Protocol and Statistical Analysis Plan

Frontal E/I balance mediation of tACS effects on behavioral flexibility

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Master Protocol Document

Title	Frontal E/I balance mediation of tACS effects on behavioral flexibility
Sub-Title	FLAME Study
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I have read, understood, and approved this version of the protocol. [electronic signatures accepted]

Principal Investigator: _____ Date: 10/02/2024

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Statement of Compliance

This study will be conducted as specified in the protocol and in accordance with the *International Conference on Harmonisation Guidelines for Good Clinical Practice* (ICH E6) and the *Code of Federal Regulations on the Protection of Human Subjects* (45 CFR Part 46).

The protocol, informed consent form(s), recruitment materials, and all participant materials will be submitted to the *Institutional Review Board* (IRB) for review and approval. Approval of both the protocol and the consent form must be obtained before any participant is enrolled. Any amendment to the protocol will require review and approval by the IRB before the changes are implemented to the study. In addition, all changes to the consent form will be IRB-approved; a determination will be made regarding whether a new consent needs to be obtained from participants who provided consent, using a previously approved consent form.

If required by the IRB, the master protocol document, informed consent form(s), recruitment materials, and all participant materials will be submitted to the *Scientific Review Committee* (SRC) prior to IRB review (research.unc.edu/clinical-trials/src).

The statistical analysis plans will be consistent with guidance in CONSORT Statement [1] or STROBE Statement [2], ICMJE recommendations [3], the 2016 and 2019 statements of the American Statistical Association [4,5], and recommendations in Nature [6,7].*

All personnel involved in the conduct of this study have completed human subjects protection training.

* [1] www.consort-statement.org
[2] www.strobe-statement.org
[3] www.icmje.org
[4] Wasserstein RL, et al. (2016), The ASA's Statement on p-Values, ***The American Statistician***, 70:2, 129-133
[5] Wasserstein RL, et al. (2019), Moving to a World Beyond p < 0.05, ***The American Statistician***, 73:sup1, 1-19
[6] Amrhein, et al. (2019) Scientists rise up against statistical significance, ***Nature*** 567, 305-307
[7] Editorial (2019) It's time to talk about ditching statistical significance: Looking beyond a much used and abused measure would make science harder, but better. ***Nature*** 567, 283-283.

Table of Abbreviations

AE / SAE	adverse event / serious adverse event
aIC	anterior insular cortex
AIE	adolescent binge drinking
ANCOVA	analysis of covariance
AUD	alcohol use disorder
CFR	U.S. Code of Federal Regulations (www.eCFR.gov)
CI	confidence interval
CITI	Collaborative Institutional Training Initiative
CON	control group
CONN	functional connectivity toolbox (web.conn-toolbox.org)
CONSORT	Consolidated Standards of Reporting Trials (www.consort-statement.org)
CZ	central midline in the 10/20 EEG system
DOI	digital object identifier
dLPFC	dorsolateral prefrontal cortex
DSA	Data Submission Agreement
DSMB	data and safety monitoring board
EEG	electroencephalogram
E/I	excitatory/inhibitory
F3	left dorsolateral prefrontal cortex in the 10/20 EEG system
F4	right dorsolateral prefrontal cortex in the 10/20 EEG system
FC	functional connectivity
FDA	U.S. Food and Drug Administration (www.fda.gov)
FDR	false discovery rate
fMRI	functional magnetic resonance imaging
GABA	Gamma-amino-butyric acid
Glx	glutamate + glutamine
GUID	Global Unique Identifier
HABIT	Hidden Association Between Images Task
ICMJE	International Committee of Medical Journal Editors
IL-6	interleukin-6
IRB	institutional review board
MPD	master protocol document
MR	magnetic resonance
MRI	magnetic resonance imaging
MRS	magnetic resonance spectroscopy
N	number of enrolled participants
NADIA	Neurobiology of Adolescent Drinking in Adulthood
NDA	NIMH Data Archive
NIAAA	National Institute on Alcohol Abuse and Alcoholism
$NIAAA_{DA}$	NIAAA Data Archive
NIH	National Institutes of Health
NIMH	National Institute of Mental Health
NoA	Notice of Award
PHI	protected health information
PI	principal investigator
QA	quality assurance
QC	quality control
REDCap	Research Electronic Data Capture system
ROI	region of interest
RPPR	Research Performance Progress Report
SRC	UNC Scientific Review Committee (research.unc.edu/clinical-trials/src)
STROBE	STrengthening the Reporting of OBservational studies in Epidemiology.
SUD	substance use disorder
tACS	transcranial alternating current stimulation
TNF-alpha	tumor necrosis factor alpha

UNC	The University of North Carolina
UNCCH	UNC Chapel Hill
WHO	World Health Organization

1. Protocol Synopsis

Title	Frontal E/I balance mediation of tACS effects on behavioral flexibility
Study Description	This proposal is designed to probe the role of excitatory and inhibitory (E/I) signaling in key nodes of control circuitry in mediating the relationship between alcohol misuse and inflexible behavior. In addition, we aim to determine whether adolescent binge alcohol exposure amplifies the effects of binge exposure in adulthood. We will accomplish this goal via a single multi-session study. Participants (n=66) will comprise three groups: adults self-reporting high risk drinking (WHO risk levels 2, 3, or 4), with (n=22) or without (n=22) a history of adolescent alcohol misuse (AIE), and lifetime low risk drinking adults (WHO risk levels 0 or 1; n=22). Design: a 3-session study that includes an initial screening session and behavioral training (Session 1), behavioral testing and an MRI scan session (Session 2), bifrontal 10Hz-tACS (true or sham) during behavioral testing with pre- and post-EEG recording in a resting-state, followed by a second MRI scan session (Session 3). We predict that adolescent and adult binge history will have interacting effects on E/I balance indices derived from EEG and MR-spectroscopy and on behavioral flexibility measured in the HABIT Test and that E/I balance indices will mediate the relationship between alcohol misuse and behavioral flexibility. We also propose to test a causal relationship between E/I balance and behavioral flexibility by testing whether 10Hz-tACS to bilateral dlPFC alters habitual action selection in the HABIT Test in proportion to its effects on the dlPFC 1/f EEG slope and/or the MRS-derived GABA/Glx ratio. We predict that changes in indices of E/I balance induced by tACS will inversely associate with changes in habitual response selection. We will collect a small amount of blood from a finger prick in Sessions 1 and 3 and will use the collected dried blood spots to measure inflammatory markers.
Specific Aims **(objectives)**	**Aim 1.** Compare groups in terms of Session 2 (baseline) perseverative errorsin the HABIT Test and MRS-derived GABA:Glx, and Session 3 baseline 1/f EEG slope. **Aim 2.** Change behavioral flexibility via (transcranial alternating current stimulation (tACS), and test whether changes in E/I measures mediate this change. **Aim 3.** Test whether MRS-derived GABA:Glx, or 1/f EEG slope in fronto-limbic nodes correlate with functional MRI connectivity of these nodes.
Target Population	**Inclusion Criteria** • 22-50 years old • Medically healthy • Fluent in English • High school educated • Group 1 (CON): no AIE and current (past month) WHO risk drinking levels of either 0 or 1 (abstinent or low-risk) • Group 2 (RISK): no AIE and current WHO level 2, 3 or 4 (medium risk, high risk, or very high risk) • Group 3 (RISK+AIE): history of adolescent binge drinking (4 or more binge episodes before age 18; per NIAAA, a

	binge episode = 4+ drinks in a 2-hr period for females and 5+ drinks in a 2-hr period for males) and current WHO risk level 2, 3, or 4. **Exclusion Criteria** • Lifetime history of an SUD (including nicotine) but participants will not be excluded for an AUD. • Neurological disease such as dementia, seizures or head trauma • History of psychosis or psychotic episodes • Diagnosis of ADHD • Systemic disease such as cancer, cardiovascular or inflammatory disease which could influence cognitive functioning • Motor or visual disturbance (including color blindness) which precludes them from performing the tasks • Current (past month) use of psychoactive drugs (aside from caffeine or alcohol) • Current (past month) use of psychotropic medication including antidepressants, mood stabilizers, antipsychotics, anxiolytics, stimulants, or hypnotics with the exception of stable doses of antidepressants for one month or more. • Past month use of therapeutic brain stimulation (e.g. TMS or ECT) • Any MRI contraindication based on BRIC's MRI safety checklist • History of brain surgery • History of migraine headaches • Pregnancy • Brain implants/devices • First degree relative with primary epilepsy • Claustrophobia • Breath alcohol above 0.0% at time of session • Positive urine drug screen at time of session
Numbers of Enrollees	A total of N = 66 eligible individuals will be enrolled. We anticipate that at least n = 64 of the enrollees will complete all aspects of the protocol and have complete data.
Interventions	In this double-blinded randomized design, participants will receive a single session of 10 Hz bi-frontal tACS or active Sham tACS during the second half of the HABIT Test in Session 3. 10 Hz bi-frontal tACS: Alternating current stimulation is delivered by an XCSITE 100 device (Pulvinar Neuro, Chapel Hill, NC), through three conductive carbon-rubber electrodes. Electrodes are placed over the apex of the head (Cz) and the prefrontal cortex bilaterally (F3 and F4). Stimulation parameters: 2mA peak-to-peak 10Hz sine-wave flanked by 10 second linear envelope ramps in and out for a total duration of 30 min and 20 seconds. Sham tACS: The procedure for sham stimulation will be identical, but stimulation will last for 2 minutes within the "stimulation" period of 30 minutes.

Outcome Measures	**For Aim 1.** 1) Habitual action selection, calculated as the proportion of perseverative errors after Stimulus-Response contingency change for highly trained (Familiar) stimuli in the HABIT. 2) Prefrontal GABA:Glx ratio measured via single voxel MRS. 3) Power spectral slope (1/f) of the EEG signal localized to the dlPFC. 4) Concentrations of circulating inflammatory markers, including C-reactive protein, IL-6, and TNF-α measured from dried blood spots. (Secondary outcome) **For Aim 2.** 1) Change in habitual action selection after 10Hz- or sham-tACS. 2) Change in prefrontal GABA:Glx ratio after 10Hz- or sham-tACS. 3) Change in 1/f slope of the EEG signal localized to the dlPFC after 10Hz- or sham-tACS. 4) Change in concentrations of circulating inflammatory markers, including C-reactive protein, IL-6, and TNF-α after 10Hz- or sham-tACS. **For Aim 3.** Change Z-transformed Pearson correlation between bilateral dlPFC and striatum after 10Hz- or sham-tACS.
Statistical Analysis Plans for Each Aim	**Aim 1 Plans.** To test the prediction that E/I balance indices will differ from low-risk drinkers more among high-risk drinkers with adolescent (<18 years old) binge history, we will analyze the baseline (i.e. pre-tACS) data collected in Sessions 2 and 3. We will conduct Analyses of Covariance (ANCOVA) to compare groups in terms of Session 2 (baseline) perseverative errors in the HABIT Test and MRS-derived GABA:Glx, and Session 3 baseline 1/f EEG slope, controlling for age, sex, education, psychiatric diagnoses, and medication status. We will use similar experimental designs to those described to evaluate a number of secondary outcomes. These include C-reactive protein measured from blood spots and other immune activation markers (TNF-α and IL-1β) derived from salivary microvesicles, as well as exploratory/alternative group comparison analyses. The latter include effects of tACS on GABA:Glx ratios in the dlPFC and aIC, and on EEG 1/*f* slope. **Aim 2 Plans.** Effects of tACS on habitual action selection in the HABIT task (primary outcome, Aim 2) will be assessed based on the proportion of perseverative errors (relative to all errors) when the participant must replace a highly trained response to a set of familiar stimuli. We will use a general linear model that includes group and stimulation condition as between-subjects factors; we will also include baseline perseverative error, age, sex, handedness, and anxiety or mood disorder diagnoses as covariates. As for Aim 1, we will use similar experimental designs to those described to evaluate a number of secondary outcome responses to tACS. These include C-reactive protein measured from blood spots and other immune activation markers (TNF-α and IL-1β) derived from salivary microvesicles, as well as exploratory/alternative outcome analyses. The latter include effects

	of tACS on GABA:Glx ratios in the dlPFC and aIC, and on EEG 1/f slope. **Aim 3 Plans.** We will test whether the indirect effect of tACS on HABIT perseverative error is mediated by resting-state functional connectivity. For our mediation analyses, functional connectivity (FC) values derived from a priori ROI-to-ROI (e.g., dlPFC, aIC, and limbic striatum) analyses will be used as the mediating variable; change in HABIT perseverative errors will serve as the dependent variable and will be adjusted for sex, age, handedness, and anxiety or mood disorder diagnoses as covariates; tACS condition (stim/sham) will serve as the independent variable; and AIE and current alcohol misuse scores will be included as moderators. We will also conduct exploratory analyses testing for associations between indices of E/I balance in the dlPFC and dlPFC FC at baseline, and whether changes in E/I balance indices associate with changes in dlPFC FC.
Study Duration	We anticipate the study duration to last 4 years.
Participation Duration	We anticipate the participation duration to include 3 sessions over the course of 3 days (minimum) or within 2 weeks.
Enrollment Duration	Slightly less than 4 years.

2. Introduction

2.1. Background Information

Problematic alcohol use is a major health crisis in the United States, with approximately 13% of Americans meeting the diagnostic criteria for an AUD (Grant et al., 2017; Grant et al., 2015). AUD diagnoses are associated with significant decreases in quality of life, as well as increased risk of early mortality (Hasin et al., 2007; Rehm et al., 2016). Even below the clinical threshold, binge drinking contributes significantly to risk of death, head injuries, hospitalizations and other severe consequences – particularly in adolescents (CDC, 2012; Siqueira & Smith, 2015). The development of tolerable and efficacious interventions for risky drinking would benefit a large portion of the American population, especially given the current gaps in treatment utilization for individuals with AUDs (Cohen et al., 2007).

A major risk factor for later development of an AUD Is binge drinking during adolescence (Siqueira & Smith, 2015; Viner & Taylor, 2007). Preclinical and human literature both indicate that alcohol use during adolescence can result in lasting changes to the brain (Crews et al., 2016; Ewing et al., 2014; Guerri & Pacual, 2010). Individuals who engage in binge drinking during adolescence are likely to continue these patterns into early adulthood (Degenhardt et al., 2013). Adolescent alcohol use may even increase vulnerability to future assaults, particularly those resulting from heavy drinking in adulthood.

One behavioral feature associated with heavy alcohol or substance use is overreliance on habitual action selection, as opposed to goal-directed decision-making (Ersche et al., 2016; McKim et al., 2016). The regions involved in top-down modulation of behavior are commonly referred to as the cognitive control circuitry (Friedman & Robbins, 2022; Miller & Cohen, 2001). The dlPFC is one element of this fronto-striatal circuitry involved in habit formation and decision-making (Boettiger, 2005; Brosnan & Wiegand, 2017; Smittenaar et al., 2013). The involvement of the dlPFC in adaptive decision-making has been repeatedly demonstrated in the human literature, as has involvement of the analogous rodent

prelimbic cortex in stimulus-response tasks (Smittenaar et al., 2013; Wood & Rünger, 2016). The dlPFC is a major hub of the cognitive control circuitry involved in adaptive decision-making, and it represents a good target for non-invasive neuromodulation due to its proximity to the scalp (Gbadeyan et al., 2016; Vanderhasselt et al., 2013).

Chronic alcohol exposure modifies GABAergic activity in the CNS long-term, causing plastic changes that suppress inhibition mediated by GABA receptors (Davies, 2003; Olsen & Liang, 2017). Unpublished MRS data from our lab indicate that binge drinking in adulthood is associated with decreased levels of GABA in the dlPFC. Binge drinking was also associated with higher levels of glutamate in the region, resulting in a disrupted balance between excitatory and inhibitory firing. These data indicate that heavy drinking may shift E/I balance toward excitation. Behaviorally, frontal glutamate to GABA ratio was positively correlated with habitual action selection. This is consistent with the literature, in which higher E/I balance has been associated with impulsivity in decision making (Lam et al., 2022). We hypothesize that E/I balance in control circuitry hubs may be shifted toward excitation by repeated binges during a critical developmental period, and that this shift results in overreliance on habitual action selection. This shift in E/I balance may make one more susceptible to problematic drinking in adulthood through disruptions in adaptive and goal-based decision making. This process may be a positive feedback cycle in which shifted E/I balance leads to more drinking, and more drinking further shifts E/I balance and thus habitual tendencies.

Previous research has demonstrated that non-invasive neurostimulation of the dlPFC can modulate decision-making (Ostlund, 2005). However, the literature as a whole is equivocal on the efficacy of non-invasive neurostimulation to modulate behavior (Brunye et al., 2020; Sellers et al., 2015). The mechanisms of neurostimulation remain poorly understood, but a reasonable assumption is that efficacy and directionality of effects would be dependent on a number of factors, including scalp-to-cortex distance, priming, and individual differences in circuitry (Brunye et al., 2020; Kearney-Ramos et al., 2018). Data from our lab showed that controls and individuals with a history of SUD were differentially affected by 10Hz tACS bilaterally to the dlPFC, such that controls showed an increase in habitual behavior, whereas individuals with a history of SUD trended toward improved adaptive behavior (McKim et al., 2021). We hypothesize that the directionality of behavioral modulation by dlPFC tACS may be dependent on E/I balance at baseline.

The main purpose of this study is to examine the role of frontal excitatory/inhibitory (E/I) balance in manifesting the adult behavioral phenotypes associated with adolescent binge drinking, as well as determining the potential efficacy of tACS to modulate E/I balance and associated behaviors. We hypothesize that risky drinking in adulthood will shift resting dlPFC E/I balance toward excitation, and that adolescent (before age 18) alcohol use will exacerbate this shift. We predict that this shift will mediate behavior such that higher E/I ratios will be associated with an increase in perseverative errors in an adaptive learning task, and that tACS-induced changes in E/I balance will mediate behavioral changes after stimulation.

2.2. Scientific Rationale

We have selected 10Hz bilateral tACS to the dlPFC as an intervention to target disrupted E/I balance resulting from adolescent binge drinking and contributing to an excessively habitual behavioral profile and risky drinking in adulthood. Transcranial electrical stimulation is a non-invasive, temporary intervention that is generally well-tolerated and has a very low risk of adverse side effects (Matsumoto & Ugawa, 2017). As described in Background, our lab previously performed 10Hz bilateral tACS to the dlPFC in a sample of individuals with a history of SUD and saw a trend toward improvement in performance on the HABIT task (McKim et al., 2021). In this study we aim to examine the role of stimulation-induced shifts in frontal E/I balance in these behavioral changes, specifically by recording resting-state EEG immediately prior to and immediately after stimulation. We will also be examining the lasting effects of tACS (on the scale of hours) using baseline and post-stimulation fMRI scanning. The literature indicates that tACS allows for entrainment of brain activity to a specific frequency, although

circuit effects are poorly understood (Herrmann et al., 2013; Zaehle et al., 2010). Additionally, this study will provide the first test of modulating E/I balance – determined through resting-state EEG 1/f slope and MRS – using 10Hz tACS. Our lab has performed hundreds of sessions of tACS using the Pulvinar Neuro XSCITE100 system with no adverse events reported.

3. Specific Aims

3.1. Aim 1

Compare groups in terms of Session 2 (baseline) perseverative errors in the HABIT Test and MRS-derived GABA:Glx, and Session 3 baseline 1/f EEG slope.

1a: Compare perseverative error rates in the HABIT test at session 2 (pre-tACS) across all 3 groups

1b: Compare MRS-derived GABA:Glx at session 2 (pre-tACS) across all 3 groups

1c: Compare Session 3 baseline 1/f EEG slope across all 3 groups

Hypothesis 1: Perseverative error rates will be higher in the Hazardous drinking groups, with the highest rates in the adolescent binge alcohol exposure group, and GABA:Glx ratio and 1/f EEG slope will be lower in the hazardous drinking groups, with the lowest ratios and slopes in the group with adolescent binge alcohol exposure, tracking with the theory that hazardous alcohol use, especially in the adolescent period, drives reduced inhibitory signaling in executive control circuitry, shifting E/I balance and reducing behavioral flexibility.

3.2. Aim 2

Change behavioral flexibility via (transcranial alternating current stimulation (tACS), and test whether changes in E/I measures mediate this change.

2a: Compare 10Hz and sham tACS effects on perseverative error in the HABIT test (session 3) compared to baseline (session 2).

2b: Compare 10Hz and sham tACS effects on GBA:Glx ratio and 1/f EEG slope (session 3) compared to baseline.

2c: Test whether tACS induced changes in GABA:Glx of1/f EEG slope mediate changes in behavioral flexibility after tACS.

Hypothesis 2: 10Hz-tACS to bilateral dlPFC will change habitual action selection in the HABIT Test in proportion to its effects on the dlPFC 1/f EEG slope and/or the MRS-derived GABA/Glx ratio. We predict that changes in reductions in 1/f slope and in MRS-derived GABA:Glx induced by 10Hz-tACS will associate with increased habitual response selection.

3.3. Aim 3

Change functional MRI connectivity (fcMRI) of fronto-limbic circuitry via tACS.

3a: Compare 10Hz and sham tACS effects on fcMRI of *a priori* ROI (dlPFC, aIC, and limbic striatum) in session 3 compared to baseline (session 2).

3b: Conduct mediation analyses with fcMRI changes to identify connections that mediate the effects of tACS on habitual responding.

3c: Conduct exploratory analyses testing for associations between indices of E/I balance in the dlPFC and dlPFC fcMRI at baseline, and whether changes in E/I balance indices associate with changes in dlPFC fcMRI.

Hypothesis 3: fcMRI of the dlPFC will correlate with indices of E/I balance and habitual action selection before and after 10Hz-tACS to bilateral dlPFC

4. Investigational Plan

Study Design: This is a randomized, double-blind, sham-controlled tACS study.

Brief overview of study events: Potential participants will complete eligibility screening for the study. Participants meeting eligibility criteria will complete a baseline session including further eligibility screening, training on the computerized HABIT task, and collection of urine, saliva, and blood samples. Participants will then complete a session involving the full version of the HABIT task and MRI scanning. The final session will involve the full HABIT task, resting-state EEG, sham or active tACS, MRI, and collection of another blood spot. Participants will also complete a series of online questionnaires outside of their scheduled sessions. See Figure 1 for depiction of study procedures.

Study Duration: 4 years

Target Number of Participants: 66 adults comprising three groups: high risk drinkers with (n=22) or without (n=22) a history of adolescent alcohol misuse, and lifetime low risk drinkers.

***tACS*:** For Session 3, participants will complete a HABIT Test Session in the Howell Hall Neurostimulation Core Lab or EEG Core Lab, which is located one floor below the Boettiger Lab. Participants will receive either 10Hz bi-frontal tACS or sham tACS.

10 Hz bi-frontal tACS: Alternating current stimulation is delivered by an XCSITE 100 device (Pulvinar Neuro, Chapel Hill, NC), through three conductive carbon-rubber electrodes. Electrodes are placed over the apex of the head (Cz) and the prefrontal cortex bilaterally (F3 and F4). Stimulation is delivered during the second half of the HABIT Test session. Stimulation parameters: 2mA peak-to-peak 10Hz sine-wave flanked by 10 second linear envelope ramps in and out for a total duration of 30 min and 20 seconds.

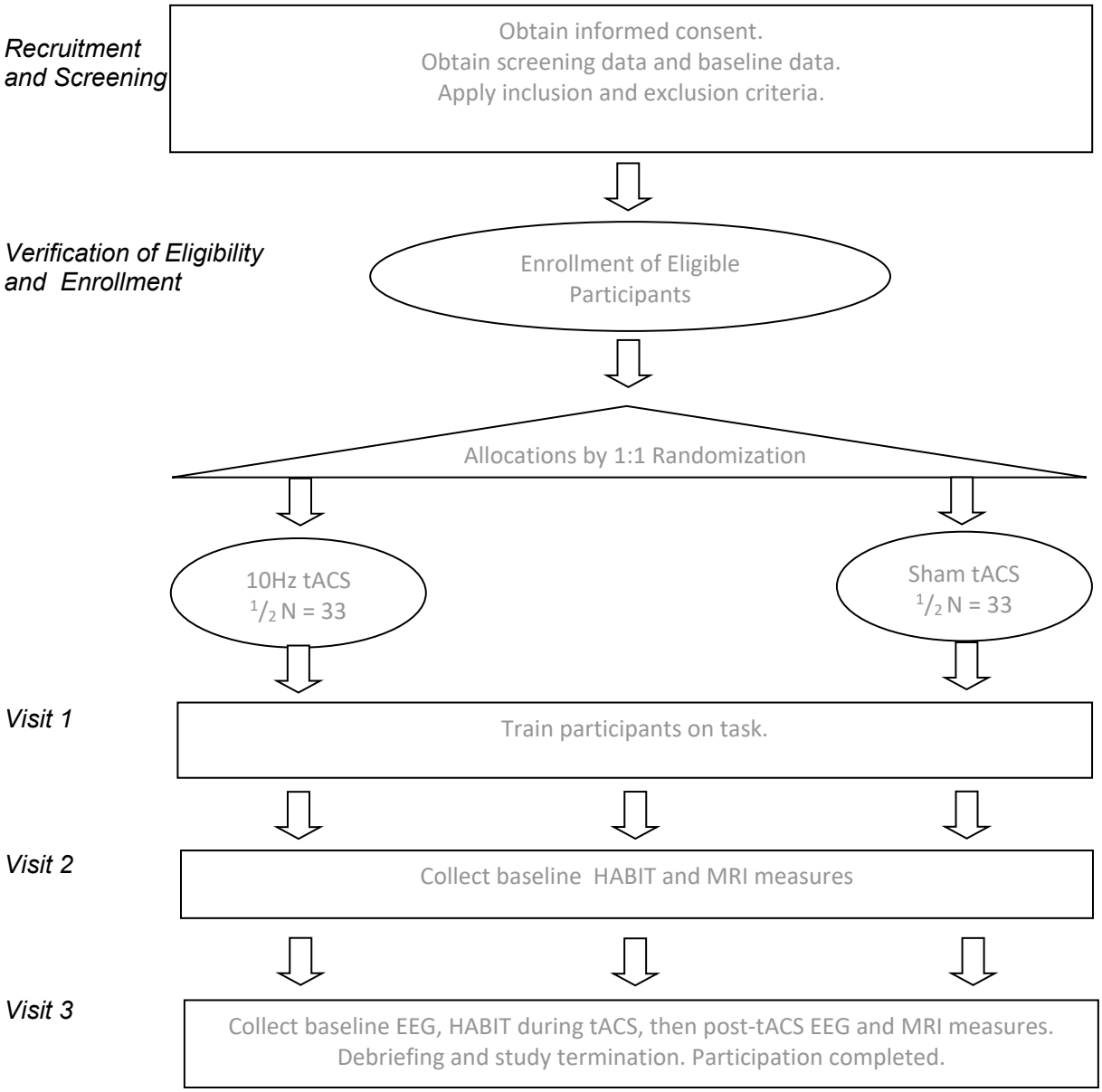
Sham tACS: The procedure for sham stimulation will be identical, but the actual stimulation will last for 2 minutes instead of 30 minutes. Participants generally report that stimulation is felt most strongly at the beginning of active stimulation, before they adjust to the sensation. Sham stimulation is meant to mimic this progression in terms of tactile salience. Stimulation is delivered for 2 minutes at the beginning of the HABIT reversal task, flanked by 10 second linear envelope ramps. The stimulating electrodes are left on the head until completion of the HABIT task, as is the case in active stimulation. There is no visual or auditory indication to the participant or researcher when the 2-minute sham stimulation period has ended, allowing the sham stimulation condition to feel similar to active stimulation. The XSCITE 100 device allows for the programming of stimulation codes corresponding to sham or active stimulation, both of which are set to a total duration of 30 minutes, allowing for reliable double-blinding.

HABIT Task: The HABIT task is a computerized measure of behavioral flexibility; it has been used by our lab in multiple previous studies. The task is composed of two parts: the adaptive learning task and the reversal task. The HABIT is also preceded by a necessary training module. The task is composed of a series of visual patterns defined by consistent color blocking. Patterns come in sets and participants learn to pair each pattern within a set of two with a specific button-press response by pressing buttons and receiving auditory and visual feedback. During training, participants are presented with one set of two patterns and are allowed to learn the button-press responses associated with each pattern. This is the familiar set and will reappear throughout the task. During the adaptive learning portion of the task,

participants are presented with two or three new sets of patterns and are allowed to learn the button-press responses corresponding to the patterns within each set. These are the novel sets – they will only be presented during this iteration of the adaptive learning and reversal tasks. During the reversal task (part 2), the novel and familiar sets are presented again, but the button-press responses have changed for one familiar set and one novel set. The adaptive learning portion of the task is intended to measure one's ability to learn stimulus-response associations. The reversal portion of the task, performance on which is our primary behavioral interest in this study, is intended to measure behavioral flexibility, or one's ability to update stimulus-response associations that have changed by adapting to feedback.

Timing of Visits: Eligibility screening will occur up to two weeks before session one. Session 1 will occur on day 0, and session 2 will occur at least one night's sleep after session one to allow for memory consolidation of HABIT training sets (max 2 weeks). Session 3 can occur at any time after the completion of session 2, ideally within 2 weeks, and the online questionnaires can be completed any time after session 1 and prior to session 3. We anticipate most participants will choose to complete the questionnaires between sessions at the time and location of their choosing; if not they will be given the option to complete them onsite during session one, two, or three. If the online questionnaires are not completed prior to session 3, they will be completed by the participant onsite at the end of session 3 prior to payment.

Figure 1. Randomized Protocol Schematic.



5. Study Participants

5.1. Numbers of Participants

We plan to enroll 66 individuals total (22 in each group). With possible attrition or loss of data, we expect 64 individuals to complete the study.

5.2. Eligibility Criteria

We chose the criteria described here in order to ensure participant safety, minimize confounds, and optimize recruitment opportunities. The following exclusion criteria are meant to exclude individuals who may be at an elevated risk for adverse events during tACS: history of brain surgery, history of migraine

headaches, family history of epilepsy, or pregnancy. The safety of tACS for these populations has not yet been determined, and our lab has administered hundreds of sessions of tACS with no adverse events using these exclusion criteria. The following exclusion criteria are meant to exclude individuals who should not enter the MRI scanner: presence of any brain implants or devices, or claustrophobia.

In order to reduce the number of confounding variables, we have chosen to exclude individuals whose task performance, brain activity, or inflammatory markers may be affected by external factors unrelated to drinking history. Diagnosis of any neurological disease or ADHD, symptoms of psychosis, diagnosis of systemic or inflammatory disease, presence of motor or visual disturbances, or use of psychotropic medications or psychoactive drugs will all be exclusionary for this reason. A high school diploma or equivalent and fluency in English will also be required to control for confounds in task performance. Although use of psychotropic medications will generally be exclusionary, individuals who have been on a stable dose of an antidepressant for 1 month or more will still be eligible. We believe this will allow for the recruitment of a sample that is more representative of Americans who drink alcohol.

In order to ensure that individuals are fully lucid and able to consent to participate in this study, a breath alcohol level above 0.0% or a positive urine drug screen at the beginning of any visit will be exclusionary. If this occurs, the participant will be given the option to reschedule their visit.

There is currently no established threshold for what constitutes meaningful adolescent alcohol exposure in the human literature. For this reason, we have chosen to define our AIE group as those who report 4+ binge episodes before the age of 18 based on previous data from our lab (Elton et al, 2021). We are using the established WHO risk drinking level criteria to define levels of adult drinking (low risk vs. high risk).

5.2.1. Inclusion Criteria

All of the below criteria will be self-reported.

In order to be eligible to participate in this ssitudy, an individual must meet all of the following criteria:

- 22-50 years old
- Have a high school diploma or equivalent
- Medically healthy
- Fluent in English

For **RISK** group only:

- High risk alcohol use in the past month (WHO risk level 2-4)
- No history of adolescent binge drinking

For **RISK+AIE** group only:

- High risk alcohol use in the past month (WHO risk level 2-4)
- High levels of adolescent alcohol use (4 or more binge drinking episodes before age 18)

For **CON** group only:

- Low risk alcohol use throughout the lifetime (WHO risk level 0-1)
- No history of adolescent binge drinking
- No lifetime history of AUD

5.2.2. Exclusion Criteria

Any individual who meets one or more of the following criteria will be excluded from participation (excluding BAC, urine drug screen, psychiatric diagnoses, and color blindness, all will be self-reported):

- Lifetime history of an SUD (including nicotine) but participants will not be excluded for an AUD.
- Neurological disease such as dementia, seizures or head trauma
- History of psychosis or psychotic episodes
- Diagnosis of ADHD
- Any systemic or inflammatory disease that could affect cognitive functioning (e.g., cancer, cardiovascular disease)
- Any motor or visual disturbances that could hinder task performance (e.g., color blindness)
- Use of psychoactive recreational drugs in the past month (excluding caffeine and alcohol)
- Use of psychotropic medications in the past month including antidepressants, mood stabilizers, antipsychotics, anxiolytics, stimulants, or hypnotics (excluding antidepressant use when dosage has been stable for 1 month or longer)
- Use of therapeutic brain stimulation (e.g. TMS or ECT) in the past month
- Any history of brain surgery
- History of migraine headaches
- Pregnancy
- Any brain implants or devices
- First degree relative with primary epilepsy
- Claustrophobia
- Any MRI contraindication based on BRIC's MRI safety checklist
- Breath alcohol above 0.0% at time of session
- Positive urine drug screen at time of session

If a participant should have an exclusion criterion arise in the course of their participation (e.g. pregnancy or psychotic episode), their participation in the study will end unless the circumstances are transitory in nature (e.g. positive breath alcohol or urine drug screen).

5.3. Randomization and Blinding

Our study design consists of three groups of participants:

1. (CON): no adolescent binge drinking (AIE) and current (past month) World Health Organization (WHO) risk drinking levels of either 0 or 1 (abstinent or low-risk)
2. (RISK): no AIE and current WHO level 2, 3, or 4 (medium risk, high risk, or very high risk)
3. (RISK+AIE): history of adolescent binge drinking and current WHO risk level 2, 3, or 4.

Within each group (n=22), participants will be randomly assigned to either the 10Hz tACS (stim) group or the sham tACS (sham) group.

Our study design requires randomly assigning individuals to a stimulation group after completing Session 1. Prior to study onset, Dr. Boettiger will create a randomization table in which stimulation type (true or sham) is pseudo randomly ordered within groups, stratified by sex. Dr. Boettiger will provide assignment to the study team based on this predefined randomization table in order to ensure balanced sex and group for each stimulation type. The study team will be provided a numeric code to enter into the XCSITE 100 tACS system, which will conceal the stimulation parameters from the study team, ensuring double blinding.

5.4. Recruitment and Retention

The research team has extensive experience in successfully recruiting participants into multi-session research studies. Dr. Boettiger has conducted many such studies, including those with challenging populations (e.g. alcohol or substance use disorders), or with challenging interventions (e.g. lengthy pharmacological challenges or brain stimulation studies). She has been the PI on 8 different challenge protocols, has been continuously NIH-funded for 14 years, and is currently the Co-PI of an NIAAA-funded (P60AA011605) 4-5 session study investigating the neural circuit bases of behavioral flexibility in adults (22-40). That study entails an EEG and behavioral training session, three transcranial brain stimulation sessions, and an MRI scanning session, with a similar scan protocol to that proposed here. Moreover, the Boettiger laboratory has extensive expertise in working with individuals with a history of hazardous alcohol use. Dr. Boettiger's team is trained in all technical aspects of the proposed study, including screening, cognitive testing, EEG, fMRI and MRS, and tACS. Thus, the team is highly qualified and prepared to recruit and retain sufficient sample sizes for the proposed study.

Recruitment
Participants will be recruited through IRB-approved flyers and advertisements distributed in the local community, which includes the UNC campus and the larger "Research Triangle" area, including Durham, Orange, and Chatham counties. No Protected Health Information (PHI) will be accessed prior to contacting participants. Potential subjects who respond to these advertisements will be contacted initially via telephone or encrypted email. If contacted via email, subjects will be scheduled for an initial telephone screening to determine eligibility status. Those who meet inclusion criteria based on the phone interview will be scheduled for all study sessions. We have completed data collection for a 4-5 session study(n=66) that includes multiple brain stimulation sessions with a drop out rate of only 4%. Moreover, that study included three groups similar to those proposed here but with additional exclusion criteria. Thus, we do not anticipate difficulties with either participant recruitment or retention. Participants are free to discontinue the experiment at any time. If a participant does not complete the entire experiment, we will recruit a replacement participant.

Target Communities for Recruitment: UNC Chapel Hill, North Carolina Central University (an HBCU), Duke University, Durham, Carrboro, Hillsborough, Pittsboro, Apex, Morrisville, and Cary, NC.

Retention
We will promote retention by building rapport with study participants during study sessions, by maximizing ease of participation through flexible session scheduling and by fairly compensating participants for their time. Approaches used by our team in other studies will be used to maximize retention including bonuses for exceeding task performance targets and a study completion bonus. We will also obtain several types of contact information, including phone, email, and social media during the initial screening and at subsequent study sessions. Participants will be paid a bonus of $70 for completing all study sessions. Participants will also receive an email reminder from the study team 24 hours prior to each scheduled session. Given that this study involves only three visits and that the protocols are minimally invasive and generally well-tolerated, we anticipate a high retention rate.

6. Schedule of Activities and Procedures

6.1. Table of Events

Table 3. Schedule of activities and procedures

Procedure		Screening by phone	Visit 1	Visit 2	Visit 3	Early Stop
	Informed consent	X	X	X	X	

Recruit a Sample of Participants	Eligibility assessments	X	X			
	Enrollment		X			
Methods	Electroencephalography (EEG)				X	
	Transcranial alternating current stimulation (tACS) – real or sham				X	
	Magnetic resonance imaging (MRI) – MRS and resting state fMRI			X	X	
Safety Monitoring	Review contraindications to procedures	X	X	X	X	
	Medical history	X	X			X
	Assessment of AEs		X	X	X	X
Screening Labs	Pregnancy test		X	X	X	X
	Urine drug test		X	X	X	X
	Breathalyzer		X	X	X	X
Research Labs	Dried Blood Spot collection		X		X	
	Saliva collection		X			
	Urine collection		X		X	
Computerized Tasks	HABIT training		X			
	HABIT testing			X	X	
	REDCap Questionnaires		X	X		

6.2. Screening

Individuals who are interested in participating can contact the research team via email or phone. These individuals will complete an optional online prescreening (collected with REDCap) followed by a required phone screening. Individuals may also choose to complete only the phone screening. The online prescreening includes a short e-consent form, followed by questions concerning limited demographic information, pregnancy, medical history, history of alcohol use, and current alcohol and substance use. Individuals who appear to meet the eligibility criteria will be contacted via phone to complete the full phone screening, up to 14 days prior to enrollment. The phone screening includes a brief description of study purpose and requirements, followed by collection of verbal consent. The phone screening includes questions pertaining to demographic information, pregnancy, medical history, medication use, contraindications to MRI or tACS, history of alcohol use, psychiatric symptoms, and current use of alcohol and other psychoactive substances. The phone screening will also involve scheduling for the first in-person session.

The purpose of screening is to identify individuals who are members of the target population/s.

6.3. Enrollment

Eligibility will be determined using the screening process described in 6.2. A number of additional eligibility checks will also occur during session one. These include a color blindness test, a survey assessing health history, and administration of the MINI to rule out symptoms of psychosis. At the beginning of every session the participant will be asked to undergo an alcohol breath test and urine drug screen, and will be asked by the research team if there have been any changes to their health since their last session or phone screen.

“Baseline” metrics will be collected throughout the study, as the tACS intervention will not occur until session three. A blood spot for baseline inflammatory markers will be collected during session one, a baseline fMRI scan will be collected during session two, and baseline resting-state EEG data will be collected immediately prior to stimulation during session three.

6.4. Study Visit Procedures

Visit 0 (Day –14 to Day –1): Eligibility screening

- Explanation of study requirements

- Verbal consent to continue with screening
- Eligibility questions
- Scheduling the first session

Visit 1 (Day 0)

- Informed consent
- Alcohol breath test + urine drug screening
    - Aliquoting of urine sample for future analysis
- Ishihara color blindness test
- MINI
- Subject information sheet
- Collection of saliva samples
- Collection of blood spot
- HABIT task training
- Participant departure
- Participant completes questionnaires at home prior to final session

Visit 2 (Day 1 at earliest, Day 7 at latest)

- Informed consent
- Alcohol breath test + urine drug screening
- HABIT practice
- HABIT task
- HABIT reversal task
- Screening for metal prior to entering the magnet
- MRI scan session
- Participant departure

Visit 3 (Day 2 at earliest, Day 14 at latest)

- Informed consent
- Alcohol breath test + urine screening
- HABIT practice
- HABIT task
- Head measurements for tACS electrode placement
- Placement of tACS electrodes
- Placement of EEG net
- Resting-state EEG
- tACS stimulation during HABIT reversal task
- Resting-state EEG
- Removal of tACS electrodes + EEG net
- Collection of blood spot
- Screening for metal prior to entering the magnet
- MRI scan session
- Participant payment
- Participant departure

6.5. Final Visit

At the conclusion of the final testing session, the purposes and predictions of the research will be clearly explained to the participants and any questions they have will be answered.

6.6. Phone Contacts

Considering the complexity of activities, the study team will frequently communicate with participants to remind them about study visits. Study visit reminders will be sent once a day starting 2 days before the study visit via text messaging.

6.7. Enrollees May Drop Out

Participants may voluntarily withdraw from participation at any time, for any reason, with no penalty or loss of rights. The reasons for drop-out and missing data will be documented in the database.

7. Statistical Analysis Plans

7.1. Strategies that Apply to all the Aims

All statistical procedures will be performed in SPSS, and in-house programs written in MATLAB, R, and Python. The PI, Dr. Charlotte Boettiger, currently serves at the biostatistics consultant for the NIAAA-funded NADIA Consortium led by the Bowles Center for Alcohol Studies at the University of North Carolina at Chapel Hill. As such, she will oversee all data analyses. When needed, she will consult with statistical collaborators, including Dr. Hongtu Zhu, or Dr. Daniel Bauer.

- To help ensure replicability of the research, the analysis plans have been finalized prior to collection of data (*a priori*). For each specific aim, the analysis plans specify detailed steps for obtaining estimates of the population parameters of interest (e.g., treatment effects) and for making inferences.
- For each aim, sensitivity analyses will be performed to assess the robustness/fragility of the main results as indicated by their sensitivity to reasonable perturbations of the choices of the methods and assumptions used. Any question about the optimal choice of methods and assumptions are best handled by relegating competing approaches to roles in the domain of sensitivity analyses. Results of the sensitivity analyses will be used to guide our level of trust in the main results.
- Human studies are prone to drop-out, missing data, and interval-censored values. The reasons for drop-outs, missing / censored data values, and protocol departures will be documented in the database. Best practices for dealing with incomplete data will depend on the documented causes of those occurrences. In the analysis plans established *a priori*, the strategies for coping with incomplete data will be based on anticipated causes. Alternative methods for dealing with incomplete data will play important roles in the sensitivity analyses.
- The analyses for each aim will focus on the magnitude and direction of point- and interval-estimates of the population parameters of interest. To indicate precision, all statistical estimates of population parameters will be tabulated along with corresponding confidence intervals (CI). The CI will be interpreted as the set of potential values of the population parameter that are most compatible with the observed data.
- All hypothesis tests yielding large p-values will be reported as being inconclusive. For all sample sizes, all hypothesis test procedures are (by design) incapable of establishing that the null hypothesis is true.
- If p-values are computed they will be reported to several decimal places without categorizing or dichotomizing the p-value; that is, the words “significant” and “non-significant” should be avoided. The p-value should be reported and interpreted as a continuous measure indicating the availability of information against the (null) hypothesis being tested.

- The proposed statistical analysis strategy acknowledges that no p-value can reveal the plausibility, presence, truth, or importance of an association or effect –which is consistent with the statements of the American Statistical Association [4,5], the recommendations in Nature [6,7], and guidance, such as the CONSORT Statement [1], STROBE Statement [2], and ICMJE guidance [3].†]
- The analysis plans will include outcome-dependent exploratory analyses to generate new hypotheses.
- Graphical methods such as forest plots will be used to visualize the analysis results.
- Possible study limitations are that this is a relatively small sample, and the experiments include numerous variables. As such, our analyses may prove underpowered to detect significant effects, and null findings should not be interpreted as definitive.

7.2. Statistical Design

Our study design consists of three groups of participants: 1 (**CON**): no adolescent binge drinking (AIE) and current (past month) World Health Organization (WHO) risk drinking levels of either 0 or 1 (abstinent or low-risk); 2 (**RISK**): no AIE and current WHO level 2, 3, or 4 (medium risk, high risk, or very high risk); and 3 (**RISK+AIE**): history of adolescent binge drinking and current WHO risk level 2, 3, or 4. Within each group, participants will be randomly assigned to either the 10Hz tACS (stim) group or the sham tACS (sham) group. Effects of tACS on habitual action selection in the HABIT task (primary outcome, **Aim 2**) will be assessed based on the proportion of perseverative errors (relative to all errors) when the participant must replace a highly trained response to a set of familiar stimuli. We will use a general linear model that includes group and stimulation condition as between-subjects factors; we will also include baseline perseverative error, age, sex, handedness, and anxiety or mood disorder diagnoses as covariates.

We will enroll 66 participants, divided into three groups of 22, who will be randomly assigned within group to condition (stim vs. sham, n= 11 each), each of which will be divided into cohorts, based on best practices.

Our study design requires randomly assigning individuals to a stimulation group after completing Session 1. Dr. Boettiger will provide assignment to the study team based on a predefined randomization table in order to ensure balanced sex and group in each stimulation arm.

To test the prediction that E/I balance indices will differ from low-risk drinkers more among high-risk drinkers with adolescent binge history, we will analyze the baseline (i.e. pre-tACS) data collected in Sessions 2 and 3. We will conduct Analyses of Covariance (ANCOVA) to compare groups in terms of Session 2 (baseline) perseverative errors in the HABIT Test and MRS-derived GABA:Glx, and Session 3 baseline 1/f EEG slope, controlling for age, sex, education, psychiatric diagnoses, and medication status. We may also consider using data from multiple timepoints within the HABIT Test session, for which we would either employ a mixed model ANCOVA or multilevel modeling, as we have used in our prior studies.

For **Aim 2**, we will test for effects of tACS (true vs. sham) on habitual action selection in the HABIT task (primary outcome, Aim 2) based on the proportion of perseverative errors (relative to all errors) when the participant must replace a highly trained response to a set of familiar stimuli. We will use a general linear model that includes group and stimulation condition as between-subjects factors; we will also include baseline perseverative error, age, sex, handedness, and anxiety or mood disorder diagnoses as

†[1] www.consort-statement.org , [2] www.strobe-statement.org, [3] www.icmje.org

[4] Wasserstein RL, et al. (2016), The ASA's Statement on p-Values, ***The American Statistician***, 70:2, 129-133.

[5] Wasserstein RL, et al. (2019), Moving to a World Beyond p < 0.05, ***The American Statistician***, 73:sup1, 1-19.

[6] Amrhein, et al. (2019), Scientists rise up against statistical significance, ***Nature*** 567, 305-307.

[7] Editorial (2019) It's time to talk about ditching statistical significance: … ***Nature*** 567, 283-283.

covariates. For our mediation analyses, change in E/I balance measures (GABA:Glx and 1/f EEG slope) analyses will be used as the mediating variables; change in HABIT perseverative errors will serve as the dependent variable and will be adjusted for sex, age, handedness, and anxiety or mood disorder diagnoses as covariates; tACS condition (stim/sham) will serve as the independent variable; and AIE and current alcohol misuse scores will be included as moderators.

For **Aim 3** we will test whether the indirect effect of tACS on HABIT perseverative error is mediated by resting-state functional connectivity. For our mediation analyses, functional connectivity (FC) values derived from *a priori* ROI-to-ROI (e.g., dlPFC, aIC, and limbic striatum) analyses will be used as the mediating variable; change in HABIT perseverative errors will serve as the dependent variable and will be adjusted for sex, age, handedness, and anxiety or mood disorder diagnoses as covariates; tACS condition (stim/sham) will serve as the independent variable; and AIE and current alcohol misuse scores will be included as moderators. We will use CONN for initial FC analysis of fMRI data. We will also conduct exploratory analyses testing for associations between indices of E/I balance in the dlPFC and dlPFC FC at baseline, and whether changes in E/I balance indices associate with changes in dlPFC FC. Significance testing will be based on a bootstrapping procedure and 95% CI, with FDR-correction (q=0.05) for multiple ROI-ROI connections. Additional ROI-to-ROI and seed-to-voxel exploratory analyses will also be conducted with these data in collaboration with other components.

Secondary outcomes: We will use similar experimental designs to those described to evaluate a number of secondary outcomes. These include C-reactive protein measured from blood spots and other immune activation markers (TNF-α and IL-1β) derived from salivary microvesicles, as well as exploratory/alternative outcome analyses. The latter include effects of tACS on GABA:Glx ratios in the dlPFC and aIC, and on EEG 1/*f* slope.

8. Sample Size Rationale

We will enroll 66 participants, divided into three groups of 22, who will be randomly assigned within group to condition (stim vs. sham, n= 11 each), each of which will be divided into cohorts, based on best practices. Based on Cohen's method for calculating power of a linear model, as implemented in G*Power 3.1, in order to detect a large effect size (f = 0.4), n=64 will give us 80.3% power to detect an interaction between tACS and group on habitual responding (primary outcome for Aim 2) at an alpha value of .05. Our prior study found a moderately large effect size (Cohen's f = 0.21) in a repeated measure design with a large nuisance effect of session. By changing to a between subjects design and eliminating session effects, we expect a large effect size in this study. Thus, the needed total sample to recruit is 64 participants. We propose collecting n=66 to account for possible attrition or data loss, and so that each participant subgroup contains the same number of stim/sham participants (11/11).

To test the prediction that E/I balance indices will differ from low-risk drinkers more among high-risk drinkers with adolescent binge history, we will analyze the baseline (i.e. pre-tACS) data collected in Sessions 2 and 3. We will conduct Analyses of Covariance (ANCOVA) to compare groups in terms of Session 2 (baseline) perseverative errors in the HABIT Test and MRS-derived GABA:Glx, and Session 3 baseline 1/f EEG slope, controlling for age, sex, education, psychiatric diagnoses, and medication status. A power analysis in G*Power 3.1 indicates that a sample size of 64 will allow 80.1% power to detect moderately large group differences (Cohen's f=0.4; alpha=0.05). Thus, our proposed sample size of 66 participants will also be adequate for testing the hypotheses of Aim 1. We may also consider using data from multiple timepoints within the HABIT Test session, for which we would either employ a mixed model ANCOVA or multilevel modeling, as we have used in our prior studies.

For **Aim 3** we will test whether the indirect effect of tACS on HABIT perseverative error is mediated by resting-state functional connectivity. Our previous study using a similar statistical design to evaluate brain functional connectivity measures that mediate the effect of dopamine depletion on a measure of behavioral flexibility detected statistically significant effects with a smaller sample size (*n*=34).

9. Data Collection and Management

Questionnaire Data Collection: The study data will be entered into a REDCap database developed by the study personnel. REDCap is a 21 CFR Part 11-compliant data capture system provided by the NC TraCS Institute at UNC. The data system includes password protection and internal quality checks, such as automatic range checks, to identify data that appear inconsistent, incomplete, or inaccurate. Study data will be entered directly by participants or from the source documents by study personnel.

Behavioral Task Data Collection: Behavioral task data will be collected electronically and will be stored on a password-protected lab server only accessible by IRB-approved study personnel.

MRI Data Collection: MRI data will exist in digital form on computer disk. These data will be collected at the UNC Biomedical Research Imaging Center (BRIC) and be stored securely on UNC's longleaf secure server.

EEG Data Collection: EEG Data will be collected digitally using Net Station software installed on a computer in Howell Hall. This data will be stored on a password-protected lab server only accessible by members of the IRB study team.

Data Quality and Monitoring: All data are collected by trained bachelor's-level or Master's level study staff. Data quality and safety will be monitored by the PI and research team members. Any issues will be discussed with the study PI. The study PI assumes responsibility for ensuring study staff is complying with the investigational plan and IRB regulations, as well as ensuring any changes to the protocol have received IRB approval and have been reported to the sponsor, and that accurate, complete, and timely reports are made to the IRB. A weekly study team meeting will be conducted to discuss research procedures.

- All neuroimaging (MRI or EEG) data will be recorded in the database using alphanumeric identifiers that are unrelated to any personal identifying information about the participant. Neuroimaging data are coded by subject number only on password-protected secure servers located at the BRIC or UNC's longleaf secure server.

- All sources of electronic data (i.e., data collected via UNC REDCap) are saved on a password-protected file on a secure UNC server, of which only the research team members will have access.

- All sources of non-electronic research data will be coded by a unique subject number and stored in a dedicated locked file cabinet. Materials identifying participants by name (e.g., signed consent forms) will be stored in a separate locked file cabinet. The computer file matching participant names with code numbers will be saved in a double password-protected file on a secure UNC server directory only accessible by the PI and key research team. All data will be entered into the dataset using the subject identification number only. Participants are not identified by name in any analysis of these data, or any presentation or publication resulting from the analysis of these data.

10. Collection and Management of Tissue Specimens

10.1. Use in Current and Future Studies

Three types of biological samples will be collected for use in this study: saliva samples for DNA/RNA analysis, blood spots, and urine samples.

Saliva samples: During session one, two samples of approximately 2mL saliva will be self-collected by participants with researcher instruction. These samples will be marked with the participant's study ID number (not identifiable) and stored at –80 degrees Celsius in a secure research freezer in Howell Hall.

Urine samples: At the beginning of each session participants will self-administer a urine drug screen with researcher instruction. After session one only, a member of the research team will extract a small volume of urine from this sample to be kept for later analysis. Each urine sample will be marked with the participant's study ID number and stored at –80 degrees Celsius in a secure research freezer in Howell Hall. The urine samples will be shipped to a collaborator at the UNC Nutrition Research Institute to be analyzed for the presence of certain metabolites.

Blood spots: Blood spots will be collected by a trained researcher during sessions one and three. The researcher will sanitize the participant's chosen finger, apply a finger prick, and extract a small amount of blood. The blood spots will be marked with the participant's study ID number and session number and stored at –80 degrees Celsius in a secure research freezer in Howell Hall. These samples will be analyzed in-house by our research team to quantify inflammatory markers in the blood.

The samples collected during this study will be analyzed for this study and will be stored for possible use in future research studies. The possibility of future use by our study team or others is included in our consent form, and will be explained to participants during the informed consent process during session one.

10.2. Sample Preparation

Saliva samples: The saliva samples can be kept stable for multiple years when stored at subzero temperatures in order to ensure sample integrity.

Urine samples: These samples will be stored at subzero temperatures in order to maximize sample integrity over time. A researcher will transport these samples to the lab freezer within 2 hours of collection. These samples will then be driven by a member of the study team or shipped to our collaborator in Kannapolis, NC.

Blood spots: These samples will be stored at subzero temperatures in order to maximize sample integrity over time. After collection the samples will be left to dry at room temperature, and then a researcher will transport these samples to the lab freezer promptly.

10.3. Record Keeping and Monitoring

A record of samples collected will be kept and continuously updated. A spreadsheet listing samples collected for each participant will be kept according to study ID number. This spreadsheet will be kept on a protected server only accessible to the research team and will contain no identifying information. Researchers will update this spreadsheet after each session to ensure fidelity in record keeping.

10.4. Storage and Security

As described in 10.1, all samples will be kept in a dedicated research freezer located in Howell Hall. The freezer itself is designed to constantly monitor internal temperature and door openings. In the event of a drop in temperature, an alarm sounds and the PI is notified. All samples taken during this study will be de-identified from the time of collection.

11. Safety Monitoring and Management

11.1. Risk / Benefit Assessment

Potential Risks:

- *Cognitive Testing* There are no known risks for injury associated with the neuropsychological/behavioral components of these cognitive studies. Other possible risks include frustration, boredom, or fatigue. Moreover, answering detailed questionnaires and participating in computerized cognitive tasks may make subjects anxious or uncomfortable. Based on our previous experience, it is unlikely that participants will find these potential psychological risks extreme enough to end their participation.
- *Questionnaire Data Collection* Answering questionnaires may make subjects uncomfortable and could cause emotional distress.
- *Blood sample collection via finger stick*: The risks of drawing blood via a lancet finger stick include temporary discomfort from the stick, bruising, and rarely, infection.
- *Functional MRI Studies* Potential minor risks and discomforts are associated with MRI acquisition. No known health risks are associated with these types of MRI studies, although the MRI makes loud noises as it acquires data, which could affect a participant's hearing. The only significant risk associated with MRI is the presence of ferromagnetic materials. This is a noninvasive technique involving no catheterizations or injection of exogenous tracers. A great many participants have now undergone magnetic resonance studies without apparent harmful consequences. Radiofrequency power levels and gradient switching times used in these studies are within the FDA approved ranges. Subjects must lie still in the MRI scanner for approximately 90 minutes, which subjects may find uncomfortable. Some people may become claustrophobic while inside the magnet, and will be pulled out of the scanner to end testing If this should occur. A relative contraindication to MRI studies is pregnancy, as the risk of MRI to fetuses is unknown.
- *Electroencephalography (EEG)* Risks of EEG methods are considered minimal. Potential risks include discomfort during the placement of the cap or boredom during the EEG recording.
- *Transcranial Alternating Current Stimulation (tACS)* tACS is a non-invasive brain stimulation technique in which a weak electrical current is applied to the scalp. Our tACS protocol involves the application of three surface electrodes, one serving as the cathode and the other two as anodes. The current flows from the anodes to the cathode, with some current passing along the scalp and some passing through the brain. The current passing through the brain produces small changes in the excitability of the brain regions falling within the current flow. Minimal risks are associated with tACS. tACS may cause the onset of a mild headache of short duration. The device could cause discomfort or pain to the head. The use of conductive gel to affix the electrodes to the skin may cause skin irritation and dryness. Stimulation devices may cause muscle twitching in hands or face that can be uncomfortable.
- *Confidentiality* Information collected for the purpose of this research study will be kept confidential as required by law. All copies of testing records and results will be kept in locked filing cabinets, in locked offices, or in password-protected computer files. All information will be accessible only to authorized personnel. Alphanumeric codes will be used on all data sheets and data files in place of names. No subjects will be individually identified in any report or publication about this study. No personal identifiers will be associated with any study data. Screening information from participants will be kept in a locked filing cabinet along with their consent forms. The information collected during the initial telephone screening is not retained.
- *Privacy* Telephone interviews will be conducted only in the Boettiger laboratory or office space. All laboratory staff have undergone the CITI ethics training for conduct of human subjects research. All neuropsychological and behavioral testing will occur in private testing rooms. All virtual training will require a meeting password and subjects will remain in waiting room until entry

is accepted by teacher. No mailed or emailed materials or messages will include subject specific data.

Potential Benefits: Subjects will not experience any direct benefit from participating in these studies. We anticipate that the results of these studies will improve our understanding of behavioral inflexibility in people who engage in high risk drinking. Eventually this knowledge could lead to differentiated treatment options for high risk drinkers with a history of adolescent binge drinking. Participants may benefit psychologically from knowing that they are participating in clinically motivated research.

11.2. Safety Monitoring

We propose testing the efficacy of a single session of 10 Hz transcranial alternating current stimulation (tACS) on behavioral flexibility, brain mediators of behavioral effects, and inflammatory markers. Participants will be healthy adults ages 22-50 years in one of three groups: low risk drinkers (WHO level 0 or 1) with no adolescent binge history, and adults who currently engage in hazardous alcohol use (WHO level 2, 3, or 4) with or without a history of adolescent binge drinking. Our participants are not considered to belong to a vulnerable population. Completion of computerized behavioral tasks, tACS, and MRI scanning is minimal risk. Therefore, the data and safety monitoring plan (DSMP) does not include a Data Safety Monitoring Board (DSMB), consistent with NIAAA policy: "***Phase III clinical trials must have an independent DSMB. Phase I and II studies that either involve multiple clinical sites, use high risk interventions, or involve vulnerable research participants may require a DSMB at the discretion of NIAAA***." (from https://www.niaaa.nih.gov/research/guidelines-and-resources/data-and-safety-monitoring-guidelines, accessed 6/4/2021).

The primary risks anticipated for the proposed study are psychological effects and loss of confidentiality or privacy. As detailed in the *Confidentiality and Privacy* section, comprehensive measures will be implemented to protect participants' data and anonymity.

The proposed research poses minimal risk. In the unlikely event of a Serious Unanticipated Adverse Event (AE) during the behavioral testing, tACS, or brain imaging session, study personnel will contact campus and local emergency resources and the PI will report the event to the University of North Carolina, Chapel Hill IRB. In addition, for all MRI scans, at least two trained individuals will always be present in the MRI control room, one of whom will be a trained MRI technician, and a defibrillator/monitor is available on-site in case of emergency.

Reporting Procedure for Serious Unanticipated Adverse Events
In the event of an unanticipated serious adverse event, such as hospitalization of a subject due to study enrollment, the PI will ensure that these events are reported to the UNC-CH IRB within 24 hours by phone, fax, and/or email, and we will submit a written report no more than two days later. The project staff will also use the following reporting procedures:

1. When the study staff and/or PI become aware of a serious adverse event, reporting requirements must be implemented in a timely manner.

2. Dr. Boettiger will complete an "Adverse Event Reporting Form" and submit the form to the UNC-CH IRB.
3. The UNC-CH IRB, with the input of the PI, will review the study protocol and determine what further action, if any, to take based on the best interests of the participants.

Suicidality
In addition to possible AEs from study procedures, the use of the MINI screening interview could lead to disclosure of participants' suicidal ideation which would require further action. Unfortunately, no measures are available to determine in a reliable and valid way whether someone is likely to engage in

life-threatening behavior within 48 hours. Thus, for the past 15 years, we have followed a protocol that allows us to identify and intervene in a cautious and safe manner. This protocol is based on that successfully used by Dr. Mitchell Prinstein, within our department. Dr. Prinstein is a licensed Clinical Psychologist specializing in the area of depression and self-injury in youth. This protocol is described below.

Step 1: Screening with MINI: Any participant endorsing a current state of suicidality is flagged and brought to the attention of the PI, Dr. Charlotte Boettiger.

Step 2: Contact participants: We intervene with cases determined to be at potential imminent risk by contacting participants. We do not have a therapeutic relationship with the participants, and our measures are research, NOT clinical instruments and cannot be used to detect psychological processes with absolute certainty. We therefore cannot be sure that there is reason for major concern, but we would rather be safe and alert participants about our concerns. We will explain that the responses made on MINI are quite rare, and express our concern that this might indicate a risk of suicide. Dr. Boettiger will then help them determine how to get a psychological evaluation, and encourage them to do so. These options include:

1. The UNC Department of Psychology Community Clinic, which offers sliding scale treatment to anyone in the community (919) 962-6906.

2. For University of North Carolina students: The UNC Counseling and Psychological Services, CAPS, Monday-Friday, from 8am-5pm. Their phone number is 919-966-3658. After hours call the same number to speak to ProtoCall service. For medical services for UNC students, HealthLink can be accessed 24/7 at 919-966-2218.

3. The national suicide hotline at 988, available 24/7, or the suicide text line by texting HOME to 741741.

4. Escort to the local emergency room.

Oversight

The PI is responsible for the general oversight for all study activities and will inform the Program Officer about changes and requirements for the DSMP. Yearly review of the DSMP will be conducting during the regular continuing review process, outlining the following points:

- Reassessment of the risks and benefits to study participants
- Participant recruitment, accrual, and retention
- Data quality and confidentiality
- Consideration of external scientific or therapeutic developments with impact on the safety of participants or the ethics of the study
- Review any adverse events

The PI will update the general DSMP procedures as needed.

11.3. Adverse Events and Serious Adverse Events Reporting Procedure

See 11.2 for reporting procedure.

Adverse events of any type seem highly unlikely for this study given the safety profile of the intervention being used. We will define adverse events as any medical issues arising after study enrollment. We will

define serious adverse events as any medical issues arising after study enrollment that require hospitalization. Participants will be asked by the research team at the beginning of each session whether they have had any notable changes to their medical record. If a participant reports an adverse event, it will be recorded by the research team and reported to the study PI within 24 hours. The study PI will determine whether they believe the adverse event was related to study activities and data collection will proceed accordingly. If a serious adverse event occurs, data collection for all participants will be suspended temporarily until the study PI, the study medical advisor, and the UNC-CH IRB determine resuming sessions is safe for participants.

12. Regulatory, Ethical, and Study Oversight Specifications

12.1. Informed Consent Process

Individuals interested in participating will be asked to sign an online consent form before filling out the optional RedCap pre-screening form. Potential participants, whether or not they have previously completed the optional online pre-screening form, will be asked to provide verbal consent to participate in a phone screening to determine eligibility. Acquisition of verbal consent will only occur after a member of the research team has described the study and given an overview of the screening questions to follow. Enrolled participants will be asked to sign an informed consent form at the beginning of every session. During the first session a member of the research team will review the terms of the consent form with the participant and will confirm the participant's full understanding before obtaining consent.

All subjects will participate in the informed consent process. Participants will be familiarized with the protocol by the Dr. Boettiger or qualified study personnel, including its risks and benefits, and informed consent will be documented according to the regulations governing human subject research at the University of North Carolina, Chapel Hill, which meet the standards of the NIH. A copy of the informed consent document will be given to the participants for their records. The informed consent process will be conducted, and the form signed, before the participant undergoes any study-specific procedures.

Recruiting Participants will be recruited through IRB-approved flyers and advertisements distributed in the local community, as well as through emails sent to surrounding college campus communities. No Protected Health Information (PHI) will be accessed prior to contacting participants. Potential subjects who respond to these advertisements will be contacted initially via telephone or email. If contacted via email, subjects will be called for an initial telephone screening to determine whether the subject meets our inclusion criteria. Those meeting criteria based on the phone interview will be scheduled to come to the lab to participate in the study.

Inclusion of Women and Minorities Every attempt will be made to recruit women and minorities, including posting advertisements at the Sonja Haynes Stone Center for Black Culture and History; the Lesbian Gay Bisexual, Transgender Office; the Student Union; the Carolina Women's Center; and the Medical Campus buildings.

Initial Telephone Screening Potential participants are first screened via telephone for eligibility. This process includes a complete explanation of the protocol procedures. At that time, a potential participant can decline to participate.

Obtaining Informed Consent Upon arrival to participate in Session 1, participants will read and have explained to them the informed consent document. Informed consent is obtained in a comfortable, private area in the Boettiger lab. Participants' questions about the protocol will be thoroughly answered prior to obtaining consent, with no time limit to this procedure. Upon consent, the subject is free to

withdraw that consent at any time, which will be emphasized to the subject. Consent for behavioral testing and imaging will be obtained by approved study staff.

Waiver of written consent for telephone pre-screening We will obtain a waiver of written consent for the initial telephone screening. This request derives from our need to conduct a health and medical history telephone screen in order to ensure that individuals who respond to our IRB approved recruitment efforts are eligible to participate in our research project. The health screen is preparatory to our research and its sole purpose is to ensure that participants meet our inclusion/exclusion criteria.

A screening procedure that is efficient for both volunteers and staff members is necessary. These studies have strict inclusion/exclusion criteria and the telephone screen efficiently provides the study staff with information needed to determine eligibility. To require potential study participants to come to our lab for a qualifying screen that can be done over the telephone would place an unnecessary burden on potential participants, as well as on study staff. In addition to the health screen, we have created a phone script that includes elements of informed consent and explains to potential participants how their privacy will be protected. The potential subject is informed that they may stop the phone conversation at any time. The health and medical information documented during the telephone screen is not disclosed to other investigators. Screens from participants who are excluded are destroyed. Screens from participants who are included are kept in a locked filing cabinet along with their consent form and other screening information.

12.2. Confidentiality and Privacy

Confidentiality
Information collected for the purpose of this research study will be kept confidential as required by law. All copies of testing records and results will be kept in locked filing cabinets, in locked offices, or in password-protected computer files. All information will be accessible only to authorized personnel. Alphanumeric codes will be used on all data sheets and data files in place of names. No subjects will be individually identified in any report or publication about this study. No personal identifiers will be associated with any study data. Screening information from participants will be kept in a locked filing cabinet along with their consent forms. The information collected during the initial telephone screening is not retained.

Privacy
Telephone interviews will be conducted only in the Boettiger laboratory or office space. All laboratory staff have undergone the CITI ethics training for conduct of human subjects research. All neuropsychological and behavioral testing will occur in private testing rooms. All virtual training will require a meeting password and subjects will remain in waiting room until entry is accepted by teacher. No mailed or emailed materials or messages will include subject specific data.

All participant data will be de-identified at the time it is stored on computing resources, such that only unique participant ID codes will associate the names of individuals to corresponding data. Access to the file linking these two sources of information will be maintained on a secure server that is separate from the one housing the actual MRI, biochemical, and behavioral performance data. All behavioral performance data will be stored on computers in locked rooms, password protected, and will be backed up to a secondary, secured location, all behind the firewall of the University of North Carolina, Chapel Hill. All MRI data will be uploaded to secure data servers, access to which will be restricted to personnel involved in the administration of the proposed studies.

Shortly after data upload, at least one member of the study team will perform an initial quality check to ensure that all files are intact and that there were no errors in transfer. All analyses will be conducted when the targeted sample size (n=66) is obtained. The conduct of research according to the documented study protocol will be monitored by at least two individuals from the study team, including one senior/key

personnel (Dr. Boettiger). All data analyses will also be quality checked by at least two individuals from the study team.

12.2.1. Certificate of Confidentiality

To further protect the privacy of study participants, a Certificate of Confidentiality will be issued by the National Institutes of Health (NIH). This certificate protects identifiable research information from forced disclosure. It allows the investigator and others who have access to research records to refuse to disclose identifying information on research participation in any civil, criminal, administrative, legislative, or other proceeding, whether at the federal, state, or local level. By protecting researchers and institutions from being compelled to disclose information that would identify research participants, Certificates of Confidentiality help achieve the research objectives and promote participation in studies by helping assure confidentiality and privacy to participants.

12.3. Future Use of Stored Data

Data collected for this study will be analyzed and stored at UNC Chapel Hill (UNCCH). As required by NIAAA, the de-identified, archived data will be transmitted to and stored at the $NIAAA_{DA}$ (see below) for use by other researchers including those outside of the study. Permission to transmit data to the NDA should be included in the informed consent.

When the study is completed, access to study data will be provided through the $NIAAA_{DA}$.

12.4. Key Roles and Study Governance

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Dr. Boettiger currently serves as the Statistical Consultant for the U24 Neurobiology of Adolescent Drinking in Adulthood (NADIA) Consortium.

12.5. Publication and Data Sharing Plan

DISSEMINATION PLAN

We will comply with the NIH policy on dissemination of NIH-funded clinical trial information by registering the details of this study on ClinicalTrials.gov no later than 21 days after the start of data collection. We will adhere to all reporting requirements, including posting a summary of results and outcomes within a year after the completion of data collection. Plans for disseminating results and the timeline for doing so will be included in the informed consent documents for this study. The UNC Office of Sponsored Research will ensure compliance for registering and reporting as part of their post-award monitoring.

We will also present our findings at relevant scientific conferences, including that of the Research Society on Alcoholism and will submit the results of our planned analyses for publication in peer-reviewed journals.

NIAAA Data Archive ($NIAAA_{DA}$) Data Sharing Plan (DSP)

Per NOT-AA-22-011, this study will submit and share data with NIAAA Data Archive ($NIAAA_{DA}$), a data repository housed within the NIMH Data Archive (NDA). To this end, this study will fulfill the following summary of tasks and expectations:

***Common to all studies*:**

- Obtain Informed Consent that allows for broad sharing of the research subjects' de-identified data and/or approval of the Institutional Review Board, consistent with the Data Submission Agreement (DSA)
- Collect Personally Identifiable Information (PII) from research subjects that will allow for the creation of the NDA GUID (a de-identified subject ID).
- Create an NDA Account with access to the GUID Tool.
- Complete and submit a Data Submission Agreement (DSA) within 6 months of the Notice of Award (NoA) issue date.
- Create a list of data items to be collected in the project in the Data Expected tab of the NDA Collection within 6 months of NoA issue date.
- Create a GUID for each research subject using the NDA GUID tool.
- Format and submit data according to the NDA Data Dictionary and/or work with NDA staff to define an appropriate data structure, if one does not already exist.
- Submit data on or before the NDA submission due dates (April 1 and October 1 each year) in accordance with the applicable Data Sharing Terms and Conditions of award.
- Perform QA/QC checks on data within 4 months after the submission due dates and address any issues identified by the NDA.
- Share data in accordance with the default $NIAAA_{DA}$ data sharing schedule in accordance with the applicable Data Sharing Terms and Conditions of award.
- Upload supporting documents that allow future analysts to effectively use the data, including: study protocols, assessment schedules, operating procedures, original assessment instruments (if not proprietary), and analytic/statistical algorithms used to derive variables in publications.
- Create an NDA Study, linking relevant publications to submitted data at the time of publication, and use the appropriate Digital Object Identifier (DOI) and Publication Publication template in these publications.
- Submit a statement of progress on data sharing in non-competing renewals and progress reports. Further instructions are available in the RPPR Instruction guide under section C.5.b. Resource Sharing. Refer to specific Tutorials about data submission and sharing via the NDA or refer to the data sharing planning section where the data will be shared.

Study-specific details:

Brief summary of the assessment schedule for data that will eventually be shared with the $NIAAA_{DA}$:

In this 3-visit study, we will first determine eligibility at the screening and randomization visit; dried blood spot measures of inflammatory markers will also be collected. In visit 2, we will measure

baseline habitual action selection in the HABIT, and collect structural MRI, baseline resting-state fMRI, and single-voxel MRS measuring GABA and glutatmate/glutamine. At visit 3, we will collect resting-state EEG before and after 10Hz- or sham-tACS, as well as habitual action selection in the HABIT during 10Hz- or sham-tACS, as well as repeat structural and functional MRI and MRS, and dried blood spot collection following 10Hz- or sham-tACS. Demographic, psychometric, and substance-related information will be collected via REDCap at the participant's convenience.

Listing of proposed data collection instruments (assessments) that do not currently exist in the NDA:

1. *Alcohol Related Blackout Questionnaire*
2. *Alcohol-Induced Blackout Measure-2*
3. *Munich Parasomnia Screening*
4. *Alcohol Use Questionnaire [Q10-12]*
5. *Carolina Alcohol Use Pattern Questionnaire (version 2)*
6. *Value-Driven Attention Questionnaire*
7. *Hidden Association Between Images Task (HABIT)*

Proposed schedule for running the data validation tool once data collection begins:

The PI (Boettiger) has established an NIMH Data Archive (NDA) account. Data will be uploaded to the NDA by the deadlines (June 1; Dec 1) and will be subject to quality control checks by the investigators within 4 months (Oct 1; Apr 1) of submission to the NDA. Upon notice of award, Data Expected will be submitted to the NDA, and will include (i) a data dictionary, (ii) targeted enrollment, and (iii) anticipated data submission and sharing dates. For each manuscript based on findings from this project, investigators will submit to the $NIAAA_{DA}$: data, list of variables, analytic plan, and results. We will determine who will have access to which aspects of the project data based on NIH's data access principles. We will provide access to person-level data for research purposes only, based on Data Use Certifications. Data on UNC servers will be made available to investigators with appropriate IRB approval and Data Use Agreements.

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Appendices

Appendix 1: XCSITE 100 User Guide Version 1.1 (May 2017)