OFFICIAL TITLE: AN INFLAMATORY CHALLENGE USING ENDOTOXIN

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AN INFLAMATORY CHALLENGE USING ENDOTOXIN
STUDY PROTOCOL

1. INTRODUCTION

Neuroimmune factors, expressed in neurons and glia, mediate neuroinflammation and modulate a wide range of brain functions, including neuronal activity, endocrine function, and CNS development. A growing body of literature suggests that systemic inflammation leads to neuroinflammation, and alcohol exposure increases both systemic- and neural markers of inflammation. Specifically, chronic alcohol consumption is thought to produce a sustained inflammatory state, such that individuals with alcohol use disorder (AUD) have increased inflammation throughout the brain. Alcohol-induced inflammation has been implicated in chronic alcohol seeking as well as the behavioral and neurotoxic effects of alcohol. In rodents, lipopolysaccharide (LPS)-induced inflammation produces prolonged increases in alcohol consumption, and knocking out immune signaling genes attenuates alcohol preference and self-administration. Thus, elucidating the role of inflammation in AUD has significant implications, including opportunities for the development of novel and potentially more effective therapeutics for AUD.

The proposed study consists of a randomized, double-blind, placebo-controlled trial of low dose endotoxin. The low dose endotoxin challenge induces a transient systemic inflammatory response with normalization of cytokine levels within hours. This “phasic” inflammation is distinct from chronic (“tonic”) levels of inflammation that may be present with AUD.

2. BACKGROUND AND SIGNIFICANCE

2.1. Inflammatory signaling appears to be a key component of alcohol use disorder.

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 alcohol use disorder. Neurotrophins, including glial (GDNF) and brain derived neurotrophic factor (BDNF), are essential for synaptic plasticity, neuron survival, and basic cell signaling, including midbrain dopamine transmission. 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. Conversely, increases in GDNF and BDNF signaling restores mesolimbic dopamine function, reduces alcohol self-administration, and attenuates relapse to alcohol seeking. In preclinical studies, induction of inflammation produces prolonged increases in alcohol consumption, and knocking out inflammatory signaling genes attenuates alcohol preference and self-administration. Proinflammatory signaling also mediates acute alcohol-induced motor impairment, and chronic alcohol exposure produces long-lasting increases in systemic inflammation, which in turn is associated with sustained cognitive and behavioral impairment and brain damage. Furthermore, inflammation is thought to increase vulnerability to stress-induced drug seeking and relapse.

It is hypothesized that chronic alcohol consumption produces a sustained inflammatory state, and in turn, this alcohol-induced neuroinflammation contributes to the behavioral and neurotoxic effects of alcohol. Individuals with AUD are thought to have increased neuroinflammation throughout the brain, and elevated peripheral levels of proinflammatory cytokines have been

proposed as a biomarker for AUD. 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. In sum, the role of inflammation in AUD remains opaque, particularly in human/clinical studies.

2.2. The literature on inflammatory signaling and AUD is overwhelmingly preclinical and translation to human clinical samples is sorely needed.

To date, the vast majority of the research implicating alcohol and inflammation has been preclinical. Human studies, however, have recently shown a positive correlation between serum levels of inflammatory cytokines and alcohol craving. While these associations are intriguing, it is crucial for the field to progress from correlational to experimental models that can effectively probe the role of neuroinflammation in AUD phenotypes in humans. This is particularly relevant given recent evidence that genomic responses to inflammation and inflammatory disease in mouse models cannot be extrapolated to the human condition, which "supports higher priority to focus on the more complex human conditions rather than rely on mouse models to study human inflammatory diseases." To that end, we propose to conduct an inflammatory challenge in a sample of non-treatment-seeking heavy drinkers and a comparison sample of light drinkers to examine the effects of systemic increases in inflammation on cue-induced craving for alcohol in the laboratory. Such endotoxin-induced increases in systemic inflammation are reported to induce neuroinflammation with glial activation in humans. Specifically, we propose to experimentally provoke an inflammatory response using low dose endotoxin derived from E. coli (E. coli group O:113:BB-IND 12948 to MRI) and delivered intravenously. This reference endotoxin provided by the NIH Clinical Center has demonstrated safety and has been successfully administered at UCLA by the investigative team in studies of inflammation, socioemotional processes, and depression. The proposed study will leverage these resources for a safe, effective, and well-tolerated inflammatory challenge in order to experimentally probe the role of inflammation in alcohol craving among non-treatment-seeking heavy drinkers. The scientific premise of this proposal is that if inflammation plays a causal role in inducing alcohol craving, heavy drinkers will experience greater cue-induced alcohol craving following an inflammatory challenge (via endotoxin) as compared to those given a placebo challenge. In addition to a human study showing a positive correlation between serum levels of cytokines and alcohol craving, a host of preclinical studies have shown that while endotoxin administration reduces effort, it actually boosts preference for high rewards, thus advancing craving as a plausible reward-based outcome for this study.

2.3. Significance of the proposed project hinges on the premise that while evidence is accumulating that the innate immune system and inflammation contribute to AUD, the evidence is largely preclinical and translation to human clinical samples is necessary.

The findings in humans have been mostly correlational while experimental approaches that can establish a causal link between inflammation and AUD phenotypes is lacking. The proposed study fills an important gap in the literature by examining the role of inflammation in alcohol craving in a clinical sample of non-treatment-seeking heavy drinkers and light drinking healthy controls. The successful completion of the proposed study will advance the field of alcoholism by experimentally probing the role of neuroinflammation in alcohol craving, a translational phenotype for AUD. Results from this study will inform an R01 application in which the combination of inflammatory challenge and alcohol cue-reactivity can be used in medications

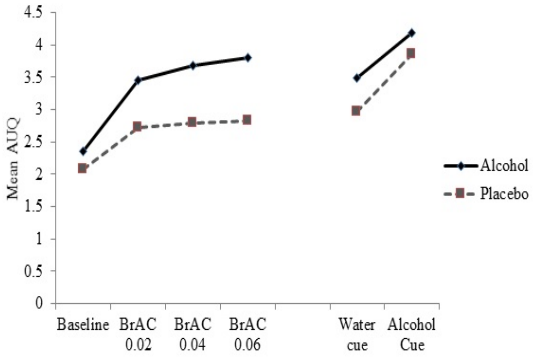
development applied to novel neuroimmune modulators with therapeutic potential for AUD. Notably, this approach has been successful in the psychoneuroimmunology (PNI) field.

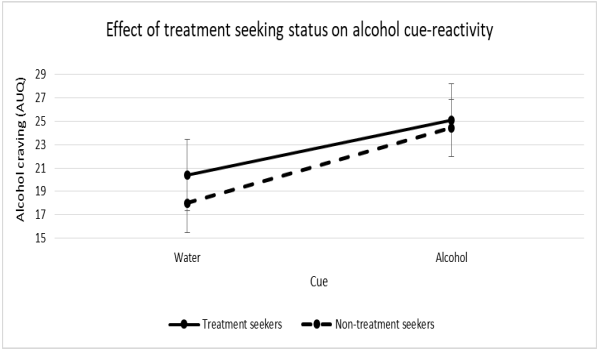
2.4. Preliminary studies conducted by the research team.

The investigative team brings together expertise in the areas of clinical neuroscience of AUD (Ray), psychoneuroimmunology (PNI) (Irwin, Eisenberger), experimental psychology (Ray, Eisenberger), and clinical psychiatry (Irwin, Miotto). The team is led by Dr. Lara Ray who routinely employs behavioral pharmacology approaches including intravenous alcohol administration and medications in her research. Dr. Ray has a growing interest in the role of inflammation in AUD. Dr. Irwin is a world-expert in PNI, including a strong publication record in the intersection between PNI, sleep, and AUD, and the use of endotoxin to probe inflammation-behavior relationships. Dr. Eisenberger is a neuroscientist with a program of research on the biological underpinnings of social connection, including PNI mechanisms. Dr. Karen Miotto is a physician scientist who has served as the medical director for Dr. Ray's studies for the past 9 years. Dr. Miotto has a strong record of caring for research patients in addiction pharmacotherapy studies. In addition, the research team is building important collaborations through publications and grant proposals under review. Lastly, all investigators hold primary faculty appointments at UCLA, which facilitates their active involvement in all aspects of the proposed study.

2.5. Cue-reactivity represents an important translational phenotype for alcoholism.

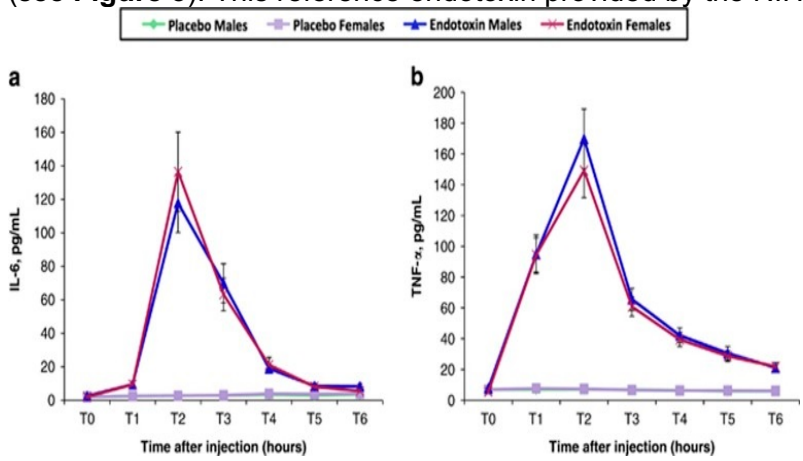
Dr. Ray has an active research program using experimental approaches to elucidating AUD etiology and treatment development. Dr. Ray's laboratory has ample expertise applying cue-reactivity methods to AUD. For example, in a recent publication, Dr. Ray and her team examined craving for alcohol during an alcohol administration (i.e., comparing I.V. alcohol vs. placebo control) as well as during a cue-reactivity paradigm (i.e., comparing water cue vs. alcohol cue) in individuals with AUD. As shown in **Figure 1**, there is a main effect of alcohol administration (vs. placebo) in eliciting craving as well as a main effect of alcohol cue (versus water cue). The cue-reactivity paradigm proposed herein is consistent with those successfully implemented in Dr. Ray's laboratory, including studies of cue-reactivity among individuals with AUD. Further, we have demonstrated the safety of these procedures, by evaluating drinking behavior following study participation. More recently, we have become interested in comparing treatment-seekers to non-treatment seekers in cue-induced craving. Preliminary analyses with a sample of 29 individuals with moderate-to-severe AUD showed no significant differences in cue-reactivity between treatment-seekers (N=15) and non-treatment seekers (N=14) ($F(2,28)=0.42$, $p= .66$) (see **Figure 2**).





2.6. Endotoxin provides a safe and reliable probe of inflammatory response.

Drs. Irwin and Eisenberger have expertise in PNI including a host of studies using endotoxin to provoke an inflammatory response. Specifically, using low dose endotoxin derived from E. coli (E. coli group O:113:BB-IND 12948 to MRI) and delivered intravenously, Drs. Eisenberger and Irwin demonstrated reliable increases in plasma levels of proinflammatory cytokines [i.e., Interleukin-6 (IL-6) and tumor necrosis factor-α (TNF- α)] which peak at 2 hours post infusion (see **Figure 3**). This reference endotoxin provided by the NIH Clinical Center has demonstrated safety and has been successfully administered at UCLA by the investigative team in studies of inflammation and depression. The proposed study will leverage these resources for a safe, effective, and well-tolerated inflammatory challenge in order to experimentally probe the role of inflammation in alcohol craving among non-treatment seeking heavy drinkers and light drinking controls.



2.7. **Acute inflammation induces neural changes detectable by neuroimaging**. Translating the role of inflammation in AUD from preclinical animal models to clinical populations has been slow. Because it is the only non-invasive technique to assay the living brain, neuroimaging represents an important tool to translate findings from preclinical studies to individuals with addictive disorders [39]. Neuroimaging provides objective quantifications of brain activation, beyond what can be gathered from subjective self-report data alone [40]. Critically, functional magnetic resonance imaging (fMRI) paradigms have been used to detect alterations in the neural signal induced by acute inflammation [13, 14, 41, 42]. In healthy individuals, acute inflammation induced by endotoxin attenuated ventral striatal activation to anticipation of a monetary reward, which was further associated with increases in depressed mood [14]. Acute inflammation enhanced neural activation to both positive and negative social feedback, such that endotoxin increased amygdala activation to negative social feedback and increased reward-related activation (ventral striatum, ventromedial prefrontal cortex) to positive social feedback [13]. These studies emphasize the complexities of the role of acute inflammation in reward processing, such that the neural response to positive social feedback is potentiated whereas it is attenuated to monetary reward.

Importantly, no studies have yet investigated the effect of acute inflammation on the neural response to alcohol cues in AUD or other addictive disorders. fMRI alcohol cue-reactivity paradigms have been commonly used to evaluate if pharmacological AUD treatments alter brain activation in circuitry associated with reward processing [43, 44], and as such are sensitive to biological changes in the body as would be present in an inflammatory state. Therefore, the proposed study will fill an important gap in the literature by examining the role of acute inflammation on the neural substrates of alcohol cue reactivity in a clinical sample of non-treatment-seeking heavy drinkers and light drinking healthy controls. The successful completion of the proposed study will advance the field of alcoholism by experimentally probing the role of neuroinflammation in alcohol craving, a translational phenotype for AUD. Results from this study will inform an R01 application in which the combination of inflammatory challenge and alcohol

cue-reactivity can be used in medications development applied to novel neuroimmune modulators with therapeutic potential for AUD [9, 45]. Notably, this approach has been successful in the psychoneuroimmunology (PNI) field [46, 47].

3. STUDY OBJECTIVES

3.1. Primary Aims

Primary Aim #1: To test whether low dose endotoxin (0.8 ng/kg of body weight) will increase cue-induced craving for alcohol in non-treatment-seeking heavy drinkers, as compared to placebo and as compared to light drinking controls. Based on the correlational findings of a positive association between proinflammatory cytokines and alcohol craving (8, 9), as well as preclinical studies (16-18), we hypothesize that endotoxin will produce greater cue-induced alcohol craving than placebo infusion.

Primary Aim #2: To test whether low dose endotoxin (0.8 ng/kg of body weight) will increase depressed mood as compared to placebo. Based on recent work by our Co-Is (13), we hypothesize that endotoxin will produce greater increases in depressed mood than placebo infusion.

3.2. Exploratory Aims

Exploratory Aim #1: To test associations between plasma levels of proinflammatory cytokines (IL-6 and TNF- α), depressed mood, and alcohol craving during the challenge.

Exploratory Aim #2: To examine sex differences in responses to the endotoxin challenge.

Exploratory Aim #3: To determine the effect of low dose endotoxin (0.8 ng/kg of body weight) on neural alcohol cue-reactivity.

Exploratory Aim #4: To determine the effect of low dose endotoxin (0.8 ng/kg of body weight) on reward responsiveness.

4. STUDY DESIGN

4.1. Design Overview

The study design consists of a randomized, double-blind, placebo-controlled trial of low dose endotoxin. The low dose endotoxin challenge induces a transient systemic inflammatory response with normalization of cytokine levels within hours. This “phasic” inflammation is distinct from chronic (“tonic”) levels of inflammation that may be present with AUD. A total of 38 non-treatment seeking heavy drinking men and women and 38 light drinking healthy controls will participate in the study. Recruitment will be monitored to ensure the two groups are matched by gender. Eligible participants will be randomly assigned to receive a single I.V. infusion of either low dose endotoxin (0.8 ng/kg of body weight) or placebo (same volume of 0.9% saline solution) at the UCLA Outpatient Clinical and Translational Research Center (CTRC). All participants will complete an alcohol cue-exposure paradigm 2 hours post infusion, which is the time of expected peak cytokine response (13, 14). Participants will complete the fMRI alcohol cue reactivity paradigm 3 hours post infusion. Plasma levels of proinflammatory cytokines [i.e.,

Interleukin-6 (IL-6) and tumor necrosis factor-α (TNF- α)], mood, reward reactivity, and alcohol craving, will be assessed at baseline and then hourly for four hours post infusion.

5. STUDY PROCEDURES

5.1. Recruitment of Subjects

Participants will be recruited from the community through online and newspaper advertisements. Campaigns in local buses and print publications (e.g., LA Weekly) will also be implemented. Targeted recruitment will also take place through a lab database of previous study participants who agreed to be contacted for future studies.

5.2. Eligibility Criteria

5.2.1. Inclusion Criteria

To be included in the study, participants must:
(1) Be between the ages of 21 and 45
(2) Be non-treatment seeking for AUD
(3) Have had at least one alcoholic beverage in the last 30 days
(4) FOR HEAVY DRINKERS: Alcohol Use Disorder Identification Test (AUDIT) score between 8 – 15; FOR LIGHT DRINKERS: AUDIT score < 4
(5) FOR HEAVY DRINKERS: Report drinking at binge levels at least 1 time in the past month (5+ drinks/day for men, 4+ drinks/day for women); FOR LIGHT DRINKERS: report no occasions of binge drinking in the past month

5.2.2. Exclusion Criteria

To be included in the study, participants must not:
(1) Have a current (last 12 months) DSM-5 diagnosis of substance use disorder for any psychoactive substances other than alcohol and nicotine
(2) Have a lifetime DSM-5 diagnosis of schizophrenia, bipolar disorder, or any psychotic disorder
(3) Have current moderate to severe depression as indicated by a score of ≥ 21 on the Beck Depression Inventory – II (BDI-II)
(4) Have current suicidal ideation or lifetime history of suicide attempt as reported on the Columbia-Suicide Severity Rating Scale (C-SSRS)
(5) Have a positive urine screen for drugs other than cannabis;
(6) Have clinically significant alcohol withdrawal symptoms as indicated by a score ≥ 8 on the Clinical Institute Withdrawal Assessment for Alcohol-Revised (CIWA-R)
(7) Have an intense fear of needles or have had any adverse reactions to needle puncture
(8) Be pregnant, nursing, or planning to become pregnant while taking part in the study; and must agree to one of the following methods of birth control (if female), unless she or partner are surgically sterile:
    - Oral contraceptives
    - Contraceptive sponge
    - Patch
    - Double barrier
    - Intrauterine contraceptive device

- Etonogestrel implant
- Medroxyprogesterone acetate contraceptive injection
- Complete abstinence from sexual intercourse
- Hormonal vaginal contraceptive ring

(9) Have a medical condition that may interfere with safe study participation (e.g., unstable cardiac, renal, or liver disease, uncontrolled hypertension or diabetes, autoimmune or inflammatory disease)
(10) Have clinically significant abnormal EKG
(11) Have ≥ Grade 2 laboratory abnormalities, based on FDA Guidance Document "Toxicity Grading Scale for Healthy Adult and Adolescent Volunteers Enrolled in Preventive Vaccine Clinical Trials"
(12) Have any other circumstances that, in the opinion of the investigators, compromises participant safety
(13) Have non-removable ferromagnetic objects in body
(14) Have claustrophobia
(15) Have serious head injury or prolonged period of unconsciousness (>30 minutes)

5.2.3. Exclusion Criteria for Inflammatory Challenge Visits

To participate in the inflammatory challenge, participants must not show any of the following upon arrival to the inflammatory challenge study visit:
(a) BrAC > 0.000 g/dl
(b) clinical withdrawal (CIWA-R) score ≥ 8
(c) blood pressure ≤ 90/60 or ≥ 160/120
(d) resting pulse ≤ 50 beats/minute or > 100 beats/minute
(e) temperature ≥ 99.5°F
(f) recent (past 2 weeks) acute illness or vaccination
(g) score of 10+ on Physical Sickness Symptoms Assessment

5.3. Screening Period

5.3.1. Telephone Screen

Individuals who call the lab (in response to flyers and advertisements) expressing interest in the study will receive detailed information about the study procedures, and if they remain interested they will complete a telephone screen performed by a trained research assistant for self-reported inclusion and exclusion criteria. Those who appear eligible will be invited to the laboratory for an initial in-person screening session.

5.3.2. Initial Screening Visit

Prior to conducting any research related procedures, research staff will conduct the informed consent process, which details the procedures to take place during the screening visit. The initial screening visit may take place in-person or virtually over Zoom. At an in-person visit, informed consent will be a three-part process. First, participants will be asked to read and provide verbal consent for breathalyzer. If the breathalyzer is above 0.000, the visit will be stopped and the participant will not be compensated. The participant will be given an opportunity to reschedule the visit for another day. If the breathalyzer test is negative, the written informed consent form will be reviewed and signed by the participant and study staff

outlining procedures for the initial screening visit. At the initial screening visit, subjects will be asked to provide a urine sample to test for drugs of abuse and pregnancy (if female), and will complete a series of individual differences measures (described in detail below). This visit should take approximately 2 hours.

Initial screening visits may also take place remotely. In this case, participants will be provided with a Zoom link to meet with a researcher. Participants will be asked to confirm that they have not consumed alcohol or any illicit substances prior to the study visit. They will not provide breathalyzer or urine samples in a virtual visit, but these elements will be included in the medical screening visit if participants are otherwise eligible. In the event of a virtual screening visit, participants will be emailed a PDF copy of the initial visit consent form. After reviewing the consent form with study staff, participants will type their names onto a signature page to indicate their agreement and consent to participate in the screening visit. Typing their name on the signature page will serve as an electronic signature.

Following the initial screening visit, the study coordinator will meet with the PI to determine if the participant is eligible to continue to the medical screening based on study inclusion/exclusion criteria. A second written consent form will be reviewed and signed in the presence of the study physician or nurse practitioner at the medical screening visit if the participant is found eligible to continue to that visit.

5.3.3. Medical Screening Visit

Those participants who appear to be eligible after the initial screening visit, will then be scheduled for a second screening visit. This visit will be conducted in the research lab and CTRC by the study physician (MD) or nurse practitioner (NP) and will start with a breathalyzer test. If the breathalyzer is above 0.000, the visit will be stopped and the participant will not be compensated. The participant will be given an opportunity to reschedule the visit for another day. If the breathalyzer test is negative, the MD or NP will conduct the second written (experimental) consent; medical history interview and physical exam. In addition, a urine drug screen test will be repeated. At the CTRC, a blood specimen will be collected for a Comprehensive Metabolic Panel and Complete Blood Count to evaluate overall health; and will obtain an EKG to screen for medical conditions that could make study participation medically unsafe. The MD or NP will review each participant's medical history, vital signs, weight, review of systems, and laboratory tests, including liver function tests (LFTs), drug screen, chemistry screen, and urine pregnancy screen to determine if it is medically safe for the participant to take the study medication.

Any subject who is excluded from the study will be compensated for their time in the screening session and will be offered referrals for alcohol treatment in the community.

5.4. Experimental Visit

5.4.1. Randomization

Participants who are eligible after the physical exam will be randomized to an experimental condition (i.e., endotoxin vs. placebo) within an appropriate amount of time based on study physician discretion. Urn randomization will be used to balance the two groups by gender and BDI-II severity. The UCLA Research Pharmacy will manage the blind. The two treatment conditions will not be different in appearance or method of administration. However, it is

possible that participants undergoing the endotoxin challenge who feel very symptomatic may suspect that they are in the active condition. For this reason, we have decided to exclude individuals who experience severe sickness symptoms as described below (which is expected to be less than 5% of the sample). If participants are withdrawn from the study due to sickness symptoms, their data will be retained for the purposes of reporting adverse events and safety data during the trial. Additional subjects will be recruited to replace the withdrawn individuals.

5.4.2. Inflammatory Challenge (endotoxin or matched placebo)

Upon arrival to the CTRC, eligibility for the inflammatory challenge will be reviewed to ensure that none of the exclusion criteria have been met as described above. A nurse, who will be blind to the condition, will insert a catheter with a heparin lock into the non-dominant forearm for drug administration and hourly blood draws. Each participant will be randomly assigned to receive either low-dose endotoxin (0.8 ng/kg of body weight administered) or placebo (same volume of 0.9% saline), which will be administered by the nurse as an intravenous bolus. The endotoxin will be derived from *Escherichia coli (E. Coli* group O:113: BB-IND 12948 to M.R.I) and will be provided by the National Institutes of Health Clinical Center as a reference endotoxin for studies of experimental inflammation in humans. Participants will complete assessments as outlined below at baseline and every hour for 4 hours post-infusion. One standard meal and one snack will be provided by the CTRC to each participant during the experimental visit, and participants will be provided oral hydration throughout the visit. At the end of the experimental period, participants will have the catheter removed and will be discharged with instructions to abstain from consuming alcohol for 24 hours after discharge. A follow-up phone call will be conducted the day after the inflammatory challenge and again 1-2 weeks later to assess for any adverse events.

5.4.3. Adverse Events and Study Stopping Criteria

The NP will manage any adverse events during the inflammatory challenge and will consult with co-investigators, Dr. Miotto or Dr. Irwin, as needed to manage adverse events. In the event that significant medical problems are encountered, the blind will be broken and appropriate medical treatment will be provided. Individuals who meet the following stopping criteria will discontinue study-related data collection procedures (cytokine assays and cue exposure paradigm):

a. ≥1 SAE at least possibly related to endotoxin administration
b. ≥2 Grade 3 (severe) adverse events at least possibly related to endotoxin administration, based on the FDA Guidance Document “Toxicity Grading Scale for Healthy Adult and Adolescent Volunteers Enrolled in Preventive Vaccine Clinical Trials”

5.4.4. Plasma Cytokine Assays

Whole blood samples will be collected in two lavender EDTA tubes at baseline and every hour for 4 hours post-infusion. After collection, the samples will be centrifuged at 4°C, plasma will be harvested into multiple aliquots, and then stored in a -70 °C freezer until the completion of the study. Peripheral markers of inflammation (TNF-α, IL-6, IL-8, IL-10, IFNγ) will be assessed using a multiplex assay.

5.4.5. Alcohol Cue Exposure

Participants will complete an alcohol cue-exposure paradigm at baseline (T0) during the experimental visit and again approximately 2 hours after receiving intravenous endotoxin (or matched placebo). Alcohol cue exposure will follow well-established experimental procedures. Sessions will begin with a 3-minute relaxation period. Participants will then hold and smell a glass of water for 3 minutes to control for the effects of simple exposure to any potable liquid. Next, participants will hold and smell a glass of their preferred alcoholic beverage for 3 minutes. Order is not counterbalanced because of carryover effects that are known to occur. Participants (who are smokers) will be allowed a smoke break immediately prior to and immediately after the CR assessment. After every 3 minute period, participants will rate their urge to drink on the Alcohol Urge Questionnaire.

5.4.6. Neuroimaging

Approximately 3 hours after receiving the intravenous endotoxin or matched placebo, participants will complete a functional magnetic resonance imaging (fMRI) scan. The scan will take place at the Center for Cognitive Neuroscience. Participants will be asked to lie down on a padded table, with their head placed in the center of a large, metal doughnut-shaped magnet. While the machine is running, the participant will hear loud banging noises and will be offered earplugs to reduce the noise made by the magnet. Head and back support will also be provided to minimize discomfort. During the imaging session we will utilize several structural and functional scans. The structural protocol will consist of a high-resolution, matched-bandwidth (MBW) scan and a structural magnetization-prepared rapid-acquisition gradient echo (MPRAGE) scan. The functional scan will include a fMRI alcohol-cue reactivity paradigm previously reported to elicit blood oxygen level dependent (BOLD) response in mesocorticolimbic regions (Schacht et al., 2011).

The visual alcohol cue reactivity paradigm is a well-validated, with strong reliability and within-participant stability (1). The alcohol cues task consists of viewing alcohol, negative and neutral cues, modified from the work of Schacht and colleagues (2011). There are four types of visual cues: alcoholic beverages, non-alcoholic (neutral) beverages, negative images, and a fixation cross. Stimuli are presented in six 120-s epochs (total scan duration: 12 minutes), with each epoch consisting of four 24-s blocks (one block of alcohol cues, one block of neutral cues, one block of negative images, and one block of fixation). During each 24-s block, 5 individual pictures will be displayed for ~4.8 seconds each. Alcohol blocks will be specific to beverage type (beer, wine, or liquor), with two blocks of each beverage type. Each block will be followed by a 6-s washout period, which allows the hemodynamic response from the previous block to decline. Prior to scanning, participants will be shown how to provide ratings of their cravings using an optically isolated universal serial bus (USB) interface, which consists of a four-button (1= low to 4= high) response box. Subjects will provide ratings of their craving immediately following each cue block.

5.5. Compensation for Participation

Participants will be compensated up to $240 for their time and effort as follows:

Initial Screening Visit:	$20
Initial Screening Bonus:	$10
Medical Screening Visit:	$20
Medical Screening Bonus:	$10

Inflammatory Challenge Visit: $100
Neuroimaging Scan: $50
Reward Task (computer game): $10-20
Inflammatory Challenge Bonus: $10

Participants can earn a $10 bonus at each visit for arriving to the lab within 15 minutes of the scheduled appointment time without having to reschedule. Participants will also have the opportunity to earn $5-10 during each iteration of the Probabilistic Reward Task, for a total of $10-20. All participants will be provided with free parking validation or bus tokens for attendance to each study visit. Participants are free to discontinue participation at any time and will receive compensation for the amount of time they participated.

6. ASSESSMENTS

6.1. Schedule of Assessments Table

STUDY VISIT:	Initial Screening	Medical Screening	Experimental Visit (Baseline-T0)	Experimental Visit Hourly (T1-T4)
Adverse Events			x	x
Alcohol Breathalyzer	x	x	x	
Alcohol Purchase Task			x	x**
Alcohol Urge Questionnaire (AUQ)	x	x	x	x
Alcohol Use Disorder Identification Test (AUDIT)	x			
Barratt Impulsiveness Scale (BIS-11)	x			
Beck Depression Inventory (BDI-II)	x			
Birth Control Assessment	x			
Brief AUD Severity Scale (BASS)	x			
Cannabis Use Disorder Identification Test-Revised (CUDIT-R)	x			
Clinical Institute Withdrawal Assessment (CIWA-AR)	x	x	x	
Columbia Suicide Severity Rating Scale (C-SSRS)	x		x	x*
Comprehensive Metabolic Panel/Complete Blood Count		x		
Concomitant Medications	x	x	x	
Cue Exposure Paradigm			x	x**
Cytokine assays			x	x
Demographics	x			
Distress Tolerance Scale (DTS)	x			
Drug Screen	x	x	x	
Electrocardiogram (EKG)		x		
Fagerstrom Test for Nicotine Dependence (FTND)	x			
Family Tree Questionnaire (FTQ)	x			
Hospital Anxiety and Depression Scale (HADS)	x			
ImBIBe	x			
Inflammation and Behavior Questionnaire			x	
Inflammatory Challenge Eligibility Checklist			x	
Locator Form	x			
Medical History		x		
MRI Screening Form	x		x	
Neuroimaging Scan				x***

Penn Alcohol Craving Scale (PACS)	x	x	x	
Physical Exam		x		
Physical Sickness Symptoms Assessment			x	x
Pittsburgh Sleep Quality Index (PSQI)	x			
Pregnancy Test	x	x	x	
Probabilistic Reward Task			x	x**
Profile of Mood States (POMS)	x	x	x	x
Reasons for Heavy Drinking Questionnaire (RHDQ)	x			
Reward, Relief, Habit Drinking Scale (RRHDS)	x			
Reward Responsiveness Scale			x	x**
Social Connection Measure			x	x**
Social Disconnection Scale		x	x	x**
Structured Clinical Interview (SCID) for DSM-5 Screener and AUD Module	x			
Timeline Follow Back (TLFB)	x	x	x	
Toxicity Grading Scale Checklist		x	x	x
UPPS-P Impulsive Behavior Scale	x			
Vital Signs	x	x	x	x

* To be completed at T4 only
** To be completed at T2 only
*** To be completed at T3 only

6.2. Description of Assessments

6.2.1. Adverse Events (AE) and Serious Adverse Events (SAE)

The study physician and/or nurse practitioner are responsible for the detection, documentation, classification, reporting, and follow up of events meeting the definition of an AE or SAE. Adverse Events will be assessed at the experimental visit. However, SAEs will be collected from the time of informed consent onward. General symptoms will be collected via an open-ended question: “How have you been feeling since your last visit or the last time we spoke?”

Adverse Events will be recorded on the AE Log using accepted medical terms and/or the diagnoses that accurately characterize the event. When a diagnosis is known, the AE term recorded on the eCRF will be the diagnosis rather than a constellation of symptoms. The MD or NP will assess all AEs for seriousness, relationship to study medication, and severity. When an event has not resolved by study closure, it will be documented on the AE Log as “ongoing”.

If a woman has a positive or borderline pregnancy test after enrollment, the pregnancy will be recorded as an AE. The site will contact the subject at least monthly and document the subject’s status until the pregnancy has been terminated or completed. The outcome of the pregnancy (e.g., normal birth, death, abortion, congenital anomaly, or other disabling or life-threatening complication to the mother or newborn) will be recorded.

All AEs, including clinically significant abnormal findings on laboratory evaluations, regardless of severity, will be followed by MD or NP until satisfactory resolution (the event either resolved or stabilized and is not expected to resolve in the near term). All SAE’s will be reported per requirements.

6.2.1.1. Adverse Event (AE) Definition

An AE is any untoward medical occurrence in a participant who has been administered a pharmaceutical product and may not necessarily have a causal relationship with the administered treatment. An AE can therefore be any unfavorable and unintended sign (including a clinically significant laboratory abnormality), symptom, or disease temporally associated with the use of the study medication, whether or not related to the medication. Pre-existing conditions, diseases, or disorders are not considered AEs unless there is a change in severity or frequency.

6.2.1.1.1. Classification of Adverse Event Intensity and Relationship to Study Medication

For each recorded AE or SAE, the MD or NP must make an assessment of severity based on the following criteria:

- Mild: An event that is usually transient, requiring no special treatment, and does not generally interfere with the subject's daily activities.
- Moderate: An event that interferes with usual activities of daily living, causing discomfort but poses no significant or permanent risk of harm to the subject. The event is usually ameliorated with additional specific therapeutic intervention.
- Severe: An event that interrupts usual activities of daily living or significantly affects clinical status. The event poses a significant risk of harm to the subject and hospitalization may be required, and typically requires intensive therapeutic intervention.
- Life-threatening: An event that puts the subject into imminent risk of death without intervention.

The MD or NP must also make an assessment of relationship to the investigational product based on the following criteria:

- Unrelated: The subject did not receive the investigational product, the temporal sequence of the AE/SAE onset relative to administration of the investigational product is not reasonable, or there is another obvious cause of the AE/SAE.
- Unlikely: There is evidence of exposure to the investigational product but there is another more likely cause of the AE/SAE.
- Possible: There is evidence of exposure to the investigational product, the temporal sequence of the AE/SAE onset relative to administration of the investigational product is reasonable, but the AE/SAE could have been due to another equally likely cause.
- Probable: There is evidence of exposure to the investigational product, the temporal sequence of the AE/SAE onset relative to administration of the investigational product is reasonable, and the AE/SAE is more likely explained by the investigational product than by any other cause.
- Definite: There is evidence of exposure to the investigational product, the temporal sequence of the AE/SAE onset relative to administration of the investigational product is reasonable, the AE/SAE is more likely explained by the investigational product than by any other cause, and the AE/SAE shows a pattern consistent with previous knowledge of the investigational product or investigational product class.

6.2.1.1.2. Outcomes and Actions Taken

All unresolved AEs will be followed for a minimum of 14 days (unless the AE is an ongoing pregnancy which must be followed to conclusion) after the subject's final study visit, unless the investigator's judgment dictates otherwise, the event has resolved or stabilized prior to the 14-day period, or the subject is lost to follow-up. Investigators are not obligated to actively seek AEs or SAEs in former study subjects that occur following the follow-up period.

For each recorded AE or SAE, the investigator must make an assessment of outcome at the time of last observation, as follows:

- Fatal: The subject died.
- Resolved without Sequelae: The AE or SAE has ended.
- Resolved with Sequelae: The AE or SAE has ended but changes are noted from baseline.
- Unresolved – Ongoing: The AE has not ended and is ongoing at the end of the reporting period (i.e., 14 days after the final Follow-up visit) and the investigator deems that further follow up is not medically required
- Unknown – Lost to Follow-up: Lost to follow-up after repeated unsuccessful attempts to contact the subject.

Actions taken with respect to study medication (discontinuation or not) will also be recorded. In addition, if the AE was treated (medications or other physical measures), this will also be recorded.

6.2.1.2. Serious Adverse Event (SAE) Definition

An SAE is any untoward medical occurrence that meets one of the following:

- Results in death
- Is life-threatening (at the time of the event)
- Requires inpatient hospitalization or prolongation of existing hospitalization
- Results in persistent or significant disability/incapacity
- Is a congenital anomaly/birth defect

A serious and unexpected AE is an SAE that is not identified in nature, intensity, or frequency in the risk information included in the Product Label for the drug. Important medical events that may not result in death, be life-threatening, or require hospitalization may be considered a serious adverse event when, based upon appropriate medical judgment, they may jeopardize the study subject and may require medical or surgical intervention to prevent one of the outcomes listed in the definition.

6.2.2. Alcohol Breathalyzer

An alcohol breathalyzer will be administered at consent, and at every study visit as a safety measure. BrAC must be equal to 0.000 prior to performing any study assessments. Results will be recorded on the paper checklist, and later entered into the database.

6.2.3. Alcohol Purchase Task

The Alcohol Purchase Task is a 16-item scale that uses hypothetical situations regarding alcohol purchases and consumption at varying prices in order to generate several indices of

alcohol-related reinforcement. The APT is a self-report measure that will completed by the participant electronically during the experimental visit, specifically at T0 and T2.

6.2.4. Alcohol Urge Questionnaire (AUQ)

The Alcohol Urge Questionnaire (AUQ) is comprised of eight items rated on a 7-point Likert scale with items related to the subjective experience of alcohol craving. The AUQ has demonstrated high reliability in experimental studies of state alcohol craving and will be completed at both screening visits and multiple times during the experimental sessions; baseline, hourly, and after every 3 minute trial during the cue exposure paradigm.

6.2.5. Alcohol Use Disorders Identification Test (AUDIT)

The Alcohol Use Disorders Identification Test is 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. The AUDIT is a self-report measure that will be completed by the participant electronically at the initial screening visit.

6.2.6. Barratt Impulsiveness Scale (BIS-11)

The Barratt Impulsiveness Scale, Version 11 (BIS-11) captures self-reported impulsivity on three subscales (Attentional, Motor, and Non-Planning). The BIS-11 will be completed at the initial screening visit.

6.2.7. Beck Depression Inventory (BDI-II)

The Beck Depression Inventory, Revised (BDI-II) captures depressive symptomatology. The BDI-II will be completed at the initial screening visit.

6.2.8. Birth Control Assessment

The Birth Control Assessment is designed to confirm a female subject's compliance with the birth control specifications detailed in the inclusion criteria. Birth Control Assessment information will be recorded on the checklist at the initial screening visit for participant safety purposes.

6.2.9. Brief AUD Severity Scale (BASS)

The Brief AUD Severity Scale (BASS) is a 9-item self-report measure used to assess alcohol use disorder severity. The BASS will be completed at the initial screening visit.

6.2.10. Cannabis Use Disorder Identification Test-Revised (CUDIT-R)

The Cannabis Use Disorders Identification Test-Revised is used to identify persons with hazardous and harmful patterns of cannabis consumption. The CUDIT was developed as a simple method of screening for excessive cannabis use. The CUDIT is a self-report measure that will be completed by the participant electronically at the initial screening visit.

6.2.11. Clinical Institute Withdrawal Assessment for Alcohol (CIWA-Ar)

The CIWA-AR 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. This questionnaire will be administered on paper by appropriately trained staff during the initial and medical screening, and at the experimental visit. Participant responses will then be entered electronically.

6.2.12. Columbia Suicide Severity Rating Scale

The C-SSRS is a 4-page form asking questions about suicidal ideation, intensity of ideation, and suicidal behavior developed by Posner and collaborators at the New York State Psychiatric Institute. This scale is intended for use by trained administrators. The questions contained in the C-SSRS are suggested probes. Ultimately, the determination of the presence of suicidality depends on clinical judgment. Training is required before administering the C-SSRS through a 30-minute interactive slide presentation followed by a question-answer session through the Columbia University Medical Center. Those completing the training are certified to administer the C-SSRS, and will receive a training certificate. This scale will be used to assess lifetime and current suicidal ideation at screening and the experimental visit, baseline and discharge, and will be administered by a trained staff member with responses recorded on paper first, then entered electronically.

6.2.13. Comprehensive Metabolic Panel/Complete Blood Count

Blood will be drawn for a comprehensive metabolic panel and complete drug count during the medical screening to assess for participant safety. The total blood volume to be collected is approximately 8 mL. Additional laboratory samples may be taken at the discretion of the study physician and/or nurse practitioner if the results of any tests fall outside reference ranges or clinical symptoms necessitate testing to ensure safety.

6.2.14. Concomitant Medications

All medications taken by the participant 2-weeks prior to the start of screening and through the final experimental visit, collected via participant self-report will be recorded on a source document and later entered electronically.

6.2.15. Cytokine Assays

Blood samples will be collected at 5 time points during the experimental visit (baseline, T1, T2, T3 and T4) as described above.

6.2.16. Demographics

Demographics data include the participant's age, gender, race/ethnicity, marital status, education, employment pattern, occupation, and income level. This questionnaire will be administered electronically at the initial screening visit.

6.2.17. Distress Tolerance Scale (DTS)

The DTS is a 15-item, self-report questionnaire that measures distress tolerance along with 4 subscales: Tolerance, Absorption, Appraisal, and Regulation. Questions are scaled from 1 to 5. Participants will complete this measure at the initial screening.

6.2.18. Drug Screen

An FDA cleared, CLIA waived urine drug test card will be used at all visits to assess for recent use of opioids, cocaine, amphetamines, methamphetamine, THC, buprenorphine, methadone or benzodiazepines. Subjects must be negative for all substances except THC. Results will be recorded on the visit checklist first and then entered into the database.

6.2.19. Electrocardiogram (EKG)

A 12-lead resting EKG will be obtained at the medical screening visit to assess for medical safety. Any abnormalities will be noted and an assessment of clinical significance will be made by the study physician and/or nurse practitioner.

6.2.20. Fagerström Test for Nicotine Dependence (FTND)

The Fagerström Test for Nicotine Dependence will be used to assess smoking status and motivation to change smoking behavior at screening. This questionnaire will be completed by the subject electronically.

6.2.21. Family Tree Questionnaire (FTQ)

Information on family history of alcohol problems will be collected using the Family Tree Questionnaire. The questionnaire provides subjects with a family tree listing of relatives to identify blood relatives with alcohol problems. This questionnaire will be completed by the subject electronically at the initial screening visit.

6.2.22. Hospital Anxiety and Depression Scale (HADS)

The HADS is a self-report assessment to measure anxiety and depression levels used widely in clinical trials. The HADS will be completed at the initial screening visit.

6.2.23. ImBIBe

The ImBIBe is a 15-item questionnaire in which the subject responds on a 5-point scale responses to questions on the consequences of alcohol use. The ImBIBe will be completed at screening.

6.2.24. Inflammation and Behavior Questionnaire

The Inflammation and Behavior Questionnaire will be used to collect relevant inflammatory information, including if the subject has recently been ill, had any vaccinations, taken any anti-inflammatory medications, consumed caffeine, and exercised. This questionnaire will be completed at the experimental visit.

6.2.25. Inflammatory Challenge Eligibility Checklist

Eligibility for the Inflammatory Challenge will be determined at the beginning of the experimental visit to ensure participant safety immediately prior to the challenge.

6.2.26. Locator Form

The Locator Form asks participant to provide his/her name, address, and phone number and to provide names, addresses, and phone numbers of friends and family members who can be contacted if the subject cannot be located. This information is essential and will be collected during the initial screening, and will be updated throughout the study as necessary.

6.2.27. MRI Screening Form

The MRI Screening Form will be completed at the initial screening visit and again at the Inflammatory Challenge visit to ensure participant is safe to enter the scanner. The form provided by the Staglin Center for Cognitive Neuroscience and is required of anyone who enters the MR environment at the CCN.

6.2.28. Medical History

A Medical History interview will be conducted by the study physician or nurse practitioner at the medical screening visit and will screen for medical conditions that would make participation unsafe.

6.2.29. Penn Alcohol Craving Scale (PACS)

The PACS 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. Each question is scaled from 0 to 6. Participants will complete this scale at initial screening, medical screening, and at the experimental visit.

6.2.30. Physical Exam

A physical examination of the oral cavity, head, eyes, ears, nose, and throat, cardiovascular system, lungs, abdomen, extremities, skin, neuropsychiatric mental status and sensory/motor status, musculoskeletal system and general appearance will be performed during the medical screening visit.

6.2.31. Physical Sickness Symptoms Assessment

Symptoms of physical sickness will be self-reported during each experimental visit on a scale from 0 (symptom not present) to 4 (very severe) at multiple time points (baseline, T1, T2, T3 and T4). At the baseline assessment, participants must have a total score below 10 in order to move forward with the experimental challenge. Scores of 10 or above will result in the visit being rescheduled when the participant is feeling better. Participants who rate any symptom as a ‘4’ at the T1 – T4 assessment will be withdrawn from the study prior to the cue-reactivity paradigm. Research staff will contact the study physician and/or nurse practitioner on call to manage any severe sickness symptoms reported during the experimental session.

6.2.32. Pittsburgh Sleep Quality Index (PSQI)

The PSQI measures sleep quality using a 19-item questionnaire that will be self-administered electronically at the initial screening visit.

6.2.33. Pregnancy Test

An FDA approved rapid result urine pregnancy test will be used (i.e., dipstick test) to assess for pregnancy in female participants at each visit. If applicable, participants will be asked to sign a release of information form for study personnel to access medical records to obtain information regarding the outcome of a pregnancy that occurred during the study.

6.2.34. Probabilistic Reward Task (PRT)

A Probabilistic Reward Task (PRT) is a 25-minute task that will be administered at baseline and T2. The task assesses behavioral modulation as a function of reward-based reinforcement (i.e. reward seeking) by asking participants to respond to stimuli, eliciting a response bias by introducing an asymmetric reinforcer ratio.

6.2.35. Profile of Mood States (POMS)

The POMS measures dimensions of mood and will be completed electronically at screening and at 5 timepoints during the experimental visit (baseline, T1, T2, T3 and T4).

6.2.36. Reasons for Heavy Drinking Questionnaire (RHDQ)

The Reasons for Heavy Drinking Questionnaire (RHDQ) measures self-reported drinking motivation on two subscales (Reinforcement and Normalizing). The RHDQ will be completed at the initial screening visit.

6.2.37. Reward, Relief, Habit Drinking Scale (RRHDS)

The Reward, Relief, Habit Drinking Scale (RRHDS) categorizes subjects into reward, relief, or habit drinking subgroups. The RRHDS will be completed at the initial screening visit.

6.2.38. Reward Responsiveness Scale (RRS)

The RRS is a self-report questionnaire that measures reward responsiveness by asking subjects to rate their agreement with various statements on a 4-point Likert scale and will be administered at baseline and T2.

6.2.39. Social Connection Measure

The Social Connection Measure asks participants to identify a support figure that they could go to for help or comfort. Before the experimental study, participants are asked to rate on a 1-7 Likert scale how much they can rely on this support figure. The pre-study part of the measure will be administered at the medical screening visit.
During the experimental visit, participants are asked to rate on a 1-7 Likert scale how much they “feel like being around” their support figure at the time. This measure will be administered at baseline and T2.

6.2.40. Social Disconnection Scale

The Social Disconnection Scale asks participants to rate their agreement with 12 social connection/disconnection - related statements on a 1-5 Likert scale. This measure will be administered at baseline and T2.

6.2.41. Structured Clinical Interview for DSM-5 Disorders (SCID-5)

The SCID is a semi-structured interview for making the major DSM-5 diagnoses. The SCID screener assessment and alcohol module, used to assess current (past 12-month) AUD diagnosis will be completed by appropriately trained research personnel. Any positive responses on the SCID screener will be further assessed by the PI or designee to determine eligibility.

6.2.42. Timeline Follow Back (TLFB)

The Time Line Follow Back will be administered to assess quantity and frequency of alcohol, cigarette and marijuana use and will be completed at the initial screening (for the 30 days prior to that visit) and at each subsequent visit to gather data for every day prior to and including the last visit. Information obtained in this interview will be recorded on the TLFB Calendar and transcribed to the database.

6.2.43. Toxicity Grading Scale Checklist

Guidance document published by the US Department of Health and Human Services Food and Drug Administration titled “Toxicity Grading Scale for Healthy Adult and Adolescent Volunteers Enrolled in Preventive Vaccine Clinical Trials” will be used as a guideline for determining eligibility with regard to lab results and as part of the established study stopping protocol.

6.2.44. Vital Signs

Vital signs include sitting blood pressure, pulse rate (after sitting for at least 3 minutes), weight and body temperature. Values will be recorded on the visit checklist and entered into the database. Vital signs will be collected at screening and at 5 time points during the experimental visit (baseline, T1, T2, T3 and T4).

7. SAFETY MONITORING PLAN

Safety monitoring will be conducted throughout the study; therefore safety concerns will be identified by continuous review of the data by the PI, medical personnel internal quality assurance checks, and DSMB.

7.1. PI and Medical Personnel Safety Monitoring

Participants will be given a 24-hour telephone number for calling the physician to discuss side effects, and physician office hours will be available as needed. Adverse events, including signs of sickness will be collected in an open-ended way at each in-person study visit. Vital signs will be monitored at multiple time points during the experimental study visit. Alcohol withdrawal will be monitored at each visit through administration of the CIWA, and any significant withdrawal, as indicated by a score of 8+ on the CIWA will be reported to the study physician and/or nurse

practitioner immediately. In the event that significant medical problems are encountered, the study blind will be broken and appropriate medical treatment will be provided.

7.2. Internal Quality Assurance Monitoring

The PI will designate appropriately qualified personnel to periodically perform quality assurance checks at mutually convenient times during and after the study. These monitoring visits provide the opportunity to evaluate the progress of the study and to obtain information about potential problems. The monitor will assure that data are accurate and in agreement with any paper source documentation used, verify that subjects' consent for study participation has been properly obtained and documented, confirm that research subjects entered into the study meet inclusion and exclusion criteria, verify that study procedures are being conducted according to the protocol guidelines, monitor review AEs and SAEs, and assure that all essential documentation required by Good Clinical Practices (GCP) guidelines are appropriately filed. At the end of the study, they will confirm that the site has the appropriate essential documents on file, and advise on storage of study records.

7.3. Data and Safety Monitoring Board (DSMB)

An independent DSMB of external advisors will meet prior to the start of the study, annually during enrollment and follow-up and at trial end to review safety data. The Board will be blinded to subjects' actual randomized group assignments but may request at any time that the blind be broken, if concerns arise from the blinded data. In addition to annual meetings, the DSMB will meet after half of the subjects (38) have been randomized to review safety data and the integrity of the study (i.e., an evaluation of the dropout rate and impact on the planned statistical analysis of the data) and make a formal recommendation to the PI on the continuation or early stopping of the study due to safety concerns. *Ad hoc* meetings will be convened if SAEs occur that are considered at least possibly related to the study procedures.

8. STATISTICAL METHODS AND POWER CONSIDERATIONS

To confirm the efficacy of random assignment and to check for group equivalence across demographics, drinking history, and other relevant baseline measures we will perform one-way ANOVAs or Kruskal-Wallis tests on continuous variables and chi-square tests or Fisher's exact tests on categorical items. Descriptive statistics will be computed for continuous variables, and frequencies/proportions for categorical variables to summarize the data. Transformations of the continuous outcome variables will be performed as necessary (i.e. when data do not meet the assumptions of parametric statistical tests).

8.1. Statistical Power

A power analysis was conducted for the study based on results from a previous human endotoxin challenge carried out in a control sample by our collaborators (Eisenberger et al., 2010). Effect size was calculated based on Eisenberger et al.'s reported sample size of N = 39 and F-statistic of 8.13 for increase in negative mood at T2 after endotoxin infusion, yielding Cohen's d = 0.4687. Power analysis was conducted according to Cohen's guidelines to determine the sample size needed to achieve a power ≥ 0.80 (i.e. 80%) at an alpha level of .05. Using the program G*Power version 3.1 and selecting the repeated measures ANOVA, within-between interactions design, where alpha = .05, effect size f = 0.234, we arrived at a total N = 76 completers (38 heavy drinkers and 38 healthy controls). Specifically, we powered the study

to detect differences from baseline to peak (T2) inflammatory response, given that the remaining data points post-peak tend to be similar and may obscure the effects of the inflammatory challenge. Individuals who experience severe sickness symptoms will be excluded (expected to be <5% of the sample).

8.2. Aim 1: To test that low dose endotoxin will increase cue-induced craving for alcohol in heavy drinkers as compared to light drinking controls, and as compared to placebo.

Repeated measures analyses of variance (ANOVAs) will be conducted using PROC GLM in SAS Statistical Software. Specifically, in the repeated measures ANOVA models, Group (Heavy drinking versus light drinking) will be a two level between-subjects factor, Treatment (endotoxin vs. placebo) will be a two level between-subjects factor, and Cue (water cues versus alcohol cues) will be a between-subjects factor. The dependent measure will be self-reported alcohol craving captured by the Alcohol Craving Questionnaire. Sickness symptoms and drinking history (i.e., drinks per day and drinks per drinking day in past year) will be used as covariates in these analyses.

8.3. Aim 2: To test whether low dose endotoxin (0.8 ng/kg of body weight) will increase depressed mood as compared to placebo.

Repeated measures ANOVAs in which Group (Heavy drinking versus light drinking) will be a two level between-subjects factor, and Treatment (endotoxin vs. placebo) is a two level between-subjects factor. The outcome measure is depressed mood (captured by the POMS). Sickness symptoms and drinking history will also be included as covariates in these analyses.

8.4. Exploratory Analysis: To test associations between plasma levels of proinflammatory cytokines (IL-6 and TNF-α), depressed mood, and alcohol craving during the challenge; and examine sex differences in responses to the endotoxin challenge.

Repeated measures ANOVAs will be used to capture the effects of Treatment (endotoxin vs. placebo) by Time (T0 to T2) for the proposed planned outcomes (i.e. proinflammatory cytokines, hourly craving scores) as well as models that include sex as a between-subjects factor. As with the primary aims, sickness symptoms and drinking history will be entered in these models as covariates.

8.5. Exploratory Analysis: To test the effect of low dose endotoxin on neural alcohol cue-reactivity

<u>fMRI Data Processing:</u> FSL 6.0 (www.fmrib.ox.ac.uk/fsl) will be used for the neuroimaging analyses. Motion correction will be carried out using the Motion Correction Linear Image Registration Tool (McFLIRT, Version 5.0) with the estimated motion parameters entered as covariates in the general linear model. Brain Extraction Tool (BET) will be used for non-brain tissue/skull removal. The images will be smoothed using a FWHM Gaussian kernel (5 mm) and high-pass filtered (100s cutoff) in the temporal domain with the FMRI Expert Analysis Tool (FEAT, Version 6.0). The EPI images will first be registered to the MBW, then to the MPRAGE using affine linear transformations, and into standard MNI space. Registration to standard space will be refined by FSL's FNIRT nonlinear registration.

fMRI Data Analysis: Explanatory variables for the alcohol cues task will be created by convolving delta functions representing the onset of experimental events (alcohol, non-alcohol, blurred, and fixation cues; 24-s duration) with a double-gamma hemodynamic response function in FEAT. Temporal derivatives will be included as covariates. A 2X2 ANOVA in which Group (heavy drinker versus light drinker) will be a two level between-subjects factor and Treatment (endotoxin vs. placebo) is a two level between-subjects factor. The main contrast of interest will be activation during alcohol vs. non-alcohol blocks (ALC vs. BEV). Z-statistic images will be thresholded with cluster-based corrections for multiple comparisons based on the theory of Gaussian Random Fields with a cluster-forming threshold of $Z>3.1$ and a cluster-probability threshold of $p<0.05$. Sickness symptoms will also be included as covariates in these analyses.

8.6. Exploratory Analysis: To determine the effect of low dose on reward responsiveness

Repeated measures ANOVAs in which Group (Heavy drinking versus light drinking) will be a two level between-subjects factor, and Treatment (endotoxin vs. placebo) is a two level between-subjects factor. The outcome measure is reward responsiveness (captured by the Probabilistic Reward Task and Reward Responsiveness Scale). Sickness symptoms and drinking history will also be included as covariates in these analyses.

9. ETHICS

9.1. IRB Review

The study will be conducted under a protocol reviewed by the UCLA IRB; the study is to be conducted by scientifically and medically qualified persons; the benefits of the study are in proportion to the risks; the rights and welfare of the subjects will be respected; the physicians conducting the study will ensure that the hazards do not outweigh the potential benefits; the results to be reported will be accurate; subjects will give their informed consent and will be competent to do so and not under duress; and all study staff will comply with the ethical principles in 21 Code of Federal Regulations (CFR) Part 50 and the Belmont Principles.

9.1.1. Protocol Modifications

All necessary protocol changes will be submitted in writing as protocol amendments to the IRB by the PI for approval prior to implementation.

9.1.2. Protocol Deviation Reporting Procedures

All subject-specific deviations from the protocol are to be documented. The PI or designee will be responsible for identifying and reporting all deviations, which are occurrences involving a procedure that did not follow the study protocol. Any protocol deviation that adversely affects the safety or rights of a subject or scientific integrity of the study is considered a major deviation and will be reported immediately to the UCLA IRB.

9.2. Ethical Conduct of the Study

This study will be conducted in accordance with all applicable Federal human research protections requirements and the Belmont Principles of respect for persons, beneficence, and justice. The procedures set out in this study are designed to ensure that all study personnel

abide by the principles of the ICH GCP Guideline and the Code of Federal Regulations (CFR). The PI confirms this by signing FDA Form 1572.

9.2.1. Confidentiality of Data and Subject Records

To maintain subject confidentiality, all laboratory specimens, eCRFs, reports and other records will be identified by a subject number only. Research and clinical records will be stored in a locked cabinet. Only research staff, and other required regulatory representatives will have access to the records. Subject information will not be released without written permission. The PI has received a Certificate of Confidentiality for this study.

9.2.2. Compensation for Participation

Subjects will be compensated for travel expenses and for time contributed to this research study in the form of cash. Compensation will be provided at each subject visit and is detailed in the informed consent form.

9.2.3. Written Informed Consent

The informed consent process and document will be reviewed and approved by the IRB and prior to initiation of the study. The consent document contains a full explanation of the possible risks, advantages, and alternate treatment options, and availability of treatment in the case of injury, in accordance with 21 CFR Part 50. The consent document indicates that by signature, the subject, permits access to relevant medical records as described above. A written informed consent document, in compliance with 21 CFR Part 50, 32 CFR Part 219, and the Belmont Principles, and HIPAA Authorization will be signed by the subject before any study-related procedures are initiated for each subject. All potential subjects for the study will be given a current copy of the Informed Consent Form to read. All aspects of the study and informed consent will be explained in lay language to the subject by either the investigator, or a medically trained designee. Any subject who is unable to demonstrate understanding of the information contained in the informed consent will be excluded from study participation.
All study subjects will be given a copy of the signed informed consent.

9.2.4. Delegation of Responsibilities and Adequate Resources

The PI should have adequate time to conduct the study properly and should have an adequate number of qualified staff to assist with the conduct of the study. The term “investigator” used throughout this protocol refers to the PI and/or qualified Sub-investigators. The PI may delegate responsibilities to other study site personnel. The PI shall delegate tasks only to individuals qualified by education, training, and experience to perform the delegated tasks. The PI shall have direct oversight of all delegated activities and shall document delegation of responsibilities. The PI is responsible for ensuring all delegated staff has been properly trained on the protocol and their assigned study responsibilities. A delegation log identifying all delegated duties and the individual to whom they have been delegated will be maintained at the study site.

9.2.5. Financial Disclosure

Clinical investigators are required to provide financial disclosure information for the submission of certification or disclosure statements required under 21 CFR § 54. As defined in 21 CFR §54.2, a clinical investigator is a listed or identified investigator or sub-investigator who is

directly involved in the treatment or evaluation of research subjects. The term also includes the spouse and each dependent child of the investigator. In addition, investigators must promptly update financial disclosure information if any relevant changes occur during the course of the investigation and for 1 year following completion of the study.

10. DATA HANDLING AND RECORD KEEPING

Source documents include but are not limited to original documents, data and records such as hospital/medical records (including electronic health records), clinic charts, laboratory results, data recorded in automated instruments, and pharmacy records, etc. This study will use an electronic data capture (EDC) eCRF system (Qualtrics) and paper source documents. Data will be transcribed from source documentation directly into a statistical program such as SPSS. Only questionnaire data will be entered directly into eCRF (i.e., without prior written or electronic record of data). Paper copies of the eCRFs will be available in the event that the EDC is not accessible at the time the questionnaire is being completed. The transcribed data will be consistent with the source documents or the discrepancies will be explained. All entries, corrections, and alterations will be made by the investigator or other authorized study personnel. The EDC system maintains a full audit trail of data entry, data corrections, and data queries.

10.1. Subject Identification and Confidentiality

Subjects will be identified on eCRFs and paper source documents by a unique subject number. No personal identifier will be used in any publication or communication used to support this research study. The subject number will be used if it becomes necessary to identify data specific to a single subject. Regulatory bodies, such as the FDA and IRB, are eligible to review medical and research records related to this study as a part of their responsibility to protect human subjects in clinical research. Personal identifiers will be removed from photocopied or electronic medical and research records.

10.2. Retention of Records

The investigator is responsible for creating and/or maintaining all study documentation required by Title 21 Code of Federal Regulations (21CFR) Parts 50, 54, 56, and 312, ICH E6 section 8, as well as any other documentation defined in the protocol. Federal and local regulations require that the investigator retain a copy of all regulatory documents and records that support the data for this study for whichever of the following is the longest period of time:

- A period of 2 years following the final date of approval by the FDA or other regulatory agency of the study drug for the purposes that were the subject of the investigation; or
- A period of 5 years following the date on which the results of the investigation were submitted to the FDA or other regulatory agency in support of, or as part of, an application for a research or marketing permit for the study drug for the purposes that were the subject of the investigation.

If the investigator retires, relocates, or for other reasons withdraws from the responsibility of keeping the study records, custody must be transferred to a person who will accept the responsibility.