Experimentally Evaluating the Hypothesized Mechanism of Action of N-acetylcysteine for Bipolar Disorder

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Protocol

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Specific Aims

Despite 70 years of medication development, bipolar disorder (BD) remains one of the leading drivers of global disease burden. Though existing BD medications are partially effective in treating the symptoms and sequela of BD, they often leave patients with significant residual mood symptoms and cognitive dysfunction. These impairments may be due to cellular damage caused by excessive oxidative stress (e.g., increased lipid peroxidation, DNA/RNA damage) that is implicated in the neuroprogression of BD. Glutathione (GSH), the brain's first line of defense against oxidative stress, neutralizes free radicals and reactive oxidants, while providing protection and redox regulation of cellular thiol proteins via sglutathionylation. We compared dorsal Anterior Cingulate Cortex (dACC) GSH levels of BD and healthy control (HC) participants from the PI's NIH K23 study and, consistent with findings from a recently published meta-analysis, found that BD had significantly higher dACC GSH levels than did HC participants (p=0.026, Cohen's d=0.66), interpreted as compensatory GSH upregulation in response to mild-tomoderate levels of oxidative stress. Despite this endogenous compensation, however, individuals with BD continue to experience pathologically elevated levels of oxidative stress.

N-acetylcysteine (NAC), a safe and well-tolerated medication, supplements endogenous GSH levels by providing cells with the rate-limiting component of GSH synthesis (i.e., cysteine) in exchange for glutamate. Though research has long demonstrated that NAC increases peripheral GSH levels, investigation of the effects of NAC on brain GSH levels has been limited to a few studies in people with Parkinson's disease and early psychosis, which found that NAC significantly increased cortical GSH levels. Based on the hypothesis that NAC treats residual BD impairments by increasing brain GSH levels, a number of randomized clinical trials (RCTs) of adjunctive NAC for BD have been conducted over the past 10-15 years. A recent meta-analysis of these RCTs found a statistically-significant, moderate effect of NAC on reducing depressive symptoms; secondary data analyses further demonstrated that NAC significantly increased working memory performance. Despite this support for NAC as a promising adjunct treatment for BD, the (GSH-mediated) mechanistic hypothesis on which these studies have been predicated has never been tested in BD individuals, and a plausible (glutamate-mediated) alternative hypothesis exists.

It is against this background that the proposed randomized, double-blind, placebo-controlled, crossover MRI study aims to measure and experimentally-increase GSH levels in dACC (selected among candidate brain regions given its central role in guiding motivated human behavior), using NAC (3g/day), in BD participants. Twelve BD participants will complete two, 2-week experimental conditions (NAC, placebo) in a randomized order, separated by a 2-week washout. The study will evaluate the following hypotheses:

Primary**:** NAC treatment will significantly increase dACC GSH levels, relative to placebo, in BD individuals. ***Exploratory*****: 1)** NAC treatment may significantly reduce dACC glutamate levels, relative to placebo, in BD individuals. **2)** Associations of NAC-related changes in dACC GSH and glutamate levels with peripheral biomarkers of oxidative stress (plasma GSH, s-glutathionylation), mood, and cognition will be explored.

The proposed study will be conducted by a BD research team with a successful 10+ year record of collaboration, using cutting-edge proton magnetic resonance spectroscopy methods that have been optimized by the PI. Positive results would directly support the hypothesized mechanism of NAC treatment for BD. Perhaps more importantly, positive results would provide evidence for a novel neurobiological treatment target for BD, which would support the development of medications designed to increase brain GSH levels (i.e., with greater specificity and efficacy than NAC [e.g., γ-glutamylcysteine ethyl ester]) and their evaluation using the proposed experimental neuroimaging framework. Finally, the proposed study will add to the literature on the relative associations of changes in brain GSH and glutamate levels, peripheral biomarkers of oxidative stress, and BD clinical phenomenology (i.e., mood symptoms and cognitive impairments).

A. SIGNIFICANCE

A.1. Overview. Despite 70 years of medication development, bipolar disorder (BD) remains one of the leading drivers of global disease burden and mortality (Ferrari, 2016). Though existing BD medications are partially effective in treating the symptoms and sequela of BD, they often leave patients with significant residual cognitive dysfunction (Jensen, 2016), depressive symptoms, and other functional impairments (Perlis, 2006; Miskowiak, 2017). Convergent evidence supports brain glutathione (GSH) as a promising interventional target for adjunctive treatment in BD and N-acetylcysteine (NAC) as the best available drug to experimentally engage this target.

A.2. Brain GSH levels as a Promising Treatment Target in BD. Cellular damage caused by oxidative stress (OS; e.g., increased lipid peroxidation, DNA/RNA damage, nitric oxide) has been implicated in the neuroprogression of BD (Brown, 2014). BD individuals reliably demonstrate both mood-episode dependent (e.g., thiobarbituric acid reactive substances [TBARS], uric acid) and independent (e.g., malondialdehyde, total nitrites) elevations in OS biomarkers (relative to healthy control [HC] participants; Jimenez-Fernandez; 2021) that are associated with more years of BD illness (e.g., 3-Nitrotyrosine, Andreazza, 2009) and a greater number of lifetime manic episodes (e,g., DNA oxidation; Soeiro-de-Souza, 2013). There are a number of, likely interacting, causes of the pro-oxidative neural environment found in BD, including mitochondrial dysfunction (Scaini, 2016), and abnormal glutamatergic (Sattler, 2001) and dopaminergic (Hastings, 2009) transmission. GSH, the brain's primary antioxidant, confers a critical first-line of defense against OS-induced cellular damage. Aside from neutralizing radicals and reactive oxidants, GSH provides protection and redox regulation of cellular thiol proteins via s-glutathionylation, a post-translational modification (Townsend, 2007). Though brain GSH levels become depleted under extreme neurological conditions (e.g., Alzheimer's/Parkinson's diseases [Mazzetti, 2015]), GSH levels are *upregulated* in response to mild-moderate OS (e.g., healthy aging [Tong, 2016], mild cognitive impairment [Duffy, 2014], early psychosis [Wood, 2009], drug/toxin exposure [Hoffman, 2005; Smith, 2005]), and elevated GSH levels are associated with increased depressive symptoms (Duffy, 2015) and impaired executive functioning (Duffy, 2014) in older adults.

We compared dorsal Anterior Cingulate Cortex (dACC) GSH levels from BD (*n*=29) and HC (*n*=21) participants from the PI's K23 study (AA020842) and found that BD had significantly *higher* dACC GSH levels than did HC participants (*t*=2.30, *p*=0.026, Cohen's *d*=0.66; *Figure 1*), consistent with compensatory upregulation to moderate OS (Tong, 2016). As described in Prisciandaro (2017), GSH levels were acquired via 2D J-resolved PRESS, fit via the ProFit algorithm (Prescot, 2013), normalized to unsuppressed water, and corrected for within-voxel cerebrospinal fluid (CSF) fraction. BD and HC participants did not differ on demographics (*Table 1*) or gray matter (GM) to brain matter (GM+WM) proportion (both *M*s=0.61, *p*=0.714). Consistent with Tong (2016), age was positively associated with dACC GSH levels (*r*=0.33, *p*=0.023), and covarying age increased the statistical reliability of the association of BD with GSH levels (*F*=7.38, *p*=0.009). Within BD participants, GSH levels did not differ by subtype (*t*=0.77, *p*=0.447) and were not associated with Young Mania (YMRS; Young, 1978) (*r*=0.25, *p*=0.197) or Montgomery-Asberg Depression (MADRS; Montgomery, 1979) (*r*=-0.22, *p*=0.259) Rating Scale scores, possibly due to range restrictions on these measures. Medications, evaluated overall (taking/not-taking any) and

	Group		*p*
	Bipolar Disorder (*n* = 29)	**Healthy Control** (*n* = 21)	
Age	36.21(12.12)	37.95(9.71)	0.588
Sex (% female, n)	51.7(15)	52.4(11)	0.963
Smoking status (%, n)	17.2(5)	33.3(7)	0.189
YMRS	1.82(2.72)	0.33(0.80)	0.010
MADRS	7.32(6.20)	1.05(2.04)	<0.001
BD subtype (% I, n)	62.1(18)	n/a	n/a
Medication (%, n)			
Lithium	27.6(8)	n/a	n/a
Antipsychotic	58.6(17)	n/a	n/a
Anticonvulsant	41.4(12)	n/a	n/a
Antidepressant	37.9(11)	n/a	n/a

Table 1. Baseline variables by group. Smoking status, ≥10 cigarettes/day;MADRS, Montgomery-Asberg Depression Rating Scale (RS); YMRS, Young Mania RS.

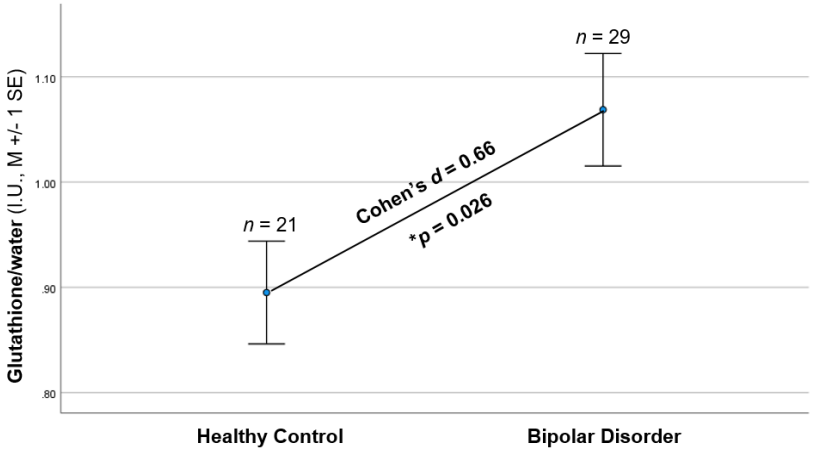

Figure 1. dACC glutathione/water, in Institutional Units (I.U.), by group.

by class (list in *Table 1*), were not associated with GSH levels (*p*s=0.275-0.964). Consistent with our findings, a meta-analysis of ACC GSH levels in BD versus HC participants confirmed that BD have significantly *higher* levels of ACC GSH than HC participants, and that BD medication is not associated with ACC GSH levels (Das, 2019).

In sum, BD have elevated dACC GSH levels, interpreted as compensatory upregulation in response to excess OS. Despite this compensation, however, they maintain pathologically-elevated levels of OS.

A.3. NAC as the Best Available Drug to Experimentally Modulate Brain GSH levels in BD. NAC (the N-acetyl derivative of L-cysteine) has been safely used for >30 years to treat acetaminophen overdose and lung conditions requiring mucolytic therapy (FDA Insert). NAC increases GSH levels by providing cells with cysteine, the rate-limiting component of GSH synthesis (Ballatori, 2009). Though studies have consistently demonstrated that NAC increases peripheral GSH levels, there have been few published investigations of NAC effects on brain GSH levels, in part because measuring brain GSH levels *in vivo* requires specialized methods (Sanaei, 2017). A study in people with Parkinson's disease found that NAC infusion significantly increased occipital GSH levels (Holmay, 2013), while another study in people with early psychosis found that chronic oral NAC dosing significantly increased medial frontal GSH levels and improved cognitive processing speed (Conus, 2018).

Based on the mechanistic hypothesis that NAC improves residual affective and cognitive impairments in BD individuals by increasing their brain GSH levels, a number of randomized clinical trials (RCTs) of adjunctive NAC for BD have been conducted over the past 10-15 years (Nery, 2020). The original landmark RCT found that NAC significantly reduced depressive symptoms and increased quality of life relative to placebo in BD outpatients (Berk, 2008). Secondary analysis of the data further revealed that NAC significantly reduced hypo(manic) symptoms (in those who entered the trial experiencing (hypo)manic symptoms; Magalhaes, 2013) and significantly increased working memory performance (in a pooled sample of participants with BD and schizophrenia; Rapado-Castro, 2017), consistent with studies demonstrating pro-cognitive effects of NAC in people with Alzheimer's disease and traumatic brain injury (Skvarc, 2017). Berk and colleagues' (2008) findings have since been replicated by three independent research groups (Hu, 2012; Porcu, 2018; Bauer, 2018, NAC+aspirin) and, despite included recently-published negative studies (Berk, 2019; Ellegaard, 2019), a meta-analysis found that adjunctive NAC (vs. placebo) treatment conferred a statistically-significant, moderate effect on reducing depressive symptoms in BD individuals (Nery, 2020).

Despite support for NAC as a promising adjunctive treatment for BD, the (GSH-mediated) mechanistic hypothesis on which RCTs have been predicated has never been tested in BD individuals, and an equally-plausible, (glutamate-mediated) alternative hypothesis exists. Specifically, the cystine/glutamate antiporter, through which NAC increases intracellular levels of the GSH-precursor cysteine, couples uptake of one molecule of cystine with release of one molecule of glutamate (see *Figure 2)*. This NAC-induced efflux of glutamate activates presynaptic mGlu2 receptors, inhibiting glutamate release, and induces astrocytic expression of glutamate transporter 1, enhancing clearance of synaptic glutamate (Raghu, 2020). These glutamatergic effects, manifested *in vivo* as NAC-induced reductions in ACC glutamate levels (Schmaal, 2012; McQueen, 2018; Woodcock, 2021), have formed the foundation of numerous preclinical and clinical studies of NAC for treating SUD. Because OS-induced cellular damage is a mechanism of (hyper)glutamatergic excitotoxicity (Sattler, 2001), NAC may improve clinical outcomes by reducing OS, but may do so via decreasing glutamate (as opposed to increasing GSH) levels.

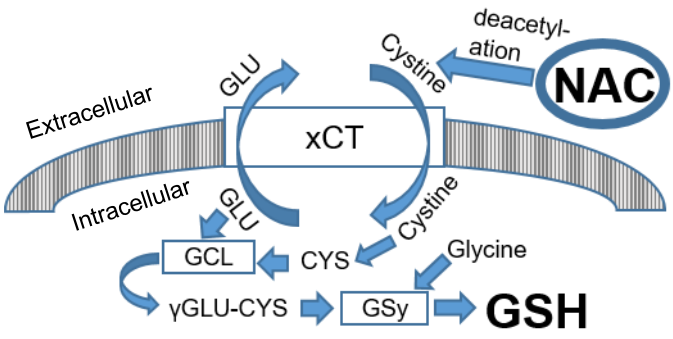

Figure 2. Metabolism of NAC to GSH. NAC, N-Acetylcysteine; xCT, cysteine/glutamate antiporter; GLU, glutamate; CYS, cysteine; GCL, glutamate-cysteine ligase; GSy, glutathione synthetase; GSH, reduced glutathione.

In sum, RCTs have supported NAC as a promising adjunctive treatment for BD, though the (GSH vs. glutamate-mediated) mechanisms through which NAC improves BD outcomes remain untested.

A.4. Conclusion. BD remains a leading driver of global disease burden, in part due to residual affective and cognitive impairments experienced by most individuals with BD. These impairments may be due to cellular damage caused by excessive OS, which persists despite compensatory upregulation of the brain's primary antioxidant, GSH. Adjunctive NAC improves residual affective and cognitive impairments in BD individuals, however the mechanisms through which these improvements occur remain untested. The proposed randomized, placebo-controlled, double-blind, crossover study aims to increase dACC GSH levels using NAC and to explore GSH- and glutamate-mediated changes in mood, cognition, and peripheral OS biomarkers in BD individuals.

B. INNOVATION

1) Although evidence supports NAC as a promising adjunctive treatment for BD (Nery, 2020), no studies have evaluated the neurobiological mechanisms of NAC treatment in BD. The proposed study will directly evaluate whether NAC engages its hypothesized neurochemical target (GSH) and/or an alternative target (glutamate) in BD individuals, and will examine relative associations of NAC-induced changes in GSH and glutamate levels with mood, cognition, and peripheral OS biomarkers (plasma GSH levels, extent of s-glutathionylated proteins). In doing so, the proposed study will be the first to examine the relationship between brain and plasma GSH levels (positively correlated in other populations, Holmay, 2013; Conus, 2018) in BD individuals.

2) The proposed study will be the first to investigate brain GSH levels in BD individuals using optimal, j-difference, ^{1}H-MRS methods (Sanaei, 2017); specifically, a TE-optimized version of the MEGA-PRESS sequence demonstrated to provide excellent SNR and test-retest reliability of dACC GSH levels (Prisciandaro, 2020).

A. RESEARCH PLAN

C.1. Overview. The proposed randomized, placebo-controlled, double-blind, crossover MRI study will evaluate the effects of 3g/day of NAC on dACC GSH levels in BD. BD participants will complete two, 14-day experimental conditions (NAC, placebo) in a randomized order, separated by a 14-day washout period. Each condition will consist of an in-person study visit for assessment and dispensing of medication (Day 1) and an MRI (Day 14). See *Figure 3* below for a study design schematic.

Figure 3. Study Design

C.2. Participants. Twelve healthy, men and women age 18-60 with BD will be enrolled across 10-months (i.e., following a 1-month study-initiation, and followed by a 1-month closeout, period). The study team has successfully recruited and retained BD participants for >10 years via clinical referral (e.g., MUSC Institute of Psychiatry, Charleston and Dorchester Mental Health Center) and advertising (e.g., radio, Facebook, Craigslist), 97% of whom have agreed to be re-contacted. This past year, we screened out ≥10 people/month who were diagnosed with BD, but failed to meet entry SUD criteria, who could have been available for this study. We anticipate approximately 35% of recruited participants will be minorities.

C.2.a. Recruitment. We will focus recruitment efforts for the proposed study on the following primary sources:

1) *Medical University of South Carolina (MUSC)*
   a. Inpatient - Over the past 10+ years, we have forged strong ties with the clinical enterprise of the MUSC Institute of Psychiatry. Study staff visit MUSC residents and attending physicians across 4 inpatient units, 3x/week. If the inpatient treatment team believes a patient may be eligible and interested in participating in the current study, they will approach the patient and gain approval for the research study staff to speak with the patient. If they agree, the patient will then be asked questions by study staff to screen for eligibility.
   b. Outpatient – We will recruit additional study participants through IRB-approved access to electronic medical records (EPIC), education and outreach to medical residents, and flyering across campus. A research data request will be submitted to indicate patients who have been diagnosed with Bipolar Disorder or Schizoaffective Disorder, Bipolar Type. Patients who have not indicated an opt-out research contact preference in their MUSC medical record will be called by study staff and screened for eligibility. Individuals who have indicated that they do wish to opt-out of research contact may be informed of the research study by their MUSC psychiatry outpatient provider if the provider feels it is appropriate. If they are interested, they will be given the study team's contact information to call or email. The study team will not cold-contact any patients who have chosen to opt-out of receiving contact about research.
2) *Other Clinical Referral Sites*
   a. Charleston Dorchester Mental Health Center (CDMH) – The CDMH provides outpatient counseling, psychiatric treatment, and support services to adults with severe mental illness through clinics in Charleston and Dorchester Counties in SC. Study staff visit the CDMH monthly, and additionally coordinate referrals and activities via phone and e-mail between visits. Study staff will not directly

approach or contact prospective subjects.

C.2.b. Inclusion Criteria. 1.) Meets DSM-5 criteria for bipolar I or II disorder. 2.) Daily use of ≥ 1 FDAapproved mood-stabilizing medication for BD (i.e., lithium, lamotrigine, divalproex sodium, 2nd generation antipsychotic); restricting the proposed study to medication-naïve individuals would represent a safety hazard, severely limit recruitment (Phillips, 2008), and would be inconsistent with the primary goal of the study, to evaluate NAC as an adjunctive medication for BD. Though BD medications have not been associated with ACC GSH levels (Das, 2019*)*, participants with medication additions, discontinuations, or dose changes of ≥ 20%, ≤ 2 weeks before testing will be excluded to further minimize the impact of medications (Swann, 2009). 3) Reports experiencing a recent prolonged mood disturbance (i.e., meets DSM-5 criteria for a mood episode, current or in partial remission), as long-term remission is characterized by relatively lower levels of oxidative stress (OS; Jimenez-Fernandez, 2021). 4.) Willing to abstain from antioxidant supplements (e.g., coenzyme Q-10) and 5.) utilize birth control (women). 6.) Aged 18 60 years, as changes in brain GSH primarily occur prior to age 18 (↓ GSH) and after age 60 (↑ GSH; Tong, 2016).

C.2.c. Exclusion Criteria. 1.) History of significant hematological, endocrine, cardiovascular, pulmonary, renal, gastrointestinal, neurological disease. 2.) Current suicidal or homicidal ideation. 3.) Severe mood disturbance conferring acute safety risk, defined as Montgomery-Asberg Depression Rating Scale (MADRS; Montgomery, 1979) > 35 or Young Mania Rating Scale (YMRS; Young, 1978) > 25. Because elevated ACC GSH levels in BD are evident across mood states (Das, 2019), participants with subthreshold mood symptom elevations will not be excluded in order to maximize clinical generalizability. 4.) Positive breathalyzer or urine drug screen. 5.) DSM-5 SUD (c.f., Tobacco) criteria met in the past 6 months. 6.) Electroconvulsive therapy in the past 3 months. 7.) Known allergic reaction to, or past month use of, NAC. 8.) Use of medications potentially hazardous if taken with NAC (e.g., carbamazepine). 9.) Hepatocellular disease as indicated by liver transaminase levels >3x normal range. 10.) Past head injury with loss of consciousness >5 minutes, presence of non-MRI safe materials, or claustrophobia.

C.3. Procedures. See *Table 2* for a summary of study events by visit.

Table 2. Schedule of events by study visit

	Baseline	Condition 1		Condition 2	
Study Visit	Visit 1	Visit 2	Visit 3	Visit 4	Visit 5
Clinician					
Informed Consent / HIPAA / BrAC contract	X				
SCID-5 / FHS	X				
History and Physical (incl. Vitals) / Metal Screen	X				
YMRS / MADRS / HAM-A / C-SSRS	X	X	X	X	X
TLFB (90 day at V1) ALL SUBSTANCES (incl. cigarettes)	X	X	X	X	X
BDRS / BR / OAS / PANSS-6			X		X
Vitals		X	X	X	X
Self-Report					
Demographics	X				
BIS-11 / SPSRQ / AUDIT / ASRS / PSQI / WHODAS / DSM-5 PID-5 / SCID-5 SPQ / CTQ / STAXI-2 (Trait) / BussPerry Aggression / SHAPS (Trait) / STAI (Trait)	X*				
BDI-II / BAI / Insomnia Severity Index / FTND / ASRM / CAPE-42 / STAXI-2 (State) / STAI (State) / CHRT-SR	X		X		X
Computer Tasks					
STOP-IT / Delay Discounting	X		X		X
NIH Toolbox (Post Scan)	X		X		X
Labs					
CMP / CBC / DNA	X				
Plasma Biomarkers	X		X		X
Riboflavin			X		X
In-House Labs					
UDS / Breathalyzer	X	X	X	X	X
Pregnancy Test	X		X		X
Saliva Drug Screen			X		X
Misc					
Actigraphy		X	X	X	X
^{1}H-MRS / fMRI			X		X
Dispense Meds		X		X	

* After eligibility confirmed via SCID-5 and H&P, whenever possible. Non-completed measures to be administered at V2, before MRI.

C.3.a. Baseline Assessment (Visit 1). Following brief screening over the phone or at a referral site, potential participants will be scheduled for formal screening at The Department of Psychiatry research clinic. They will read and sign an IRB-approved informed consent, HIPAA, and BrAC documents and will then be assessed for eligibility using the Structured Clinical Interview for DSM-V (First, 1995). Past 90-day drug and alcohol use will be assessed using the Timeline Followback method (TLFB; Sobell, 1995). Mood symptoms will be assessed using the YMRS, MADRS, Hamilton Anxiety Scale (Hamilton, 1959), the Columbia Suicide Severity Rating Scale (C-SSRS; Posner, 2011), Beck Depression Inventory-II (BDI-II; Beck, 1996), and Beck Anxiety Inventory (BAI; Beck, 1983). Cognition will be assessed using the NIH Toolbox – Cognition Battery (Gershon, 2013). Sleep will be assessed using the Pittsburgh Sleep Quality Index (PSQI; Buysse, 1989). A Metal Screening Questionnaire will assess MRI safety. Family history will be assessed with the Family History Screen (FHS; Milne, 2009). Impulsivity (BIS; Patton et al., 1995), hedonic tone (Snaith-Hamilton Pleasure Scale [SHAPS]; Snaith et al., 1995), personality pathology (Personality Inventory for DSM-5 [PID-5; Suzuki et al., 2015]), state-trait anger (State-Trait Anger Expression Inventory [STAXI-2]; Spielberger, 1995), aggression (Aggression Questionnaire; Buss & Perry, 1992), ADHD symptoms (Adult ADHD Self-Report Scale [ASRS; Kessler et al., 2005]), childhood trauma (Childhood Trauma Questionnaire [CTQ]; Bernstein et al., 1994), functional impairment (WHO Disability Assessment Scale [WHODAS; WHO, 2010]), will be measured.

Study candidates who meet diagnostic criteria will undergo a full medical history and physical exam and will provide samples for blood chemistries (Comprehensive Metabolic Panel [CMP], Complete Blood Count [CBC]) and genetics. OS biomarkers (GSH, relative to glutathione disulfide [GSSG], the product of GSH oxidation, and extent of s-glutathionylated proteins, a novel biomarker that increases under OS, using the methods detailed by Dr. Townsend (Co-I) and colleagues in Zhang, 2018). Qualitative drug screens will be performed using the Discover 12 Panel Cup® (Discover), an in vitro diagnostic test for the detection of drug or drug metabolite in urine as well as a saliva drug screen. Female participants will undergo pregnancy testing.

C.3.b. Medication Dispensing (Visits 2 and 4). On day 1 of each condition, mood symptoms (YMRS, MADRS, C-SSRS, HAM-A) and alcohol/drug consumption (TLFB, UDS) will be assessed. Medications will be packaged and dispensed by the MUSC Investigational Drug Service (IDS), a centralized research pharmacy that compounds medications. IDS will oversee blinding procedures for the study and maintain treatment assignment records. Study medication will be over-encapsulated with riboflavin (50mg/capsule, for urinary detection by fluorescence spectroscopy at each MRI), with each capsule containing either 0.5g of NAC or matching placebo dispensed in blister packs. A daily dose of 3g NAC per day was chosen because it represents the largest daily dose of NAC that has been evaluated in studies of individuals with psychiatric conditions, including the study referenced in our sample size determination (below). At each dispensing visits, participants will take their first dose (1.5g NAC or placebo) in front of study staff to ensure compliance. Following their first dose, participants will be instructed to take the study medication twice daily (to maximize compliance) for 14 days. Although NAC has an elimination half-life of 6-7 hours, with steady state concentrations achieved within 30-35 hours (Holdiness, 1991) and brain GSH increases observed in <60m following an acute dose (Holmay, 2013), a 14-day dosing period was chosen to maximize chances of finding NAC effects on GSH levels while maintaining a reasonably-minimal anticipated rate of participant dropout (<20%, estimated from experience with the population). At Visit 2, participants will be asked to wear an actigraphy watch (Actiwatch Spectrum Plus, Philips Respironics) on their non-dominant wrist for the remainder of the study. They will be instructed on its use (e.g., not to take it off when showering) and asked to keep a brief actigraph log (e.g., sleep and wake times) delivered daily via text/e-mail using REDCap.

C.3.c. MRI (Visits 3 and 5). Participants will be asked to abstain from drugs and alcohol ≥ 12 hours prior to MRI. Mood symptoms (YMRS, MADRS, HAM-A, BDI, BAI, BDRS, BR, OAS, PANSS-7, OAS), alcohol and drug consumption (Breathalyzer, TLFB, UDS, Saliva Drug Test), will be assessed upon arrival. Questionnaires evaluating cigarette and tobacco use (FTND), the presence of manic symptoms (ASRM; Altman, 1997),

psychotic symptoms (CAPE-42), suicidal ideation (CHRT; Trivedi, 2008), anxiety (STAI), anger (STAXI), and insomnia (ISI), will be evaluated. Participants will complete the STOP-IT and Delay Discounting computer tasks. Participants will take their final medication dose in front of staff, 1 hour before MRI, to ensure compliance. Unused study medications will be returned for pill counts. Participants who smoke will be allowed to have their last cigarette immediately prior to their final medication dose. Participants will provide blood and urine samples to test for gabapentin levels, riboflavin, and OS biomarkers. Total scan time is 60 min in a Siemens 3.0T Prisma (32-ch head coil) with actively shielded magnet and high-performance gradients. Participants will discontinue study medication following each MRI. To minimize potential carry-over effects, conditions will be separated by a 14-day washout period, as this has been shown to prevent carry-over effects across numerous studies featuring up to 8-weeks of NAC dosing (e.g.,Stav, 2009; Khaledifar, 2014). *1H-MRS Acquisition and Processing.* A structural scan will be taken for 1H-MRS voxel placement and tissue segmentation (256 sagittal slices; 1mm thick/50% gap). A dACC voxel will be placed on midsagittal T1-weighted images, posterior to the genu of the corpus callosum, with the ventral edge of the voxel aligned with the dorsal edge of the callosum (Hermann, 2012). This location was selected given its unique status as the primary hub within the network of brain structures responsible for the cognitive control of behavior (Shenhav, 2016), and because the vast majority of 1H-MRS investigations of brain GSH in psychiatric populations have acquired data from this region (Das, 2019). The voxel will be 2.5x2.5x3 cm3 to ensure adequate signal to noise properties. Following placement of 6 saturation bands 5mm away from the voxel faces and auto-shimming via FASTESTMAP, single-voxel water-suppressed 1H-MRS spectra will be acquired via TE-optimized MEGA-PRESS (TR=2000ms; TE-120ms; number of averages=256) using editing-pulse frequencies for GSH acquisition (4.56 [ON], 7.5 ppm [OFF]; Prisciandaro, 2020). See *Figure 4* for representative (a) dACC voxel placement and (b) fitted data, comparing GSH spectra the TE-optimized (i.e., 120ms) acquisition (right), which provided an average of 97.3% more GSH signal, relative to the more commonly used 68-80ms TE acquisition (left). Subsequently, a low-TE PRESS sequence sensitive to glutamate will be acquired (TR=2000ms; TE=40ms; number of averages=128; Mullins, 2008). Unsuppressed water spectra will be co-acquired for each sequence. MEGA-PRESS data will be analyzed using the Gannet MATLAB toolbox (Edden, 2014). PRESS data will be analyzed using LCModel 6.3 (Provencher, 1993). Metabolites with fitting uncertainties <20% will be retained. Water will be quantified from a Gaussian-Lorentzian fit to non-water-suppressed data. Within voxel tissue fractions of gray (GM) and white (WM) matter and CSF will be calculated based on automated segmentation in Statistical Parametric Mapping 12 (Wellcome Trust) using a mask generated in Gannet. Metabolite concentrations will be normalized to unsuppressed water and corrected for within-voxel CSF fraction.

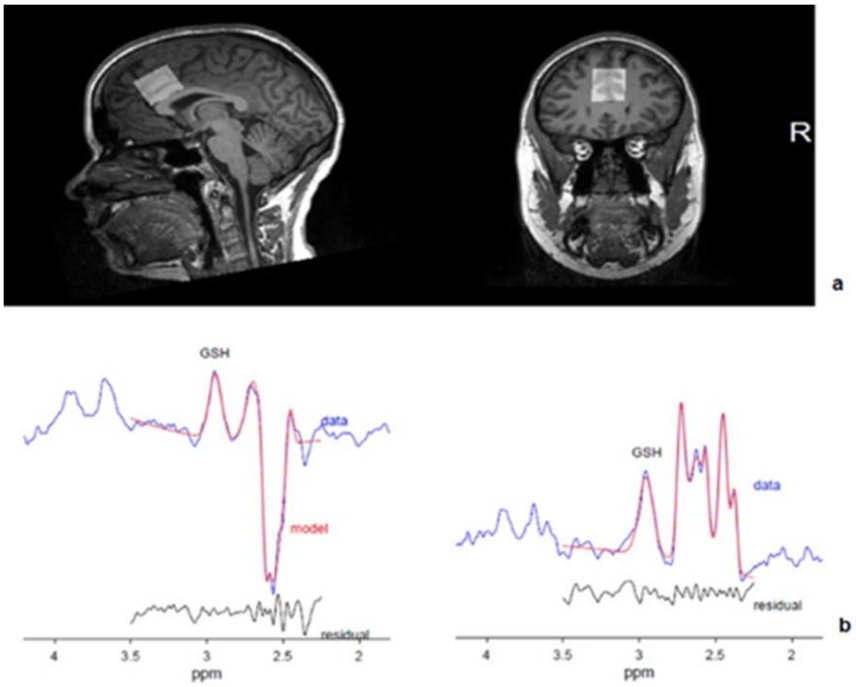

Figure 4. Sample *(a)* dACC voxel placement (top panel), and *(b)* fitted GSH spectra from 80ms (left) and 120ms (right) TE MEGA-PRESS acquisitions.

C.4. Statistical Considerations.

C.4.a. Sample Size Determination. In the only published investigation of chronic NAC dosing on brain GSH levels in a psychiatric sample, a 23% increase in medial prefrontal GSH was found in (2.7g/day) NAC-treated participants with early psychosis, in contrast to the 4.6% *decrease* found in placebo-treated participants (Conus, 2018). Our test-retest investigation of TE-optimized MEGA-PRESS for *Figure 2.* Sample *(a)* dACC voxel placement (top panel), and *(b)* fitted GSH spectra from 80ms (left) and 120ms (right) TE MEGA-PRESS acquisitions. GSH in 11 HC demonstrated excellent stability in within-subject dACC GSH levels (*M* Coefficient of Variation = 7.25%). Using variance/covariance estimates from Prisciandaro (2020), we calculated statistical power across a range of potential magnitude between-treatment changes in dACC GSH levels. With *n*=10 study

completers (*n*=12 enrolled with an estimated dropout rate of 15-20%), the proposed study would have >80% power to detect the 23% NAC-induced increase in GSH levels reported in Conus (2018) (α=0.05, two-tailed; IBM SPSS v27).

C.4.b. Data Analysis. Linear mixed effects models will be used to assess the effect of NAC on dACC GSH levels (*Hypothesis 1*). Models will be re-estimated with dACC glutamate levels entered in place of GSH levels (*Exploratory Hypothesis 1*). We predict that NAC will *increase* GSH levels, but *decrease* glutamate levels, relative to placebo. *Exploratory Hypothesis 2* will be evaluated by adding the following variables, setwise, as interacting time-varying covariates to the *Hypothesis 1* linear model: i) plasma GSH levels, extent of s-glutathionylated proteins, ii) YMRS, MADRS scores, and iii) NIH Toolbox-Cognition Battery scores. Models will contain the main effect of treatment (NAC vs. placebo), period (scan 1 vs. scan 2), and sequence (NAC 1st vs. placebo 1st) to ensure the crossover design and washout were successful. Although mood-stabilizing medications have not been associated with GSH levels (Das, 2019), we will evaluate them, along with other baseline variables that may be associated with OS (age, sex, smoking status; Keaney, 2003), as moderators across analyses to facilitate rigor and reproducibility and inform future studies. Within-voxel tissue content will be evaluated as a covariate across models. We will employ previously successful methods to minimize attrition. Where missing data cannot be avoided, mixed models yield valid inferences assuming data are missing at random.

C.5. DISCUSSION

C.5.a. Overview. Despite available efficacious treatments, BD remains a leading driver of global disease burden. Convergent evidence supports ACC GSH levels as a promising interventional target, and NAC as a candidate adjunctive treatment to increase brain GSH levels, in BD individuals. The proposed randomized, placebo-controlled, double-blind, crossover MRI study aims to increase dACC GSH levels using NAC (3g/day) in individuals with BD and to evaluate medication-related, GSH- and glutamate-mediated changes in mood, cognition, and plasma biomarkers of OS, in individuals with BD. The study will be conducted by a BD research team with a successful 10+ year record of collaboration, using imaging methods optimized by the study team. Results may support the development of increasingly-targeted medications designed to fight excessive OS through increasing brain GSH levels that would clinically benefit individuals with BD.

C.5.b. Strategies to Ensure a Robust and Unbiased Approach. The proposed study will achieve robust and unbiased results via explicit inclusion/exclusion criteria; randomization of treatment condition order and examination of order effects; placebo control; double blinding; sophisticated compliance monitoring; use of validated MRI, laboratory, and interview/self-report measures/methods; explicit hypotheses and planned statistical analyses; power estimates; planned handling of attrition and missing data; and careful consideration of potential confounds. Methodology is reported in a detailed and fully transparent manner to support replication.

C.5.c. Potential Limitations. NAC may reduce neuro-OS burden in BD individuals by increasing GSH or decreasing glutamate levels. Despite possessing multiple potential neurochemical mechanisms of action, NAC remains the best available drug for the proposed study for two reasons. First, NAC is the only drug that has been shown to modulate brain GSH levels *in vivo*. Second, though most ^{1}H-MRS NAC studies have reported *either* GSH or glutamate effects, the proposed study will acquire *both* GSH and glutamate levels within the same scan, allowing us to determine the relative extents to which NAC modulates dACC GSH and glutamate levels.

C.5.d. Future Directions. Positive results would directly support the hypothesized mechanism of NAC treatment for BD. Perhaps more importantly, positive results would provide evidence for a novel neurobiological treatment target for BD, which would support the development of medications designed to increase brain GSH levels (with greater specificity and efficacy than NAC) and their evaluation using the proposed experimental neuroimaging framework. For example, γ-glutamylcysteine, precursor for the final step of GSH synthesis, bypasses the feedback inhibition by GSH on γ-glutaminecysteine ligase, making it a potentially more efficacious glutathione prodrug than NAC. Compounds consisting of γ–glutamylcysteine alongside an attached ethyl ester moiety (to increase blood-brain-barrier permeability) have received growing interest in preclinical research on oxidative injury, cardiac disease, and neurodegenerative disorders (Pocernich, 2012; Giustarini, 2018). Finally, the proposed study will add to the literature on associations of brain GSH and glutamate levels, plasma biomarkers of OS, and BD clinical phenomenology (mood symptoms and cognitive dysfunction).

Protection of Human Subjects

1. **Risk to the Subjects**

a. Human Subject Involvement and Characteristics. A total of 12 individuals with bipolar disorder (BD) in stable medical condition will be enrolled in the study. Women and minorities will be recruited for this study.

Children and adolescents under the age of 18 will not be enrolled.

Inclusion Criteria:

1. Ages 18-60 years
2. Meet DSM-5 criteria for bipolar I or II disorder
3. Able to provide informed consent and read, understand, and accurately complete assessment instruments
4. Willing to commit to medication treatment and follow-up assessments
5. Meets DSM-5 criteria for any mood episode (i.e., Major Depressive, Hypomanic, Manic), Current or In Partial Remission
6. Prescribed daily use of at least one FDA-approved mood stabilizing medication (i.e., lithium, divalproex sodium, lamotrigine, 2nd generation antipsychotic)
7. Willing to abstain from antioxidant supplements (e.g., coenzyme Q-10, vitamin E) for the duration of the study.

Exclusion Criteria:

1. A primary psychiatric diagnosis other than BD (e.g., Schizophrenia)
2. Meet DSM-5 criteria for substance use disorder (other than Tobacco Use Disorder) within the past 6 months.
3. Any uncontrolled neurological condition (e.g., epilepsy) that could confound the results of the study
4. Any history of brain injury with loss of consciousness greater than 5 minutes
5. Any history of mental retardation, dementia, or recent electroconvulsive therapy (in the past 3 months)
6. Any uncontrolled medical condition that may adversely affect the conduct of the study or jeopardize the safety of the participant
7. Hepatocellular disease as indicated by plasma levels of liver transaminases (aspartate transaminase, alanine transaminase) greater than 3 times the normal range
8. Renal insufficiency as indicated by plasma levels of creatinine greater than 2 times the normal range
9. Concomitant use of nitroglycerine, carbamazepine, or any other medication deemed to be hazardous if taken with N-Acetylcysteine (NAC).
10. Medication dose changes of ≥ 20% ≤ 2 weeks prior to testing
11. Women of childbearing potential who are pregnant, lactating, or refuse adequate forms of contraception
12. Current suicidal or homicidal risk
13. Baseline scores greater than 35 on the Montgomery-Asberg Depression Rating Scale or greater than 25 on the Young Mania Rating Scale
14. Has taken NAC in the last month or experienced adverse effects/allergic reaction from it at any time
15. Significant claustrophobia and/or past negative experiences with MRI
16. Presence of non-MRI safe materials in the body (e.g., ferrous metal implants, pacemaker)

b. Source of Materials. Data collected from participants will include breathalyzer readings, urine and saliva drug screens, urine biomarkers (e.g., riboflavin), blood chemistries, plasma biomarkers, structural and neurochemical MRI brain images, cognitive testing results, and interviews and self-reports regarding substance use, psychiatric diagnoses, concomitant medications, and adverse events. To ensure confidentiality, all participant data will be number-coded, and only the investigators will have access to the master list of codes.

c. Compensation. To maximize participant retention, contingency management will be applied to participant compensation such that participants will be compensated significantly more for each subsequent MRI visit they attend (i.e., scan 1 = $100, scan 2 = $150), and participants will be given an additional $50 bonus for completing both MRI visits. Participant compensation will be cash. Participants will be compensated $50 for the initial psychiatric and medical evaluation appointment. Finally, participants will be compensated $25 for each additional non-MRI appointment (i.e., 2 total). Compensation will thus be $400 per participant. In our experience, this level of compensation is fair for the time commitment required without unduly coercing participants to enroll in the study despite potential concerns.

d. Potential Risks

1. Loss of Confidentiality. Individuals will be asked questions about their psychiatric symptoms. This information is highly confidential and all attempts will be made to safeguard privacy and confidentiality.
2. Medication side effects. NAC has a generally benign adverse effect profile. A meta-analysis of studies evaluating long-term oral treatment with NAC for prevention of chronic bronchitis found that NAC was well tolerated, with generally mild, most commonly gastrointestinal adverse effects that did not require treatment interruption (Grandjean et al., 2000). Common adverse effects of NAC include dry mouth, nausea, vomiting, and diarrhea. The proposed study will carefully monitor potential medication-related risks via weekly

scheduled visits and assessment of adverse events (including assessment of suicidal ideation and behavior at each and every study visit). The study physician, Dr. Tolliver (Co-I), will determine if the participant should be discontinued from the medication due to adverse drug reactions and will treat clinically as needed. Any confirmed incidence of serious adverse events that are deemed probably or definitely due to the study medication will result in immediate discontinuation of the study medication and follow-up assessments will be conducted until resolution. Subjects will be referred for treatment as necessary.

3. Drug interactions. Due to potential interactions with NAC, participants may not take nitroglycerin or carbamazepine at any time during participation in the study. As noted earlier, BD participants will be required to be taking a stable pre-existing regimen of at least one FDA-approved mood stabilizing medication treatment for BD. There are no known drug interactions with NAC and non-carbamazepine mood-stabilizing medications (i.e., lithium, lamotrigine, divalproex sodium, 2nd generation antipsychotic).
4. Unknown Medication Risks. While NAC is approved by the FDA, it is not approved for the use in this study (i.e. the treatment of Bipolar Disorder). Because of this, there may be unknown medication side effects. Study clinicians and the study physician will monitor all adverse events throughout the study as well as the likelihood that they are related to the study medication. In the event that there are any serious adverse events, the study physician will instruct the participant to discontinue the use of the study medication.
5. MRI-related risks. Individuals with non-MRI-safe medical implants or ferrous objects would be at risk for injury, if such individuals were allowed to enter the MRI scanner. Several precautions will be taken to ensure that individuals with ferrous implants or objects are not allowed to enter the MRI scanner. First, all potential participants will meet with a study physician to discuss any possible history of ferrous implants or other MRI-unsafe objects. Participants with any suspected history of ferrous implants or exposure to shrapnel will be excluded from the study. Second, participants who are deemed MRI-safe by the study physician will be screened for metal objects at the Center for Biomedical Imaging (CBI) using a handheld metal detector and a second metal detector built into the threshold of the doorway to the scanner. Participants who screen positive for metal will be asked to remove all metal objects from their person and will be rescreened. If participants continue to screen positive for metal after removing all metal objects from their person, they will be excluded from the study. Although not dangerous, participants who are claustrophobic could experience significant discomfort in the MRI scanner. As such, all participants will be assessed in terms of claustrophobia as well as past experience with MRI. Additionally, all participants will be entered into a "mock scanner" at the CBI human imaging center, which features the same dimensions of the real MRI scanner but without any of the internal machinery. Participants who report claustrophobia, past negative experience with MRI, or signification discomfort in the mock scanner will be excluded from participation. These procedures have been successfully used by our staff in previous and ongoing research studies of similar participant populations. For those participants allowed to participate in the MRI study, if abnormalities in collected brain images are found, participants will immediately be referred to an appropriate clinical care provider.
6. Mood destabilization. Exacerbation of depressive or manic symptoms during the course of the study is a risk for BD subjects regardless of treatment condition. We will minimize this risk by assessing mood symptoms, including suicidal ideation and behavior, at each and every study visit. Assessment of suicidal ideation will be conducted using primarily the Columbia-Suicide Severity Rating Scale (C-SSRS) and also the MADRS. Any participant who answers affirmatively to item 4 (SI with some intention of acting on them) or item 5 (SI with plan) on the C-SSRS or scoring > 4 on item 4 of the MADRS will be immediately removed from the study and procedures for emergent clinical assessment begun (see *2.c.* below, *Protection Against Risk*). Participants experiencing sufficient deterioration of mood stability to result in clinically significant impairment in functional capacity will be appropriately referred. Any subject exhibiting mood destabilization that is sufficient to pose an imminent danger to self or others will be accompanied by the study physician to the University Hospital Emergency Department for immediate assessment for psychiatric hospitalization.
7. Pregnancy. MRI is used in this study. Participants who are pregnant or are trying to become pregnant should not participate as to ensure there is no risk to an embryo or fetus. Pregnancy tests will be conducted to ensure participants are not pregnant during study procedures.

2. Adequacy of Protection Against Risks

a. Recruitment and Informed Consent. Recruitment will occur by clinical referral and response to advertisements. IRB-approved study staff with advanced clinical degrees (e.g., PhD, MD) will obtain informed consent. Interested individuals will be given a brief description of the study and if still interested will be scheduled for a screening visit. At the screening visit, potential participants will be provided a copy of the IRB-approved consent document to review. After providing the participant with time to read the consent, IRB-approved study

staff will review the consent document page by page with the participant and answer any questions. Only then will the participant be asked to sign the consent document. Participants will be given a copy of the signed consent document. The entire informed consent process will be documented in the research progress notes. The signed, original consent document will be maintained in the participant source record with a copy of the consent binder located at the Institute of Psychiatry, Department of Psychiatry and Behavioral Sciences, Medical University of South Carolina.

b. Safety Assessments. At every visit, IRB-approved study staff with advanced clinical degrees (e.g., PhD, MD) will clinically evaluate subjects' manic and/or depressive symptoms, including suicidal ideation. Mood symptoms will also be quantified using standardized instruments including C-SSRS, MADRS, YMRS, BDI-II, and BAI. Adverse events will be assessed at every study visit.

c. Protection Against Risk. Psychiatric Risks. The investigative team has a great deal of experience working with the study population and has the resources to make appropriate referrals as needed. Psychiatric symptoms will be assessed on a weekly basis by standardized assessments and by clinical interview. Participants answering affirmatively to item 4 (SI with some intention of acting on them) or item 5 (SI with plan) on the C-SSRS or scoring >4 on the suicide item (item 4) of the MADRS, or otherwise exhibiting potentially life-threatening decompensation in mood or other psychiatric symptoms at any study visit will be removed from the study and procedures for emergent clinical assessment begun at once. Briefly, actively suicidal participants will be accompanied to the adult ER in the main MUSC hospital by the study psychiatrist (Dr. Tolliver, co-I). One-to-one accompaniment and supervision by the study psychiatrist will continue until the participant is admitted for triage in the ER. Should a participant refuse voluntary assessment in the ER, MUSC Public Safety will be called immediately and involuntary commitment papers will be filled out by Dr. Tolliver; participants refusing voluntary assessment will be accompanied at all times by study personnel and not allowed to leave the research clinic during emergency procedures. While a rare event, over the past 15 years Dr. Tolliver has implemented these procedures successfully 10-12 times and study participants have been admitted to the Institute of Psychiatry without exception.

Medical Risks. NAC is largely excreted by a hepatic route. A blood chemistry panel will be performed and reviewed by the study physician prior to beginning the study medication. Participants with clinically significant hepatic (transaminases elevated > 3 times normal) insufficiency will not be eligible to participate in the study. Subjects will be referred for treatment as necessary.

MRI Risks. The investigative team has a great deal of experience with human MRI research and Dr. Prisciandaro is a core faculty member of the CBI at MUSC. MRI safety and comfort will be assessed at baseline and at each MRI visit. Participants with non-MRI-safe medical implants or ferrous objects will be excluded from participation as will individuals evidencing significant discomfort with MRI.

Pregnancy. NAC is rated as a *Category B* medication in terms of pregnancy. Because there are no adequate controlled studies of NAC in pregnant women, it is unknown whether the drug can cause fetal harm or affect reproductive capacity in humans. Therefore, women of childbearing potential must agree to pregnancy testing and use of adequate contraception in order to be eligible to participate in the study. Females will be given a urinary pregnancy test at the screening visit and weekly thereafter. Any female participant who becomes pregnant during the study will be discontinued from the study medication and removed from study participation. For all included females of childbearing potential, current forms of birth control and date of last menstruation will be assessed at each study visit.

Confidentiality. Records with identifying information (e.g., consent documents) will be stored in a locked file. All other non-MRI participant data will be collected via direct data capture (REDCap); MRI data will be automatically transferred to Linux-based servers managed by CBI. Both MRI and non-MRI data will be stored in restricted access directories on password-protected, encrypted servers managed by CBI and the Department of Psychiatry and Behavioral Sciences at MUSC. Participants will be given an ID number for all MRI and non-MRI data files. The master list of codes will be accessible only to the investigators, and will be stored in a locked office.

Emergencies. All study participants will be instructed how to access the 24-hour on-call system available at the Medical University of South Carolina. In the event that a participant experiences an adverse event after hours, s/he will be instructed to access the 24-hour on-call service. If it is determined that the participant needs immediate help, the participant may be advised to immediately go to the emergency room. In that event, proper medical treatment will be administered, per ER procedures. Dr. Prisciandaro and Dr. Tolliver will be available 24 hours/day, 7 days/week by pager as necessary. The Investigational Drug Service (IDS) will also be available 24 hours/day, 7 days/week for emergency identification of treatment group assignment and unblinding as

necessary.

3. Potential Benefits of the Proposed Research to the Subjects and Others

Benefits to the participants include medical and psychiatric assessments provided at no cost. Other individuals with BD are likely to benefit by the knowledge gained from the study as it may help guide future treatments

4. Importance of the Knowledge to Be Gained

Despite nearly 70 years of medication development for Bipolar Disorder (BD), BD remains one of the leading drivers of global disease burden and mortality. Existing BD medications typically leave patients with significantly impairing residual mood symptoms and cognitive dysfunction. The proposed study addresses the unmet clinical needs of BD patients using an exemplification of the modern drug discovery approach championed by the NIH and FDA: a brief, proof-of-concept study of the GSH prodrug, NAC, on neuroimaging markers of oxidative stress. Positive results may provide evidence for a novel neurobiological treatment target for BD. Such evidence would support the development and testing of medications designed to increase brain GSH levels, with potentially greater specificity and efficacy than NAC (e.g., γ-glutamylcysteine ethyl ester), using the experimental neuroimaging framework developed and validated by the proposed study. The proposed investigation's minimal risks are reasonable in relation to the importance of the knowledge to be gained from the investigation.

5. Clinicaltrials.gov Requirements

In accordance with Public Law 110-85, this project will be registered at the ClinicalTrials.gov Protocol Registration System Information Website prior to study initiation.

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