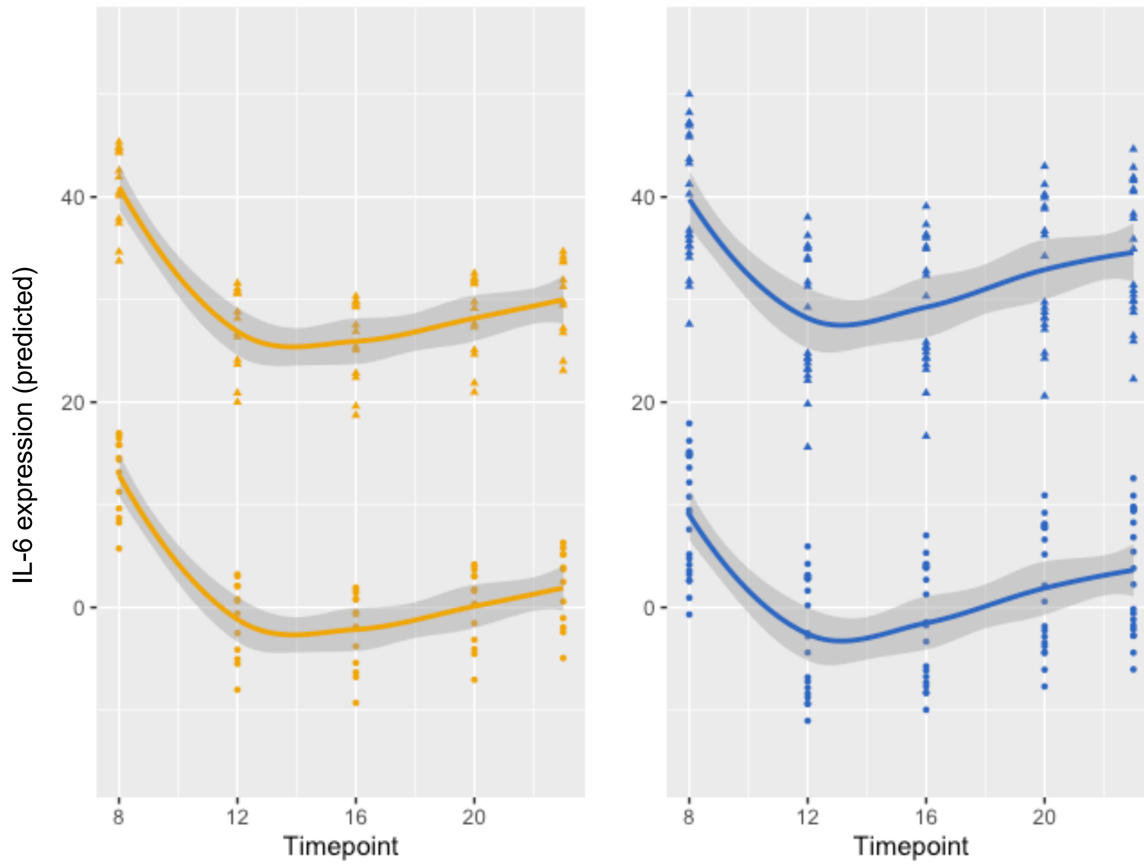


Figure 3.3. *Mixed model results predicting monocyte intracellular TNF-α / IL-6 co-expression. Yellow = control group; blue = AUD group. Circle points = unstimulated; triangle = stimulated.*

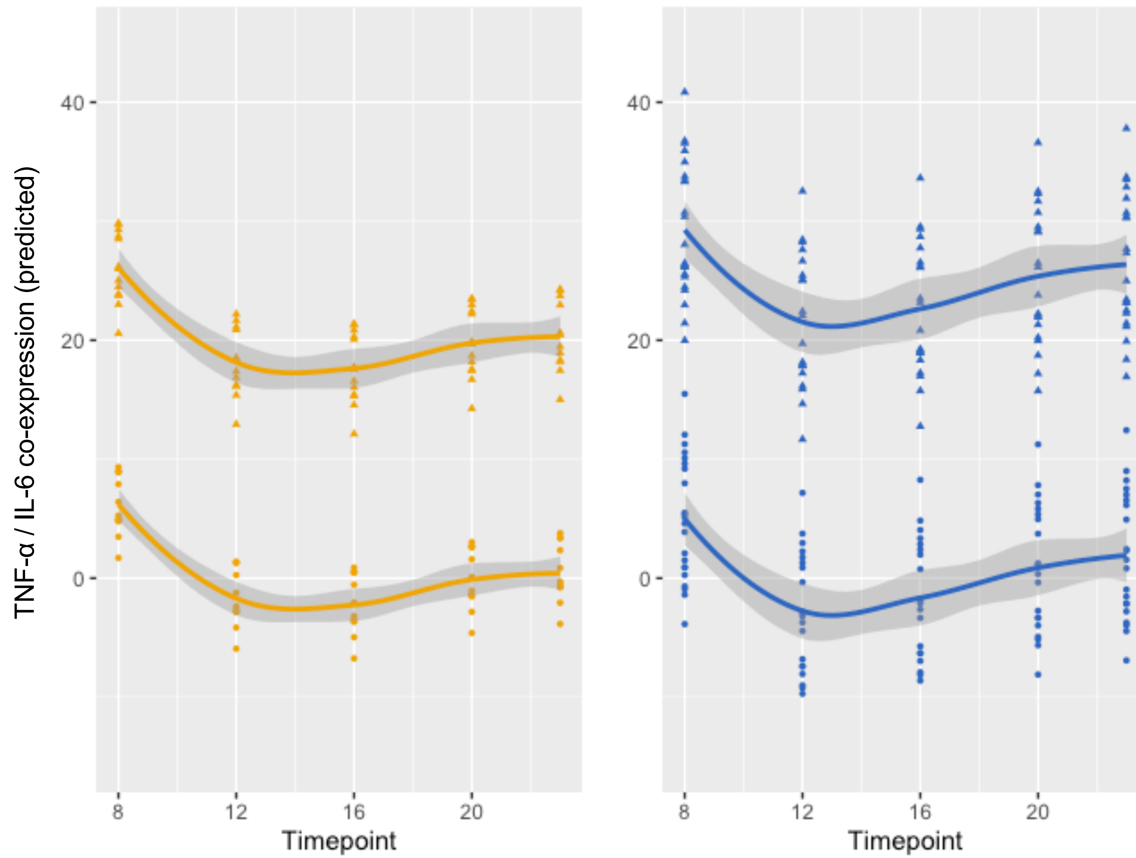
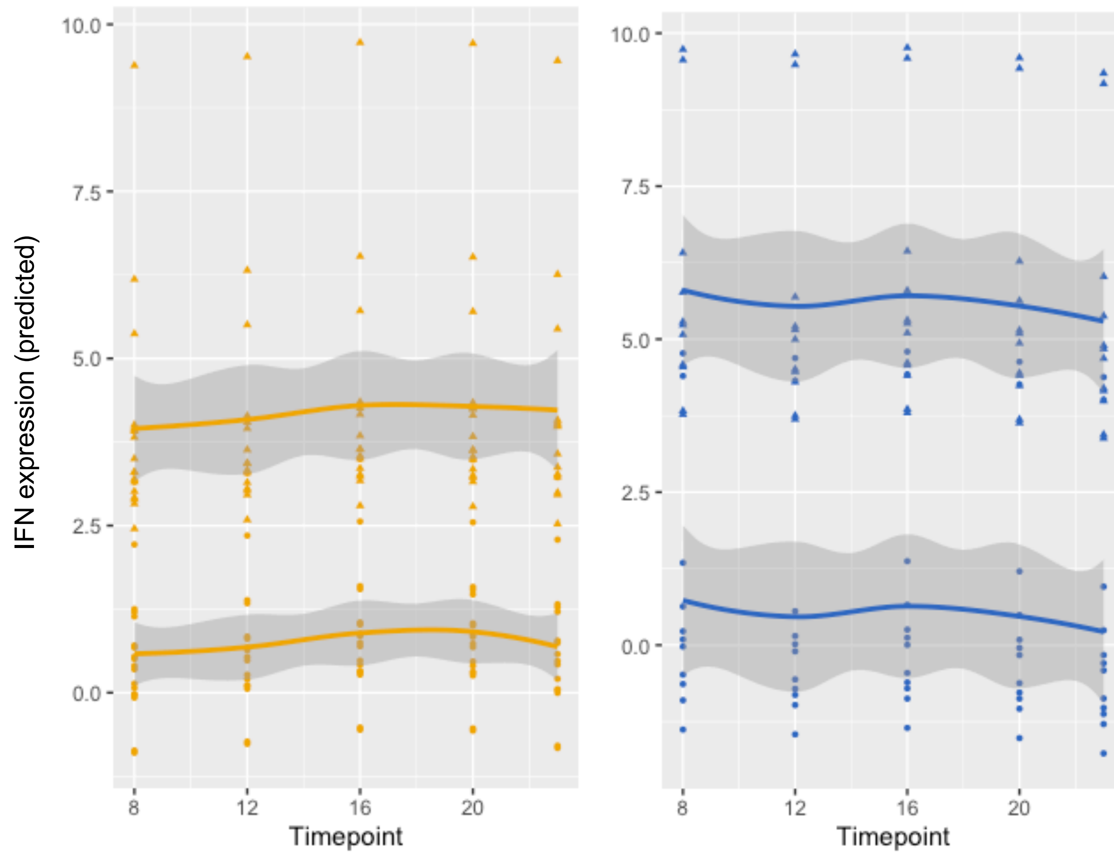


Figure 3.4. *Mixed model results predicting monocyte intracellular IFN expression. Yellow = control group; blue = AUD group. Circle points = unstimulated; triangle = stimulated.*



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CHAPTER 4

ENDOTOXIN FOR ALCOHOL RESEARCH: A CALL FOR EXPERIMENTAL MEDICINE USING LIPOPOLYSACCHARIDE CHALLENGE

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Abstract

Studies of inflammation in alcohol use disorder (AUD) are overwhelmingly preclinical, and translation to clinical samples is necessary. Endotoxin administration has been used successfully in humans to study mood disorders, offering a translational, reliable, and safe model that may be validated in AUD research. We argue for the use of endotoxin challenge to elucidate the interplay between AUD and inflammation.

Commentary

There is a great deal of interest in the role of inflammation in psychiatric disorders, including alcohol use disorder (AUD). While neuroinflammation appears to be a key component of AUD, the existing literature is overwhelmingly preclinical and findings in humans are largely correlational. Experimental approaches that establish a causal link between inflammation and AUD phenotypes are currently lacking and are necessary for the next step in the translation of this hypothesis. One reason for this limited translation of preclinical findings to human and clinical samples is the lack of reliable methods to study inflammation. Therefore, to improve the translation of preclinical findings to clinical samples, experimental manipulations which can give rise to a phasic state of high inflammation are required and may allow for experimental medicine models to be tested in clinical populations.

One promising experimental method by which transient inflammation can be provoked is through the administration of purified bacterial endotoxin (lipopolysaccharide, LPS). Endotoxin administration in humans yields a reliable, transient, and safe response, with clear dose-response relationships. LPS is administered as an intravenous infusion, usually at low doses between 0.4 – 1.0ng/kg body weight. This infusion induces a phasic inflammatory response, with peripheral cytokine levels – biomarkers of systemic immune response – peaking around two hours post-

infusion and returning to baseline within four to six hours. At these low doses, endotoxin administration has been shown to safely and briefly mimic low-grade inflammatory response, raising cytokine levels and subtly affecting neuropsychiatric symptoms, including depressed mood and anxiety, while causing limited changes in vital signs including heart rate, blood pressure, and temperature. This method has been used in many past human challenge studies and remains the World Health Organization standard for endotoxin assays used in the pharmaceutical industry (Suffredini and Noveck, 2014). While this method has been used successfully in human subjects to better understand the contribution of inflammation to mood disorders, it has not been widely implemented in the context of AUD. In this commentary, we make an argument for the use of endotoxin challenge in AUD research to elucidate the complex interplay between AUD and inflammation.

First, inflammatory signaling is significantly implicated in AUD in both preclinical and clinical models. Individuals with AUD have increased plasma levels of proinflammatory cytokines, and animal models with chronic alcohol exposure show long-lasting increases in systemic inflammation. In healthy humans, alcohol administration has been reported to modulate -- either raising (Afshar et al., 2015) or lowering (Monnig et al., 2020) -- endogenous LPS levels. Furthermore, LPS endotoxin-induced inflammation in mice produces prolonged increases in alcohol consumption. On the whole, preclinical and clinical studies show that alcohol exposure affects inflammation, and preclinical studies indicate that the reverse may be true as well. Therefore, we contend that an LPS endotoxin challenge in humans provides a valid forward translation of the role of inflammation in AUD.

In support, endotoxin challenges have been used in clinical studies of affective disorders (Lasselin et al., 2020). These studies not only offer proof of safety and reliability in human subjects, but also strengthen the reasoning that endotoxin challenge will be an effective method

for studying AUD, as there is a well-established relationship between AUD and emotion regulation. Negative mood can induce alcohol-seeking due to effects on craving, and alcohol use inhibits negative emotion regulation. This suggests that in humans, endotoxin challenge may also affect secondary symptomology of AUD through its effects on mood and behavior. To date, however, this method has not been applied broadly to AUD research, and in fact, there is some skepticism about the application of this paradigm.

Perhaps the most common criticism of the endotoxin challenge approach is driven by uncertainty regarding the degree to which central nervous system inflammatory response reflects the systemic response measured by peripheral cytokines, as well as differences between the sustained inflammatory state seen in chronic alcohol consumption and the transient state induced by endotoxin administration. However, these questions can be answered empirically. LPS administration offers the potential to explore these outstanding questions by conducting longitudinal and neuroimaging studies. Indeed, neuroimaging findings show that endotoxin-induced inflammation is linked to neural response (Lasselin et al., 2020), a promising indicator that the systemic inflammatory state induced by LPS administration is reflected in the brain. The endotoxin challenge proposed in this commentary is aimed at inducing a transient/phasic immune response distinct from and beyond the chronic/tonic levels likely present at baseline for subjects with AUD. LPS models of chronic diseases have been used successfully in animals (i.e. circulating LPS via osmotic minipump (Lindros and Järveläinen, 2005)); however, chronic administration of low-dose endotoxin in humans is hampered by endotoxin tolerance (Kiers et al., 2017). Additional preclinical and longitudinal studies will likely be necessary to investigate the chronic nature of inflammation in AUD. It is also worth noting that the only published study using endotoxin administration in the context of AUD found that LPS did not induce anxiety or alcohol craving. However, this study was conducted within a clinical trial of the neuroimmune

drug pioglitazone and was prematurely terminated after 16 subjects, with only 8 subjects undergoing the LPS challenge in the placebo group (Schwandt et al., 2020). Therefore, these findings are considered highly preliminary and indicate that endotoxin challenge requires further assessment in studies of AUD. Despite its limitations, this study provides valuable evidence that LPS administration in human subjects with AUD is indeed safe.

Importantly, both physical and behavioral responses to LPS are largely conserved across vertebrate species from animals to humans, allowing for forward- and reverse-translation of findings. Thus, endotoxin challenge presents exciting opportunities for treatment development. Given the wealth of preclinical and clinical research into neuroimmune therapies for AUD, one can envision an experimental medicine study in which an endotoxin challenge is used to elicit a transient inflammatory response as well as behavioral alcohol-related outcomes, and one such neuroimmune drug is used to either block or 'rescue' both the immune and behavioral responses. This type of approach can provide valuable proof-of-mechanism regarding these therapies for AUD.

LPS administration presents a multitude of possibilities for clinical research. One can imagine exploring the impact of inflammation in alcohol consumption, through self-administration paradigms, cue-reactivity (neuroimaging or behavioral), and secondary factors such as mood-related outcomes. This method could even shed light on the relationship between alcohol, the gut microbiome, and alcohol-associated liver disease (ALD). Studies suggest that the gut microbiome contributes to the pathogenesis of AUD (Temko et al., 2017), which in turns play an important role in ALD via inflammatory mechanisms (Hosseini et al., 2019). Furthermore, the gut microbiome might play a role in alcohol craving and seeking behaviors (Leclercq et al., 2014). This area is of high interest but is lacking in clinical mechanism-oriented

studies – thus, one can envision combining LPS endotoxin challenge with a gut microbiome study to directly interrogate these inflammatory mechanisms.

To date, the vast majority of studies implicating inflammation in AUD are preclinical in nature. Therefore, it is crucial for the field to progress to experimental models that can effectively probe the role of neuroinflammation in AUD phenotypes in humans. Endotoxin challenge offers a translational, reliable, and safe model of inflammation that may be validated through use in studies of AUD. Endotoxin administration presents a method through which the complex relationship between AUD and inflammatory signaling may be elucidated, and may aid in the development of neuroimmune treatments for AUD. We argue for the utilization of this paradigm in AUD research, with a focus on translating a large preclinical body of evidence for the relationship between inflammation and AUD into a more clinically useful and applied knowledge base.

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GENERAL DISCUSSION

1. Overview and General Summary

This dissertation explored the relationships between inflammation, reward, and alcohol use disorder (AUD). Molecular and behavioral studies suggest a central role for the innate immune system in mediating the acute and chronic effects of alcohol and generally support an inflammatory hypothesis of AUD (Mayfield et al., 2013). Neurotrophins, including glial (GDNF) and brain derived neurotrophic factor (BDNF), are essential for basic cell signaling, including midbrain dopamine transmission (Altar et al., 1992; Lin et al., 1993). In rodent models of AUD, reductions in GDNF and BDNF expression underlie dysfunctional striatal dopamine signaling, increased motivation to consume alcohol, and heightened alcohol reward (Ahmadiantehrani et al., 2014; Carnicella et al., 2009; Hensler et al., 2003). In preclinical studies, lipopolysaccharide (LPS)-induced inflammation produces prolonged increases in alcohol consumption (Blednov et al., 2011), while knocking out immune-signaling genes attenuates alcohol preference and self-administration (Blednov et al., 2012). Chronic alcohol exposure produces long-lasting increases in systemic inflammation, which in turn is associated with cognitive and behavioral impairment and brain damage (Alfonso-Loeches et al., 2010). Furthermore, inflammation increases vulnerability to stress-induced drug seeking and relapse (Frank et al., 2011).

Overall, alcohol consumption produces a sustained inflammatory state, and in turn, this alcohol-induced neuroinflammation contributes to the behavioral and neurotoxic effects of alcohol (Cui et al., 2011). Individuals with AUD are thought to have increased neuroinflammation throughout the brain (Cui et al., 2014), and elevated peripheral levels of proinflammatory cytokines have been proposed as a biomarker for AUD (Achur et al., 2010; Heberlein et al., 2014; Leclercq et al., 2014). These cytokines have been shown to cross the

blood-brain barrier (Banks et al., 1995), therefore possibly contributing to central nervous system effects.

Addiction has also been shown to be a reward deficit disorder (Joyner et al., 2016), with reward threshold heightening after substance use acting as a major reinforcing effect of substance abuse (Koob, 2013). Reward sensitivity is a marker of initial risky drinking, such that individuals with baseline higher reward sensitivity are at increased risk of problematic alcohol use (Jonker et al., 2014; Lyvers et al., 2012; Nees et al., 2012). However, in the transition from early abuse to AUD, continued alcohol use eventually impairs neuronal circuits that are involved in reward sensitivity, thereby shifting alcohol use from an innately rewarding activity into drinking to relieve withdrawal and negative symptoms (Volkow et al., 2010). Reward responsiveness is also correlated with neuroinflammation. Preclinical studies show that LPS-induced inflammation alters reward sensitivity in mice (Lasselin et al., 2020b; Vichaya et al., 2014), and in humans, neuroinflammation has been implicated in reward processing impairments in Major Depressive Disorder (Felger et al., 2016). Additionally, previous studies in human control populations indicate that brain activation in response to reward stimuli is decreased after LPS infusion (Eisenberger et al., 2010). Taken together, reward sensitivity has been associated with AUD and with inflammation, but separately. The multidirectional interplay between AUD, inflammation, and reward has not fully been explored.

1.1. Chapter 1 Summary

Study 1 examined clinical and neural characteristics of reward- and relief-motivated subtypes of AUD, as assessed by the UCLA Reward, Relief, Habit Drinking Scale (Grodin et al., 2019) and Reasons for Heavy Drinking Questionnaire (Adams et al., 2016). This study supported and extended the allostatic and incentive salience models of addiction (Koob and Schulkin, 2019; Robinson and Berridge, 1993), suggesting a consistent differentiation between motivation-based

phenotypic subtypes of alcohol use. The study also probed cue-elicited neural activation in the striatum underlying these AUD subgroups, finding that relief/habit drinkers largely showed greater dorsal striatal activation to visual alcohol cues compared to reward drinkers, although there was no significant difference in ventral striatal activation between groups. While these findings still generally align with the allostatic and incentive salience models, they may suggest that the participants were earlier in the transition from positive to negative reinforcement. Overall, the clinical and neural correlates of reward- and relief-motivated drinking found in Study 1 represent a step toward the refinement of neuroscience-informed phenotypes and eventually the development of personalized treatments for AUD.

1.2. Chapter 2 Summary

The second study investigated the effects of a neuroimmune modulator, ibudilast, on functional connectivity (i.e. temporally-correlated neural activation) within the reward circuit in response to visual alcohol cues. Ibudilast had been previously shown to diminish cue-reactivity within the ventral striatum (Grodin et al., 2021). This study supported and extended these results, finding that ibudilast also reduced correlations in activity between the ventral striatum and frontal regions, including the orbitofrontal (OFC) and anterior cingulate (ACC) cortices, which have both been heavily implicated in reward processing, decision-making, and selective attention (Volkow et al., 2011). Similarly, improvements in real-world drinking outcomes (i.e. decreased drinks per drinking day) were correlated with decreases in cue-elicited functional connectivity within these reward circuit regions from the ventral striatal seed, indicating that ibudilast's effects on reducing functional connectivity were indeed beneficial. Study 2 not only supported primary neuroimaging findings from previous research on ibudilast but expanded further on these outcomes to give an early proof-of-mechanism indicating that ibudilast's effects on drinking may be specifically related to its effects in frontostriatal reward-processing circuitry.

More broadly, Study 2's pairing of biological findings with disorder-related behavioral outcomes (i.e. drinking outcomes) represents a window into the clinical utility of neuroimaging in the development of pharmacotherapies.

1.3. Chapter 3 Summary

Study 3 evaluated relationships between AUD and toll-like receptor (TLR)-4 stimulated monocyte production of intracellular cytokines (ICCs) in response to lipopolysaccharide (LPS). As hypothesized, alcohol use disorder status (i.e., AUD versus controls) and LPS challenge were both associated with higher levels of proinflammatory markers, and interaction effects between these two factors were found, such that LPS ligation of TLR4 yielded greater monocyte expression of TNF-α, IFN, and co-production of IL-6 and TNF-α in blood samples from participants with AUD compared to controls, indicating that AUD was associated with enhanced sensitivity to cellular LPS challenge. This was, to our knowledge, the first study to investigate the relationships between alcohol use disorder and monocyte ICC production in response to cellular LPS challenge. This study extends previous preclinical and clinical findings on the roles of proinflammatory cytokines in AUD. As the cellular LPS challenge is thought to mimic a stress response, these findings suggest that individuals with AUD may mount a more robust inflammatory response to systemic stress than healthy controls without AUD or heavy drinking. This differential response, in turn, may render individuals with AUD more vulnerable to chronic alcohol use. Overall, the findings of Study 3 serve as a critical proof-of-concept for the use of this novel method in probing the neuroimmune mechanisms underlying AUD.

1.4. Chapter 4 Summary

Chapter 4 provided an argument for the use of LPS endotoxin in *in vivo* clinical studies of AUD. While LPS has been used in clinical studies of affective disorders, it has not been widely implemented in the context of AUD. Affective disorder studies not only offer proof of safety and

reliability in human subjects, but also strengthen the reasoning that endotoxin challenge will be an effective method for studying AUD, as there is a well-established relationship between AUD and emotion regulation. Given our findings in Study 3 showing that AUD is associated with enhanced sensitivity to LPS stimulation on the *in vitro* level, examining this relationship in a clinical study is warranted. Clinical LPS administration presents a method through which the complex relationship between AUD and inflammatory signaling may be elucidated, and may aid in the development of neuroimmune treatments for AUD. Chapter 4 argued for the utilization of this paradigm in AUD research, with a focus on translating a large preclinical body of evidence for the relationship between inflammation and AUD into a more clinically useful and applied knowledge base.

2. Conclusions and Future Directions

Following the discussion outlined in chapter 4, suggested future directions entail the clinical administration of LPS in the context of AUD research. In particular, given the history of clinical LPS challenge in affective disorder studies (Lasselin et al., 2020a), LPS may aid in elucidating the role that neuroinflammation plays in the well-established relationship between alcohol use and negative mood. Additional future directions may be to incorporate neuroimaging, as neuroimaging findings show that LPS endotoxin-induced inflammation is linked to neural response (Eisenberger et al., 2010; Kullmann et al., 2013). Future studies may explore the impact of inflammation in alcohol consumption through self-administration paradigms and cue-reactivity; interrogate the relationships between alcohol, the gut microbiome (Temko et al., 2017), and alcohol-associated liver disease (Hosseini et al., 2019); and / or incorporate LPS challenge into pharmacotherapy development. One can envision an experimental medicine study in which an endotoxin challenge is used to elicit a transient inflammatory response as well as behavioral alcohol-related outcomes, and a neuroimmune drug

is used to either block or 'rescue' both the immune and behavioral responses. This type of approach can provide valuable proof-of-mechanism regarding these therapies for AUD.

Together, the studies presented in this dissertation contribute to the growing body of literature implicating neuroimmune signaling in AUD, especially in association with reward response. The findings presented herein aimed to elucidate biological mechanisms related to inflammation and reward in AUD and provided clinical and neurobiological data on these relationships. On a broader scale, these studies provided critical proofs-of-concept for methods that warrant further inclusion in AUD research: namely, drinking motivation-based phenotyping as an approach toward personalized medicine, the inclusion of neuroimaging, especially functional connectivity, in pharmacotherapy development, and the use of LPS as an inflammatory challenge to probe neuroimmune mechanisms underlying AUD.

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