Connectivity between the BNST and insula

during abstinence from alcohol use disorder

By

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For Ryan,

who made this possible

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LIST OF ABBREVIATIONS

ACC	Anterior Cingulate Cortex
AUD	Alcohol Use Disorder
AUDIT	Alcohol Use Disorders Identification Test
BLA	Basolateral Amygdala
BNST	Bed Nucleus of the Stria Terminalis
BOLD	Blood-Oxygenation-Level-Dependent
CeA	Central Nucleus of the Amygdala, or Central Amygdala
CRF	Corticotropin Releasing Factor
CRH	Corticotropin Releasing Hormone
CSF	Cerebrospinal Fluid
DREADD	Designer Receptors Exclusively Activated by Designer Drugs
DSM	Diagnostic and Statistical Manual of Mental Disorders
DTI	Diffusion Tensor Imaging
EtG	Ethyl Glucuronide
FLIRT	FMRIB's Linear Image Registration Tool
fMRI	Functional Magnetic Resonance Imaging
FSL	FMRIB's Software Library
GAD	Generalized Anxiety Disorder
LDH	Lifetime Drinking History
LMM	Linear Mixed Model

MDD	Major Depressive Disorder
MINI	Mini-International Neuropsychiatric Interview
MNI	Montreal Neurological Institute
MRI	Magnetic Resonance Imaging
NAcc	Nucleus Accumbens
OFC	Orbitofrontal Cortex
PNC	Philadelphia Neurodevelopmental Cohort
QFI	Quantity Frequency Index
ROI	Region of Interest
SCID	Structure Clinical Interview of the DSM
SENSE	Sensitivity Encoding
SNRI	Serotonin-Norepinephrine Reuptake Inhibitor
SPM	Statistical Parametric Mapping
SSRI	Selective Serotonin Reuptake Inhibitor
vmPFC	Ventromedial Prefrontal Cortex

CHAPTER I

INTRODUCTION[1]

Preface

The introduction included is designed to provide the reader with the information needed for critical evaluation of the proposed thesis. The scope of this introduction is not intended to be a comprehensive review, rather, an opportunity to introduce and explore key concepts underlying the hypotheses and interpretation of the results of the original research presented. I will begin with an introduction to alcohol use disorder (AUD). Next, I will examine the leading theory of the neurobiology underlying alcohol use disorder (AUD), with a particular focus on abstinence and the extended amygdala. I will then introduce the insula and examine the role of the insula in emotions and addiction. Finally, the unifying hypothesis for the thesis and the specific aims will be introduced. This project is translational in nature, and much of the foundational addiction work has been conducted using rodent models of addiction. For this reason, the introduction will include both rodent and human literature.

[1] Parts of this chapter have been adapted from "Anxiety during abstinence from alcohol: A systematic review of rodent and human evidence for the anterior insula's role in the abstinence network", published in *Addiction Biology* and has been reproduced with the permission of the publisher and my co-authors: JR Luchsinger, MM Silveri, DG Winder, MM Benningfield, and JU Blackford

Introduction

Alcohol use disorder (AUD) is a chronic, relapsing disorder that affects more than 15 million Americans (SAMHSA, 2019). Individuals with AUD[2] experience impairment and distress from alcohol use (see Table 1), and the disorder is characterized by drinking despite negative consequences, difficulty in limiting alcohol intake, and the compulsion to seek or take alcohol (American Psychiatric Association, 2013; First, Williams, Karg, & Spitzer, 2015). Alcohol abuse and misuse is responsible for nearly 100,000 deaths annually in the United States alone and accounts for over 2.8 million years of potential life lost (Centers for Disease Control and Prevention, 2020). In addition to alcohol poisoning and accidental death, excessive alcohol intake can lead to directly and indirectly impact cardiac, hepatic, pancreatic, and nervous system health, contributing to substantial morbidity (Centers for Disease Control and Prevention, 2020). Preventing the onset and perpetuation of AUDs, therefore, has the potential to improve millions of lives.

Unfortunately, AUD is exceptionally difficult to treat, with the majority of individuals relapsing within a year of initiating treatment (Sinha et al., 2011; Willinger et al., 2002; Zywiak et al., 1996, for review see Bradizza et al., 2006). Much of this difficulty is thought to arise from the symptoms associated with abstinence from alcohol. Within 72 hours of sobriety, individuals can experience life-threatening withdrawal symptoms of autonomic dysregulation, confusion, agitation, and seizures. Fortunately, established medical protocols have greatly reduced the risk

[2] Current diagnostic criteria included for alcohol use disorder. Previous diagnostic criteria separated alcohol use disorder into alcohol abuse and alcohol dependence. For the sake of clarity, “alcohol use disorder” will be used to describe any current or previous diagnoses in the included literature.

of morbidity and mortality from these symptoms. However, as the acute and life-threatening symptoms seen in withdrawal subside after a few days, a new phase, abstinence, emerges with more chronic symptoms: heightened stress reactivity, attenuated reward processing, sleep disturbances, and anxiety (Heilig, Egli, Crabbe, & Becker, 2010). These negative affective symptoms can persist for week or months into abstinence and are thought to explain the elevated relapse risk that persists well into the first year of sobriety (Bradizza, Stasiewicz, & Paas, 2006b; Heilig et al., 2010; Willinger et al., 2002). Therefore, understanding and addressing the basis for negative affective symptoms in abstinence could improve relapse rates and patient outcomes in AUD.

Table 1: *Diagnostic and Statistical Manual of Mental Disorders (DSM)-V Criteria for Alcohol Use Disorder*

A. A problematic pattern of alcohol use leading to clinically significant impairment or distress, as manifested by at least two of the following, occurring within a 12-month period: 1. Alcohol is often taken in larger amounts or over a longer period than was intended. 2. There is a persistent desire or unsuccessful efforts to cut down or control alcohol use. 3. A great deal of time is spent in activities necessary to obtain alcohol, use alcohol, or recover from its effects. 4. Craving, or a strong desire or urge to use alcohol. 5. Recurrent alcohol use resulting in a failure to fulfill major role obligations at work, school, or home. 6. Continued alcohol use despite having persistent or recurrent social or interpersonal problems caused or exacerbated by the effects of alcohol. 7. Important social, occupational, or recreational activities are given up or reduced because of alcohol use. 8. Recurrent alcohol use in situations in which it is physically hazardous.

9. Alcohol use is continued despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by alcohol.
10. Tolerance, as defined by either of the following:
    a. A need for markedly increased amounts of alcohol to achieve intoxication or desired effect.
    b. A markedly diminished effect with continued use of the same amount of alcohol.
11. Withdrawal, as manifested by either of the following:
    a. The characteristic withdrawal syndrome for alcohol (refer to Criteria A and B of the criteria set for alcohol withdrawal, pp. 499–500).

    b. Alcohol (or a closely related substance, such as a benzodiazepine) is taken to relieve or avoid withdrawal symptoms.

Specify if:

- **In early remission:** After full criteria for alcohol use disorder were previously met, none of the criteria for alcohol use disorder have been met for at least 3 months but for less than 12 months (with the exception that Criterion A4, "Craving, or a strong desire or urge to use alcohol," may be met).
- **In sustained remission:** After full criteria for alcohol use disorder were previously met, none of the criteria for alcohol use disorder have been met at any time during a period of 12 months or longer (with the exception that Criterion A4, "Craving, or a strong desire or urge to use alcohol," may be met).

Specify if:

- **In a controlled environment:** This additional specifier is used if the individual is in an environment where access to alcohol is restricted.

Specify current severity/remission:

- **Mild:** Presence of 2–3 symptoms.
  - Mild, in early remission
  - Mild, in sustained remission
- **Moderate:** Presence of 4–5 symptoms.
  - Moderate, in early remission
  - Moderate, in sustained remission
- **Severe:** Presence of 6 or more symptoms.
  - Severe, in early remission
  - Severe, in sustained remission

Reference: American Psychiatric Association, 2013.

Alcohol use disorder and the brain

Significant advances in establishing AUD as a neurobiological disorder have been made over the past few decades. Rodent models of AUD have characterized the effects of both acute and chronic alcohol intake on the brain (Centanni, Bedse, Patel, & Winder, 2019; G. Koob, 2013; G. F. Koob & Volkow, 2016; Noori, Spanagel, & Hansson, 2012), including the neuroadaptations that occur in response to prolonged alcohol exposure. Initially alcohol acts as a rewarding substance, resulting in positive reinforcement. As alcohol consumption persists, neuroadaptations lead to allostasis, resulting in increased stress responses and shifting alcohol intake from being positively to negatively reinforced (G. F. Koob & Le Moal, 2001).

Positive reinforcement of alcohol intake

Substantial research focused on reward circuitry have demonstrated how alcohol acts as a rewarding, positively-reinforced substance. Initially, acute alcohol intake causes brain changes such as increased GABA and opioid activity in the ventral tegmental area, nucleus accumbens, and amygdala; inhibited glutamate synaptic activity in the amygdala; and increased dopamine release in the nucleus accumbens (Carrillo & Gonzales, 2011; Nestler, 2005; D. L. Robinson, Howard, McConnell, Gonzales, & Wightman, 2009; S. L. Robinson, Alexander, Bluett, Patel, & McCool, 2016; Theile, Hitoshi, A., & A., 2008). As acute intake transitions to chronic use, the brain undergoes neuroadaptation in response to constant alcohol exposure (G. Koob, 2013; G. F. Koob & Le Moal, 2001). These homeostatic changes include decreases in GABA receptor function, striatal endocannabinoid-mediated long-term depression of the synapse, and NMDR-mediated synaptic response (Abrahao et al., 2013; Devaud, Fritschy, Sieghart, & Morrow, 2002;

T. L. Kash, Baucum II, Conrad, Colbran, & Winder, 2009; G. Koob, 2013; Pandey, 2004; Xun et al., 2006). One result of these changes is a prolonged, decreased reward sensitivity (for review see Volkow et al., 2010). Increasing amounts of alcohol are needed to elicit the dopamine release associated with rewarding stimuli, a process known as tolerance. Furthermore, these reward circuitry changes are not limited to alcohol, meaning that individuals with AUD also have decreased reward sensitivity to natural rewards such as food or positive social interactions (Volkow, Fowler, Wang, & Goldstein, 2002).

The shift to negative reinforcement of alcohol intake

As tolerance increases and reward sensitivity decreases, drinking alcohol shifts from a positively-reinforced behavior to a negatively-reinforced behavior (Cho et al., 2019; G. F. Koob & Volkow, 2010). This shift results from the neuroadaptive changes seen in chronic alcohol use, where abstinence from alcohol results in negative affect, which includes anxiety, increased stress sensitivity, and depression (G. F. Koob & Le Moal, 2001; Kornetsky & Esposito, 1979; Läck, Floyd, & McCool, 2005; Lewis & June, 1990). Thus, individuals in abstinence from AUD have a strong motivation to resume drinking, as alcohol can quickly alleviate these symptoms of anxiety and depression (Brady & Sonne, 1999; Mantsch, Baker, Funk, Lê, & Shaham, 2016; Sinha et al., 2011; Zywiak et al., 1996). These symptoms of anxiety and depression during abstinence are therefore a frequent trigger of relapse. Importantly, the prolonged presentation of these symptoms is consistent with the persistently elevated relapse risk that remains even after months of abstinence (Heilig et al., 2010). This stage, characterized by heightened stress

reactivity, anxiety, and depression, has been coined the "dark side of addiction" (for review see G. F. Koob, 2008) and remains a substantial barrier to long-term treatment success.

The current abstinence model: the dark side of addiction

Defining abstinence.

A necessary component of investigating abstinence is a clarification of the terms used to describe stages of AUD. The literature surrounding AUD and abstinence is not always consistent about the terminology. The terms withdrawal and abstinence will be defined for use throughout this project. *Withdrawal:* following cessation of heavy, chronic, alcohol use, many individuals experience symptoms of nervous system hyperexcitability such as tremor, sweating, nausea, itching, and agitation that can deteriorate into seizures, hallucinations, or death. These symptoms, though potentially life threatening, are limited to the first week or two after alcohol cessation (Heilig et al., 2010). *Abstinence:* as withdrawal symptoms subside, the more chronic symptomatology emerges, in which individuals experience anxiety, depression, hyperreactivity to stress, and sleep disturbances (Heilig et al., 2010). This stage will be referred to as abstinence and can last for weeks or months into recovery from alcohol use.

Abstinence and the extended amygdala

As negative affect is increasingly recognized for its role in triggering relapse, a growing number of studies have investigated the neurobiological basis for negative affect during abstinence. In a substantial body of work, Koob and others identified the extended amygdala as critical for driving negative affect during addiction in rodents (e.g. G. Koob, 2013; G. F. Koob & Volkow, 2010). The extended amygdala consists of the bed nucleus of the stria terminalis

(BNST), central amygdala (CeA), and shell of the nucleus accumbens ($NAcc_{shell}$) (Figure 1). The BNST is a small region located at the end of the stria terminalis, a white matter tract connecting the BNST and amygdala. The CeA is a nucleus of the amygdala in the deep, medial temporal lobe. The NAcc is part of the ventral striatum and can be divided into the core and shell, of which only the $NAcc_{shell}$ is considered part of the extended amygdala (Zahm, 1998).

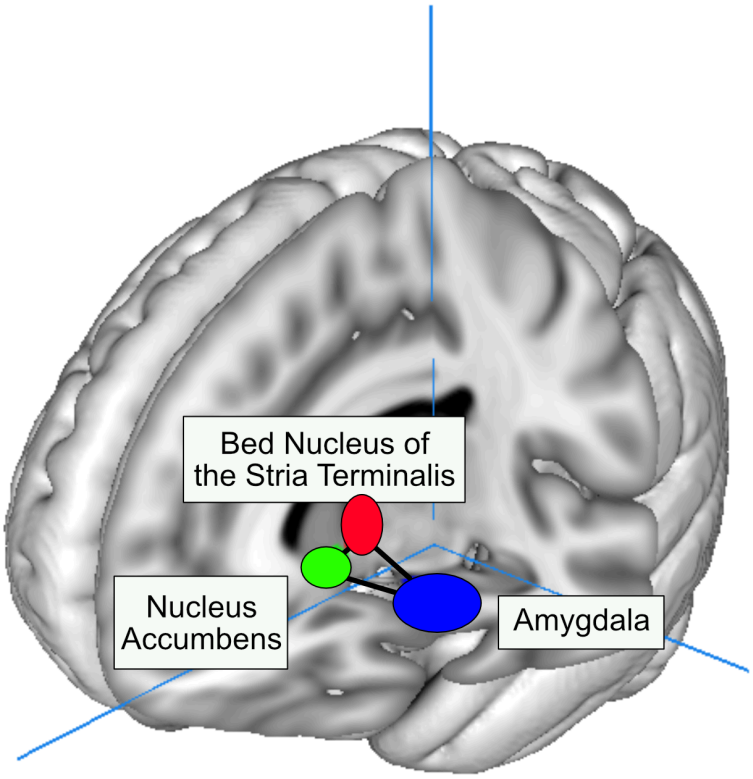

Figure 1. Current abstinence circuit: extended amygdala

The current model of the abstinence circuit includes the bed nucleus of the stria terminalis, the nucleus accumbens (specifically the shell), and the amygdala (specifically the central nucleus of the amygdala), which together form the extended amygdala.

The extended amygdala regulates behavioral responses to emotionally salient stimuli particularly associated with negative affect. Specifically, the BNST is a central hub of anxiety-related behaviors (Avery, Clauss, & Blackford, 2016; Davis, Walker, Miles, & Grillon, 2010) and mediates stress-induced reinstatement (Erb & Stewart, 1999). The CeA regulates fear processing, fear-associated behaviors, the stress response, and anxiety-related behaviors during abstinence (Erb, Salmaso, Rodaros, & Stewart, 2001). The $NAcc_{shell}$ is involved in reward processing and motivated behaviors, which contribute to its role in aversion behaviors (Cardinal, Parkinson, Hall, & Everitt, 2003; Faure, Richard, & Berridge, 2010) and drive compulsive drug seeking during abstinence (Hauser et al., 2015). Together, the BNST, CeA, and $NAcc_{shell}$ act to promote negative affect behaviors during abstinence and trigger stress-induced reinstatement.

Further evidence of the extended amygdala's role in negative affect during abstinence comes from rodent studies of corticotropin releasing factor (CRF). CRF is a neuropeptide released in response to stress and acts to initiate the brain's stress response. In the extended amygdala, administration of CRF induces anxiety-like behaviors and increases alcohol consumption (Chen, Rada, Bützler, Leibowitz, & Hoebel, 2012; Erb et al., 2001; Funk, O'Dell, Crawford, & Koob, 2006; M. M. Huang et al., 2010; Marcinkiewcz et al., 2009). Similarly, blocking CRF activity in the CeA and BNST attenuates anxiety-like behavior during abstinence (M. M. Huang et al., 2010). The effects of CRF on anxiety-related and addiction behaviors provide evidence for the link between stress, negative affect, and abstinence in the extended amygdala (for review see Silberman and Winder 2013).

Over the last few decades, mounting evidence has supported the role of the extended amygdala in abstinence. Rodent studies have demonstrated that extended amygdala physiology is altered in abstinence and signaling within the extended amygdala contributes to negative affect behaviors in abstinence. More recently, studies have provided initial translational evidence that the extended amygdala is involved during abstinence in humans as well.

Abstinence and the extended amygdala: evidence from humans

Human imaging research of the extended amygdala recapitulates findings from rodent research, including preliminary support for a role in anxiety during abstinence. Of note, due to resolution constraints associated with human imaging research, subcortical structures are rarely subdivided. Thus, human literature reviewed here will be of the amygdala and NAcc, unless the CeA or $NAcc_{shell}$ is specifically referenced. Much less research has been conducted on the BNST as methodological advancements have only recently allowed for investigation of the BNST in humans, and the field is still in its infancy (for reviews see Avery et al., 2016; Fox & Shackman, 2019).

Extended amygdala studies in humans have predominantly focused on anxiety, with less research in addiction. Evidence for greater BNST or amygdala activity in patients with anxiety disorders and in anxiety- or fear-provoking tasks has been aggregated in a number of reviews (e.g. Avery *et al.* 2016; Shackman & Fox 2016). Less is known about the NAcc in anxiety, but evidence has demonstrated NAcc activation during avoidance of anxiety-provoking situations (Levita, Hoskin, & Champi, 2012).

Few structural or functional studies have investigated the extended amygdala during abstinence in humans. In one study, the amygdala showed increased activation in response to alcohol cues in abstinent patients with AUD (Schneider et al., 2001). The NAcc has greater resting state connectivity with many cortical regions in patients with AUD who remain abstinent after six months compared to those who relapse (Camchong, Stenger, & Fein, 2013). Only a handful of studies have investigated the interaction of negative affect and addiction. Regions of the extended amygdala were evaluated in one of these studies, which found increased BNST-amygdala connectivity during fearful face viewing in abstinent patients with a history of multiple relapses of AUD (O'Daly et al., 2012). Overall, these studies support rodent findings by providing evidence for the extended amygdala's role in anxiety- and addiction-related neural processes.

Expanding the current model of abstinence

As more research supports the role of the extended amygdala during abstinence, increasingly complex methodological tools have begun to expand findings beyond the extended amygdala. Research using optogenetics and Designer Receptors Exclusively Activated by Designer Drugs (or DREADDs) have allowed for the investigation of brain regions that regulate the extended amygdala's role in abstinence-induced negative affect. In one recent example, the insula was found to be a critical region regulating BNST-mediated negative affect during abstinence (Centanni, Morris, et al., 2019). This finding parallels human investigations of the insula which describe well-characterized roles in addiction, particularly craving and relapse, and emotion (Craig, 2010b; Naqvi & Bechara, 2009). Given the role for the BNST and BNST-insula

pathway in abstinence-induced negative affect, investigating BNST-insula connectivity in humans is an important translational step for the field.

Introduction to the insula

The insula, also known as the insular cortex, the insula lobe, or the island of Reil, is a large and heterogeneous brain region, located deep to the lateral sulcus. The insula is an integrative hub, with robust connections to brain regions serving sensory, emotional, motivational, and cognitive function. In addition to a wide array of normative functions, the insula has recently become a region of great interest for its role in a variety of psychiatric and neurological disorders. Here, we will examine relevant cytoarchitecture, anatomy, connectivity, and function, with an emphasis on emotion and addiction.

Insula cytoarchitecture and anatomy

In most mammals, including humans, the insula is notable for its varied cytoarchitecture (Butti & Hof, 2010), which means the arrangement of cortical cells. The insula is typically divided into three regions, the agranular, dysgranular, and granular regions, which refer to the absence, low concentration, and high concentration of granule cells in cortical layer IV, respectively. The granule cells, most concentrated in the posterior insula, receive substantial input from specific sensory nuclei within the thalamus. Evidence from the cerebellum suggests that these cells are responsible for complex multimodal sensory integration, as inputs from different sensory modalities converge on individual granule cells (C.-C. Huang et al., 2013;

Ishikawa, Shimuta, & Häusser, 2015). These findings, along with functional studies (see below), suggest functionally distinct regions in the insula based on the presence or absence of granule cells. Importantly, the spatial organization of these regions varies between species (for review see Gogolla 2017). The human insula has a granularity gradient such that the anteroventral insula is agranular and the posterodorsal insula is granular, with dysgranular cortex between (Figure 2). Compared to humans, the rodent insula has a proportionately larger agranular zone, which comprises most of the anterior insula and extends into the posterior insula (see Figure 3, Saito et al., 2012).

Due to differences in cytoarchitectural organization and relative size, direct comparisons between human and rodent insula findings are difficult. Furthermore, many rodent studies segment the insula cytoarchitecturally; in humans, however, *in vivo* imaging methodology is unable provide information on cytoarchitecture. Another issue is that many human neuroimaging studies evaluate the insula as a whole, although some neuroimaging studies have used anatomy gleaned from structural scans or connectivity data to create subregions within the insula. A common method is to divide the insula into anterior and posterior subregions separated by an anatomical landmark: the central insula sulcus. For the purposes of this work and to facilitate translation, rodent studies of agranular cortex will be referred to as the anterior insula and studies of the granular cortex will be referred to as the posterior insula.

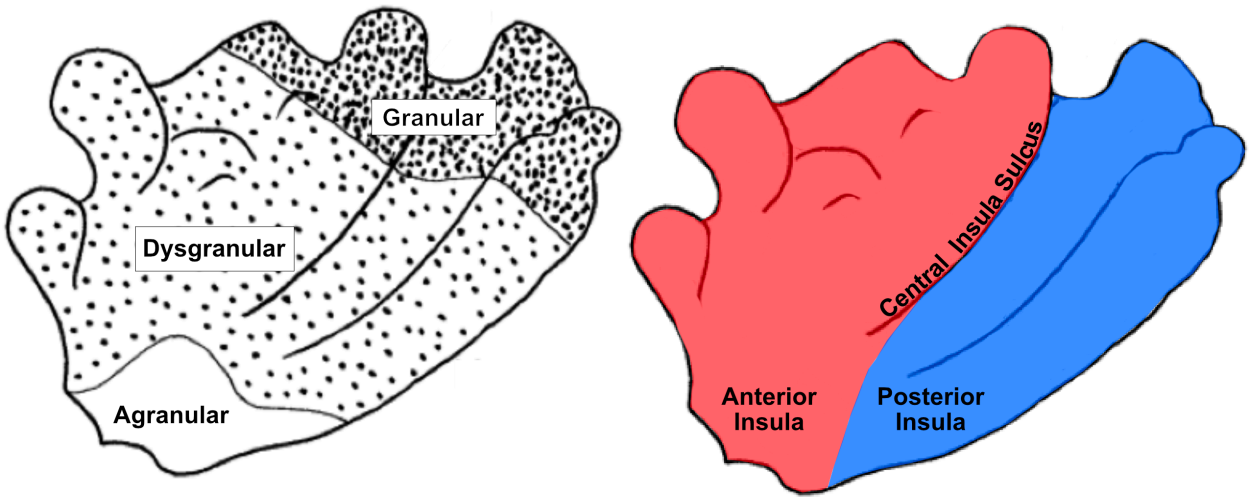

Figure 2. Cytoarchitecture and anatomy of the human insula

Left: the agranular cortex is located in the anteroventral region of the insula, the granular cortex is located in the posterodorsal region of the insula, and the dysgranular cortex is in between. Reproduced with permission from Nieuwenhuys, 2012. Right: the central insula sulcus forms a natural demarcation between the anterior and posterior insula.

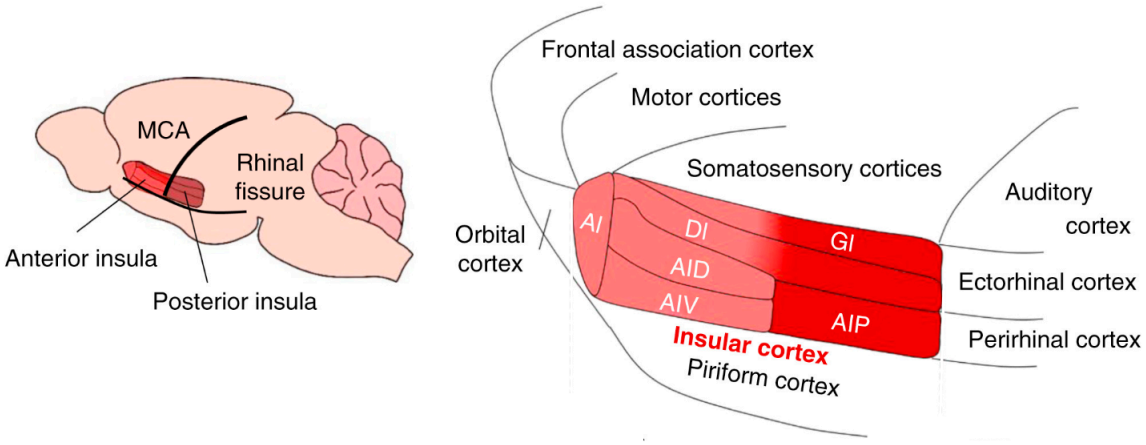

Figure 3. Cytoarchitecture and anatomy of the mouse insula

Left: the mouse insula lies superior to the rhinal fissure on the lateral surface of the brain. Right: subregions of the insula and surrounding regions with cytoarchitectural information. AI = agranular insula; AIV = ventral agranular insula; AID = dorsal agranular insula; AIP = posterior agranular insula; DI = dysgranular insula; GI = granular insula. Figure adapted from Gogolla, 2017, reproduced with permission.

Insula function and connectivity

The insula is involved in a broad variety of functions, including visceral sensory and motor responses; processing and integration of internal and external sensory information; social and emotional processing; and cognitive functioning. Although many different functional subregions within the insula have been proposed (for examples see Farb et al., 2013; Nomi et al., 2016, 2017), dividing the insula into anterior and posterior divisions is commonly recognized and has generally established functional differences. Broadly, the anterior insula is involved in social, emotional, and cognitive processes (Brass and Haggard 2010; Craig 2002, 2009; Lamm and Singer 2010); the posterior insula is involved in sensory processing, including interoception, and sensorimotor integration (Craig, 2002; Ibañez, Gleichgerrcht, & Manes, 2010). The anterior insula, accordingly, has substantial functional connectivity with regions involved in social behaviors, emotions, and cognition such as the anterior cingulate cortex and orbitofrontal cortex whereas the posterior insula is more connected with temporal and occipital regions associated with sensory processing, the posterior cingulate cortex, and the sensorimotor cortex (Cauda, D'Agata, et al., 2011; Cloutman & Lambon Ralph, 2012). Insula structural (Ghaziri et al., 2018) and functional (Cauda, Cavanna, et al., 2011; Cauda, D'Agata, et al., 2011; Weis, Huggins, Bennett, Parisi, & Larson, 2019) connectivity with the amygdala and nucleus accumbens has been shown, though the peak location of amygdala connectivity within the insula is mixed and nucleus accumbens connectivity is more concentrated in the anterior insula. Importantly, the anterior and posterior insula are highly interconnected (Failla et al., 2017; Farb et al., 2013), and many functions attributable to the anterior or posterior insula likely involve both subregions.

Insula function and connectivity in abstinence

In the last decade, the insula has increasingly received attention as a region associated with drug-related craving. This was, in part, due to a study evaluating the outcomes of individuals who were addicted to cigarette smoking prior to suffering from brain damage (Naqvi, Rudrauf, Damasio, & Bechara, 2007). Of these individuals, those with brain damage involving the insula were more likely to quit smoking without relapse or urges to smoke. Although this study did not differentiate between damage to the anterior and posterior insula, many studies of the insula in abstinence evaluate the anterior and posterior insula separately.

Both rodent and human studies have investigated a role for the anterior insula in abstinence from AUD. In rodents, abstinence following chronic alcohol use is associated with greater neuronal activity in the anterior insula (Centanni, Morris, et al., 2019). Inhibition of the anterior insula also prevents stress-induced reinstatement, providing evidence for the anterior insula's role in maintaining abstinence (Campbell et al., 2019). Specific to the BNST-insula pathway, inhibition of anterior insula inputs to the BNST attenuates BNST activity that typically increases during abstinence (Centanni, Morris, et al., 2019). In humans, insula structural differences have been observed in gray matter volume and cortical thickness. Compared to healthy controls, abstinent patients with AUD have smaller anterior insula volumes (Demirakca et al., 2011; Makris et al., 2008; Mechtcheriakov et al., 2007; Senatorov et al., 2015; Trick, Kempton, Williams, & Duka, 2014; van Holst, de Ruiter, van den Brink, Veltman, & Goudriaan, 2012; Zois et al., 2017). These decreases in volume seem to recover over the course of abstinence (Cardenas, Studholme, Gazdzinski, Durazzo, & Meyerhoff, 2007; Makris et al., 2008), suggesting that anterior insula volume may normalize during abstinence. In functional studies,

the anterior insula has heightened activity during anticipation and presentation of drug-related cues in abstinent patients with AUD (Schulte et al., 2017; Tapert, Brown, Baratta, & Brown, 2004) although exceptions exist (e.g. Huang *et al.* 2018). In abstinent AUD patients, anterior insula activation to alcohol cue presentation was also positively correlated with faster reaction times (Schulte et al., 2017). In summary, the majority of insula studies in humans have demonstrated reduced anterior insula cortical volumes in abstinent individuals with AUD, although the volume slowly increases over the course of abstinence, and heightened activity in response to alcohol-related cues.

Very few studies have specifically examined the posterior insula in abstinence. One study showed that, when compared to heavy drinkers, abstinent individuals with AUD have reduced posterior insula connectivity throughout the frontal cortex when shown alcohol cues compared to neutral cues (Strosche et al., 2021). Further studies will be important to better characterize the role of the posterior insula.

Insula function and connectivity in anxiety and depression

Decades of research have characterized the anterior insula's role in anxiety processing and regulation for both normative anxiety and pathological anxiety (for reviews see Craig 2010; Lamm & Singer 2010). A common human neuroimaging approach to studying anxiety is to examine threat anticipation, where participants learn to associate a cue or context with an upcoming, aversive stimulus. During threat anticipation, healthy controls show increased anterior insula activation (Alvarez, Chen, Bodurka, Kaplan, & Grillon, 2011; Grupe, Oathes, & Nitschke, 2013; Nitschke, Sarinopoulos, Mackiewicz, Schaefer, & Davidson, 2006; Sarinopoulos et al., 2010; Shankman et al., 2014; Simmons et al., 2011; Somerville et al., 2013). Anterior

insula activation correlates with trait anxiety during both predictable (Carlson, Greenberg, Rubin, & Mujica-Parodi, 2011) and unpredictable (Alvarez et al., 2015) threat anticipation. Further, during unpredictable threat, patients with anxiety disorders have greater anterior insula activation relative to controls (Fonzo et al., 2014; Simmons et al., 2011). Anterior insula activation also correlates with worry symptoms in healthy individuals during unpredictable threat (Grupe et al., 2013). In addition to threat anticipation, anterior insula is also associated with threat processing, such as a negative image is shown. Greater anterior insula activation is associated with increased worry when viewing negative emotional faces in patients with social anxiety disorder (Klumpp, Angstadt, & Phan, 2012) and anxiety-prone individuals (Stein et al. 2007). Anxiolytic medication attenuates anterior insula activity in both healthy controls during threat presentation (Arce, Simmons, Lovero, Stein, & Paulus, 2008; Paulus, Feinstein, Castillo, Simmons, & Stein, 2005) and patients with an anxiety disorder during both neutral and threatening stimulus presentation (Hoehn-Saric, Schlund, & Wong, 2004). Together these studies demonstrate the anterior insula is involved in normative anxiety processes, has heightened activity in individuals with an anxiety disorder, and is modulated by anxiolytics.

In humans, investigations of anterior insula connectivity with the amygdala have largely demonstrated greater connectivity associated with anxiety. For task-based anxiety connectivity studies, greater anterior insula-amygdala functional connectivity was observed during a fear-conditioned response relative to unconditioned stimuli in both healthy controls and individuals with generalized anxiety disorder (GAD) (Greenberg, Carlson, Cha, Hajcak, & Mujica-Parodi, 2013). When viewing aversive stimuli, individuals with GAD have increased anterior insula-amygdala connectivity compared to both healthy controls and individuals with GAD following

treatment (Fonzo et al., 2014). However, a meta-analysis of general emotion-related functional MRI studies did not find anterior insula-amygdala connectivity (Kober et al., 2008), suggesting the connection may not generalize to all emotions. In resting state studies, anterior insula-amygdala connectivity is stronger in patients with social anxiety disorder compared to healthy controls (Jung et al., 2018), and greater basolateral amygdala connectivity with the anterior insula is associated with greater state anxiety in healthy controls (Baur, Hänggi, Langer, & Jäncke, 2013). To our knowledge, few studies have examined BNST-insula connectivity in anxiety. One study reported greater anterior insula-BNST connectivity during threat in healthy controls (Kinnison, Padmala, Choi, & Pessoa, 2012). Specific to abstinence, one study demonstrated that higher scores of early life adversity correlated with decreased anterior insula activity during threat anticipation in abstinent AUD patients (Yang et al., 2015). Another study specifically examining the connection between the anterior insula and the basolateral amygdala found no relationship between state anxiety measures with resting state connectivity in abstinent AUD patients (Orban et al., 2013). In summary, the anterior insula has greater connectivity with the amygdala during threat processing in both healthy controls and individuals with an anxiety disorder but the evidence for anxiety during abstinence is limited.

A number of studies have demonstrated posterior insula activation and connectivity differences associated with anxiety. One study of social anxiety showed increased bilateral posterior insula activation during a social exclusion task for individuals with high compared to low social anxiety (Wang et al., 2019). An interoception task elicited greater activation in the anterior and posterior insula in individuals with GAD compared to controls (Cui et al., 2020). In a community sample selected to include a wide range of anxiety symptoms, less connectivity

between the anterior and posterior insula was associated with self-reported anxious affect and, more specifically, physiological anxiety (Bijsterbosch, Smith, Forster, John, & Bishop, 2013). Anxiety measures are also correlated with resting state connectivity between the posterior insula and right dorsal caudate, in which the correlation is negative for the individuals with an anxiety diagnosis but positive for controls (Dorfman, Benson, Farber, Pine, & Ernst, 2016). In a task-based analysis, posterior insula connectivity with the amygdala is also increased during the presentation of negative valence images in healthy controls (Denny et al., 2014). In a study of individuals with AUD in abstinence and controls, the relationship between alcohol use severity and amygdala-posterior insula connectivity was more negative when accounting for the impact of neuroticism, which includes measures of anxiety (Dean, Fede, Diazgranados, & Momenan, 2020). The findings from the posterior insula suggest increased activation and increased connectivity with the amygdala in response to anxiety.

The insula has also been investigated in depressive symptoms, which are also common during abstinence, and major depressive disorder (MDD). For example, depressive symptoms were positively correlated with anterior insula activation during threat anticipation in both healthy controls and individuals with MDD (Herwig et al., 2010). Greater posterior insula activation is seen in controls compared to individuals with MDD during a task of emotional processing of faces (Townsend et al., 2010). Connectivity studies have shown that individuals with MDD have greater resting state connectivity between the anterior insula and amygdala compared to controls (Kandilarova, Stoyanov, Kostianev, & Specht, 2018; Veer et al., 2010). Individuals with MDD have weaker insula resting state connectivity with limbic and cortical regions (Lui et al., 2011). The studies suggest greater anterior insula activation and connectivity

with limbic regions is associated with greater depressive symptoms and MDD; however, there are limited findings from the posterior insula.

Conclusions

The current model describes the neural basis for abstinence as the extended amygdala. New methodologies have facilitated the identification of other brain regions that interact with the extended amygdala during abstinence. Specifically, these methodologies paved the way for the discovery of the insula-BNST pathway's role in abstinence-induced negative affect, specifically the anterior insula. A substantial body of work in humans has characterized the insula as a region associated with addiction, anxiety, and to a lesser extent, depression. A critical next step is translating this novel BNST-insula finding from rodents into humans, as the BNST-insula pathway could reveal key information regarding relapse. The aim of this project is to address this translational gap and investigate BNST-insula connectivity in abstinent humans with AUD.

Unifying hypothesis

The paucity of human data on BNST-insula connectivity first necessitates establishing connectivity in healthy controls, which will be referred to as normative connectivity. Then, these normative patterns can be used to determine whether BNST-insula connectivity is altered during abstinence. The hypothesis of this thesis is that the anterior insula will have stronger connectivity with the BNST in humans than the posterior insula, anterior insula connectivity will

be greater in abstinent individuals than the controls, and anterior insula-BNST connectivity will be associated with anxiety and depressive symptoms in the abstinent group. Posterior insula connectivity with the BNST in abstinence will also be examined as exploratory analysis, as there is less evidence for the posterior insula's role in abstinence-induced negative affect.

Specific aims

Specific Aim 1: Determine the pattern of BNST-insula connectivity in humans.

Aim 1a. Use diffusion tensor imaging data to establish the structural connectivity between the BNST- insula (Chapter II).

Aim 1b. Use resting state functional MRI to describe the resting state connectivity between the BNST and insula (Chapter III).

Specific Aim 2: Compare BNST-insula connectivity in patients with AUD in early abstinence to healthy controls.

Aim 2a. Use diffusion tensor imaging to determine the difference in BNST-insula connectivity strength and patterns between abstinent individuals and controls (Chapter IV).

Aim 2b: Use resting state connectivity to compare the resting state BNST-insula connectivity in abstinent individuals and controls (Chapter IV).

Aim 2c: Evaluate the relationship between anxiety and depression metrics and functional and structural connectivity measures during abstinence (Chapter V).

CHAPTER II

BNST STRUCTURAL CONNECTIVITY WITH THE ANTERIOR INSULA AND POSTERIOR INSULA IN HUMANS[3]

Introduction

Investigating connectivity between the BNST and insula is critical for understanding abstinence from AUD and symptoms associated with abstinence. Tract tracing studies have demonstrated robust connections between the insula and BNST (Reynolds & Zahm, 2005; Shin, Geerling, & Loewy, 2008) with functional significance in negative affect and addiction (Centanni, Morris, et al., 2019), making a BNST-insula pathway a promising translational target. Advances in imaging methodologies, notably diffusion tensor imaging (DTI), have allowed for white matter tracts to be investigated in humans using structural connectivity. Strong structural connectivity between regions suggests that the regions share information and work in concert. Importantly, structural connectivity provides analogous evidence to tract tracing studies in rodents and other animal models, allowing for cross-species comparisons of important pathways. BNST-insula connectivity established in rodent models has translational potential to inform our understanding of abstinence. It remains unknown, however, whether the BNST and insula are structurally connected in humans.

[3] This chapter has been adapted from "BNST-insula structural connectivity in humans", published in *Neuroimage* and has been reproduced with the permission of the publisher and my co-authors: B Feola, S Avery, DG Winder, ND Woodward, S. Heckers, and JU Blackford.

Several DTI studies in humans have investigated insula structural connectivity (e.g. Cerliani et al., 2012; Cloutman and Lambon Ralph, 2012; Ghaziri et al., 2018, 2017; Nomi et al., 2018) or BNST structural connectivity (Avery et al., 2014; Krüger, Shiozawa, Kreifelts, Scheffler, & Ethofer, 2015), yet structural connectivity between the BNST and insula has not been reported. There are several potential explanations. First, most structural connectivity studies evaluate the insula as a single region, yet the insula is a large region with much heterogeneity that could be obscured by examining the insula as a whole. Due to this variation in insula anatomy, histology, and function (e.g. Nieuwenhuys, 2012), some studies divide the insula along an anatomical boundary, known as the central insular sulcus, to create an anterior and posterior insula (e.g. Ham et al., 2012). The anterior insula is primarily associated with cognition and emotion, and the posterior insula with sensory interoception (for reviews see Craig, 2010a; Gogolla, 2017). Second, in insula connectivity studies, a BNST connection might have been overlooked due to neuroimaging advancements only recently permitting the evaluation of the BNST in humans (Avery et al., 2014; Krüger et al., 2015; Theiss, Ridgewell, McHugo, Heckers, & Blackford, 2017; Torrisi et al., 2015). As a result, most *in vivo* imaging atlases don't include the BNST; thus, connectivity with the BNST could be overlooked or attributed to a neighboring brain region.

Another reason for the lack of BNST-insula structural connectivity might result from the scope of the two published BNST structural connectivity studies (Avery et al., 2014; Krüger et al., 2015). Both studies characterized only the strongest structural connections of the BNST and would not have reported more modest connections. For example, the first BNST structural connectivity study identified the BNST's most highly connected brain regions using a standard

method for segmenting the entire brain into regions (Avery et al., 2014). This segmentation included the insula as a whole region. The second study defined the three major white matter pathways of the BNST and replicated BNST connections to many of the brain regions described in the initial study (Krüger et al., 2015). Together, these first human studies revealed the most robust structural connections of the BNST, uncovering a BNST network that provides a foundation for future studies. Moving forward, the BNST network in humans can be compared to what is already known from rodents, and discrepancies, such as the BNST-insula connection, can be investigated using specific, hypothesis-driven studies.

This study aimed to determine whether BNST-insula structural connectivity exists in humans. To overcome the presented limitations of prior structural connectivity studies, we use a previously validated BNST mask (Avery et al., 2014) and divided the insula into an anterior and posterior mask (Figure 4). In addition, an analysis of BNST connectivity with the whole insula was conducted to provide an illustration of the overall pattern of connectivity. Based on previous studies in humans that show anterior insula connectivity with other limbic regions, we hypothesized that the anterior insula would have greater structural connectivity with the BNST relative to the posterior insula.

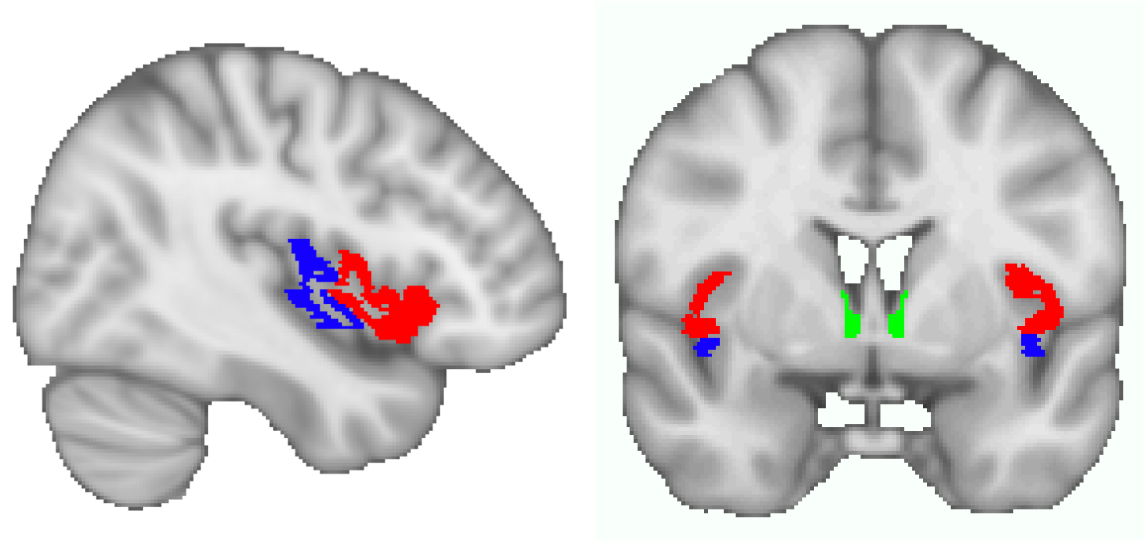

Figure 4. BNST and insula masks used for the analysis

The anterior insula (red) and posterior insula (blue) are divided by the central sulcus of the insula. Masks for the anterior and posterior insula are adapted from Farb et al., 2013. The BNST (green) is from Avery et al, 2014. Masks are shown in standard MNI space, x = 42, y = 2.

Methods

Participants

The study used the diffusion tensor imaging (DTI) scans of 81 healthy controls. Participants were aged 18 - 57 years (mean ± SD = 30 ± 11 years) and 46% female. The ethnicities of the participants were: 70% White/Caucasian, 22% Black/African-American, and 7% Asian. The original scans were collected as part of two ongoing studies. The Vanderbilt University Institutional Review Board approved the studies, and written informed consent was obtained for each participant. Participants were eligible by meeting the following criteria: 1) no current or prior mental health disorders based on evaluation with the Structured Clinical Interview for the DSM IV (SCID-IV, First, 1997); 2) no psychotropic medication use in the previous 6 months; and 3) high quality DTI data. Of the 89 participants with high quality DTI scans, 8 participants were excluded for excessive motion (> 5mm or 3 degrees of motion in any direction across the diffusion series), resulting in a final sample of 81 participants. Preprocessed scans were visually inspected for processing failures (e.g., skull stripping failure), and all failures at this stage were corrected.

Data acquisition

Diffusion magnetic resonance image (MRI) data were acquired on two identical 3 Tesla Philips Achieva MRI scanners (Philips Healthcare, Inc.). Whole-brain diffusion weighted images were acquired using a pulsed-gradient spin echo, echo planar imaging (single-shot EPI) pulse sequence, and the following parameters: 96 × 96 matrix; voxel size = 2.5 mm isotropic; number of slices = 50; TE = 65 ms; TR = 8.5 s; SENSE acceleration factor = 2. 92 diffusion directions were

acquired with a *b* value of 1600 s/mm^2 and one T2-weighted volume with a *b* value of 0 s/mm^2. High resolution T1-weighted anatomical images were collected with the following parameters: FOV = 256 mm; number of slices = 170; voxel size = 1 mm isotropic; gap = 0 mm.

Data processing

The diffusion-weighted images were preprocessed and analyzed using FMRIB Software Library (FSL, version 5.0; Oxford Centre for Functional MRI of the Brain (FMRIB), UK; http://www.fmrib.ox.ac.uk/fsl/) and Matlab (Version R2018a, The MathWorks, Inc, Natick, MA). First, Eddy Current Correct from the FMRIB FSL toolbox was applied to correct for motion and eddy current distortions (Andersson, Skare, & Ashburner, 2003). The brain extraction tool from the FMRIB FSL toolbox (Smith, 2002) was used to remove the non-brain tissue from the image. DTIFIT was then applied to align the diffusion tensors to the skull-extracted, eddy-corrected images. Scans were inspected for acquisition artifacts (i.e., ghosting, water-fat shift artifacts), excessive motion (defined as greater than 5mm or 3 degrees of motion in any direction across the diffusion series) and processing failures (e.g., skull stripping failure). Scans that could not be corrected were not included in this sample.

BEDPOSTX was used to estimate the diffusion of each voxel (samples = 5000), including the possibility of multiple crossing fibers. Seed-based probabilistic tractography was used to determine the degree of structural connectivity between the insula and the BNST, with the BNST mask as a seed and the insula masks as targets. Fiber tracking was initiated from every voxel within the BNST (samples = 5000) and the number of streamlines connecting to each insula mask was recorded. The average number of streamlines (or tracts) per voxel in each ROI

was log transformed for analysis to adjust for positive skew. Only connections between the ipsilateral BNST and insula were evaluated, as previous rodent tracer studies have demonstrated consistent but more substantial ipsilateral, relative to contralateral, BNST connections with cortical regions (Coolen & Wood, 1998; McDonald, Shammah-Lagnado, Shi, & Davis, 1999; Sun, Roberts, & Cassell, 1991; Wood & Swann, 2005). Therefore, for each participant, BNST connectivity values from both the left and right hemispheres were obtained for the 1) whole insula and 2) anterior and poster insula (see details below).

Region of interest masks

The BNST mask used for this study has been validated in previous studies (Figure 4, for details see Avery et al., 2014; Theiss et al., 2017). For the insula, we used previously published subregions masks (Farb et al., 2013) that were combined to form anterior and posterior masks, using the central sulcus of the insula as the division between the anterior and posterior insula (Figure 4). The anterior and posterior masks were combined to create the whole insula mask. For each participant, masks were transformed into participant space and reviewed in native space to evaluate anatomical accuracy.

Voxel-based connectivity

As this study is the first to investigate insula connectivity with the BNST, we also used a voxel-based approach to provide an illustration of the overall ipsilateral pattern of BNST

connectivity within the whole insula. A voxel-based approach allows for the evaluation of connectivity patterns without the a priori anatomical boundaries set by regions of interest.

Validation analysis

As a validation analysis, we compared the BNST-insula results to structural connectivity between the BNST and both a positive and negative control region. Positive and negative control regions were selected from previously published BNST structural connectivity data (Avery et al., 2014), with the central amygdala (CeA) as the positive control region and medial frontal gyrus (MFG) as the negative control region. The CeA is one of the best-established connections of the BNST, with strong translational and human evidence (Coolen & Wood, 1998; deCampo & Fudge, 2013; Dong, Petrovich, & Swanson, 2001; Oler et al., 2017), making CeA-BNST connectivity a suitable positive control for structural connectivity. The MFG was selected as the negative control, as previous studies have identified low levels of BNST-MFG connectivity (Avery et al., 2014). Negative and positive connectivity values are an important validation, as no standard value exists for determining a positive result of structural connectivity between two regions. The values from the positive and negative control regions also provide an estimate of the relative strength of the BNST-insula findings.

Statistical analyses

To determine whether BNST structural connectivity differed between the anterior and posterior insula, a linear mixed model was performed with region (anterior/posterior) and sex

(men/women) as fixed factors and participant as a random factor (α = 0.05). Hemisphere was included as a covariate of no interest. Post-hoc analyses were used to explore significant interactions (α = 0.05). Effect sizes (η^2) were computed for each main effect and interaction.

To determine the relative strength of BNST-insula connectivity, BNST connectivity values with the anterior and posterior insula in each hemisphere were compared to BNST connectivity with a positive control (central amygdala) and a negative control (MFG) using *t*-tests (α = 0.05). Effect sizes (Cohen's *d*) were calculated.

In all analyses, the effect of hemisphere was examined, as previous studies have shown connectivity differences between hemispheres (e.g. Baur et al., 2013; Gorka et al., 2017; Moran-Santa Maria et al., 2015; Onay et al., 2017; Ray et al., 2010). The effect of biological sex was examined because studies have reported sex differences in both the insula and BNST (e.g. Allen and Gorski, 1990; Avery et al., 2014; Chung et al., 2002; Lotze et al., 2019; Ruigrok et al., 2014).

Statistical analyses were performed using R (R Core Team, 2017) with the lme4 (Bates, Machler, Bolker, & Walker, 2015) package for the linear mixed models and the emmeans (Lenth, 2019) package for post-hoc analysis.

Results

<u>BNST structural connectivity by insula region (anterior vs posterior)</u>

BNST structural connectivity differed significantly by insula region ($F(1, 240) = 13.50$, $p < 0.001$, $\eta^2 = 0.05$; Figure 5), with greater connectivity in the anterior relative to posterior insula.

There was also a sex by region interaction ($F(1,240) = 5.68$, $p = 0.02$, $\eta^2 = 0.02$; Figure 5). Post-hoc analysis revealed greater anterior than posterior insula connectivity was driven by a significant region effect in males (anterior > posterior; $t = 4.48$, $p < 0.001$) but not females ($t = 0.88$, $p = 0.78$). To better characterize this interaction, post-hoc analysis was also conducted within-sex and demonstrated similar anterior insula connectivity between women and men ($t = 0.59$, $p = 0.92$) but greater posterior insula connectivity in women compared to men ($t = 2.59$, $p = 0.04$). There was no main effect of sex ($F(1, 79) = 3.07$, $\eta^2 = 0.04$, $p = 0.08$).

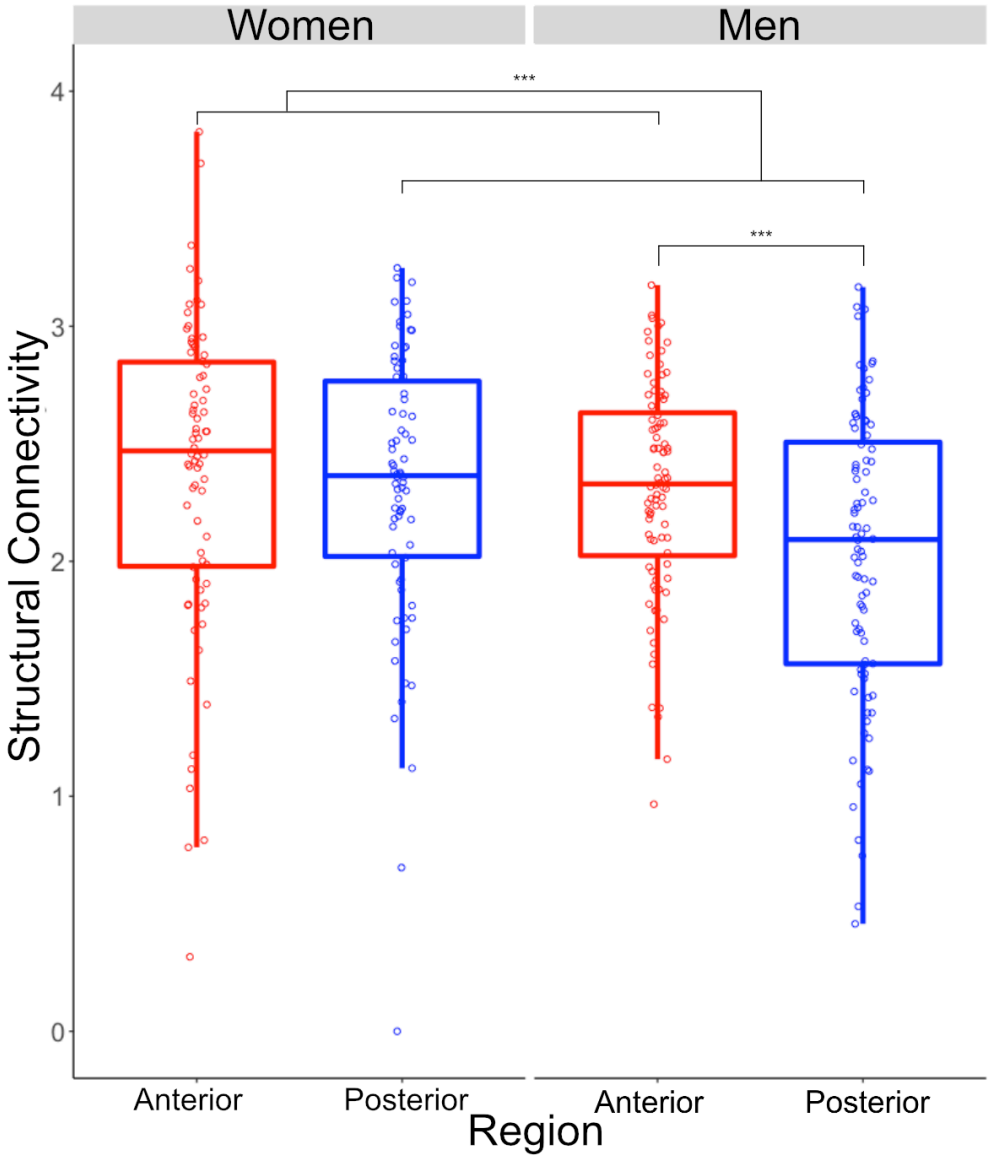

Figure 5. BNST structural connectivity with the anterior and posterior insula by sex.

Overall, anterior insula connectivity (red) was greater than posterior connectivity (blue). Post-hoc analysis revealed that men, but not women, had significantly more connectivity in the anterior compared to posterior insula. Structural connectivity values are the log-transformed, averaged number of tracts from BNST to insula regions *** $p < 0.001$

Voxel-wise analysis of BNST structural connectivity with the whole insula

To further explore BNST connectivity patterns, voxel-wise connectivity analysis was performed across the insula (Figure 6). Voxel-wise analysis were consistent with the anterior/posterior analysis; the strongest connectivity was observed in the anterior insula, specifically within the anteroventral insula.

Validation analyses

To provide a relative estimate of the strength of the observed BNST-insula connections, we compared BNST connectivity values to a negative control region (MFG) and a positive control region (CeA, Figure 7). Data for all comparisons and effect sizes are provided in Table 2. For the insula regions (anterior, posterior), the left anterior insula showed significantly greater BNST connectivity than the MFG ($p < 0.001$). All BNST-insula regions were significantly less connected than the BNST-amygdala positive control ($ps < 0.001$).

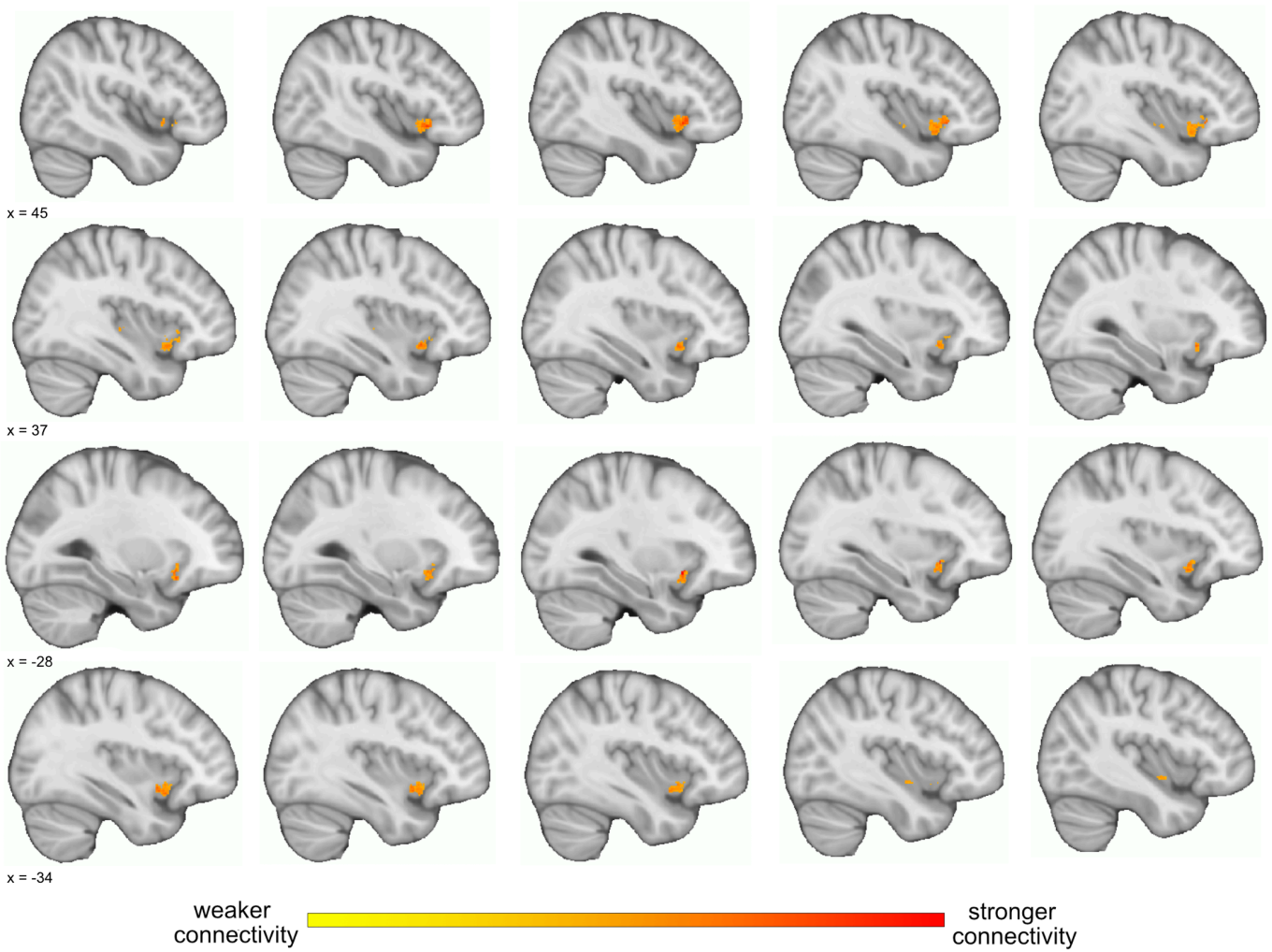

Figure 6. Voxel-wise map of BNST-insula structural connections

Structural connectivity values (log-transformed number of tracts) were thresholded to show the top 20% most connected voxels.

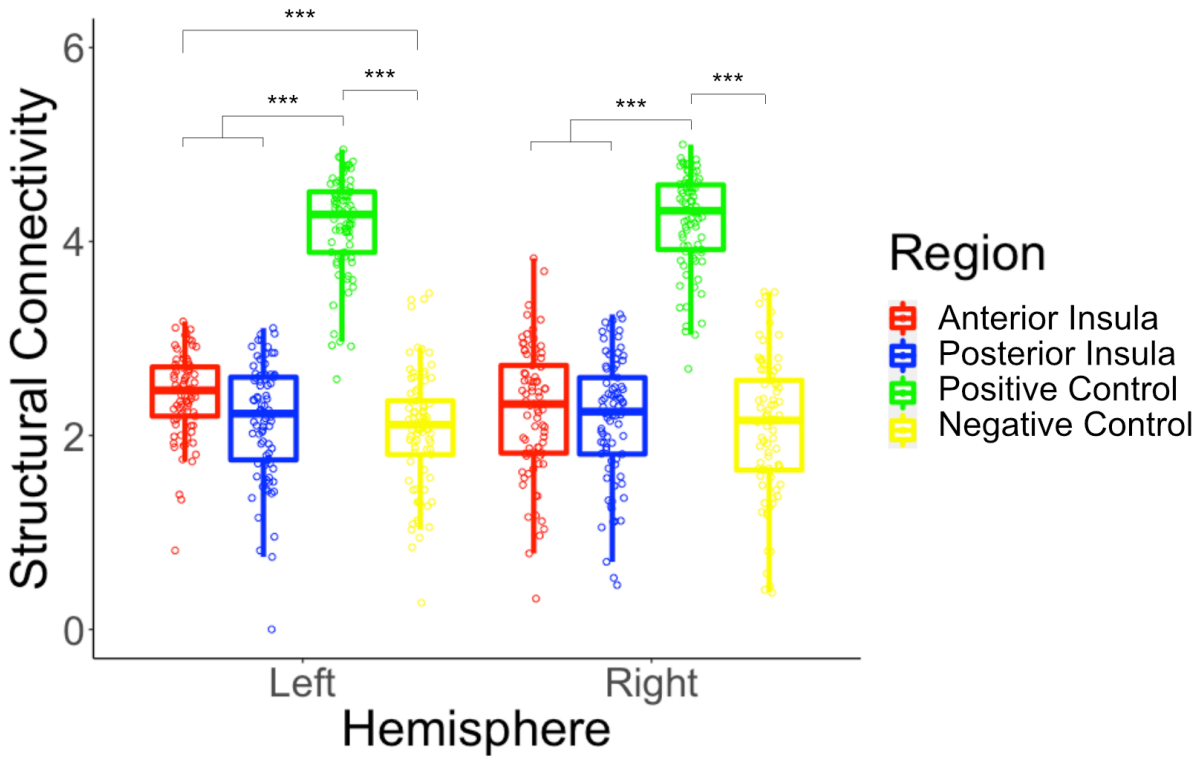

Figure 7. BNST structural connectivity with the anterior and posterior insula compared to positive and negative controls.

The anterior (red) and posterior (blue) insula in both hemispheres had less BNST structural connectivity than the positive control region (green), the central nucleus of the amygdala. In the left hemisphere, the anterior insula had greater connectivity than the negative control region (yellow), the medial frontal gyrus. Structural connectivity values are the log-transformed, averaged number of tracts between the BNST and insula regions, averaged across hemispheres.

*** $p < 0.001$

Table 2. *BNST structural connectivity of the insula regions compared to control regions*

	Positive Control (Central Amygdala)			
	Left Hemisphere		Right Hemisphere	
	t	*d*	*t*	*d*
Anterior Insula	24.17*	3.34	21.20*	2.71
Posterior Insula	23.58*	3.74	22.63*	3.18
	Negative Control (Medial Frontal Gyrus)			
	Left Hemisphere		Right Hemisphere	
	t	*d*	*t*	*d*
Anterior Insula	-4.31*	-0.56	-1.69	-0.27
Posterior Insula	-0.87	-0.12	-0.87	-0.14

Note: t values (*t*) and effect sizes (*d*) reported, negative values indicate BNST-insula connectivity > BNST-control connectivity. * = $p < 0.05$

Discussion

The results of this study provide compelling evidence for structural connectivity between the BNST and insula in humans. Using an anterior-posterior division of the insula, the BNST had stronger connectivity with the anterior relative to the posterior insula. Sex moderated these findings; BNST connectivity in men differed by region (anterior > posterior). When comparing to positive and negative control regions, the BNST has low levels of structural connectivity with most of the insula but significant connectivity with the left anterior insula. To our knowledge, this is the first study to identify BNST-insula structural connectivity in humans—a critical first step to investigating a role for BNST-insula connectivity in abstinence.

Structural connectivity between the BNST and anterior insula likely has functional relevance, as the BNST and anterior insula are involved in many of the same neural processes. The BNST and anterior insula are both involved in emotion, feeding behaviors, attention, and autonomic and threat processing (for reviews see Craig, 2010; Crestani et al., 2013; Davis et al., 2010; Menon and Uddin, 2010). A number of rodent studies have shown anterior insula projections to the BNST (e.g. Centanni et al., 2019; Reynolds and Zahm, 2005), with less evidence for reciprocal connections (Dong & Swanson, 2006) suggesting largely unidirectional flow of information from the anterior insula to the BNST with possible, weak feedback from the BNST to the anterior insula. The feedback from the BNST to the insula projects from the dorsomedial portion of the BNST, a region associated with integrating social information and influencing stress, mood, and reward circuitry (Lebow & Chen, 2016). Thus, feedback could serve to fine-tune the anterior insula's integrated emotional response to incoming stimuli. Provided this directionality is conserved across species, the human anterior insula would have

more influence over BNST activity than the reciprocal. In humans, the anterior insula has been associated with emotional regulation, suggesting that inputs from the insula to the BNST could initiate behavioral responses by translating emotional states into BNST-modulated behavioral changes including a fight-or-flight response or changes in motivated behaviors.

The strongest cluster of BNST structural connectivity was in the anteroventral insula, consistent with what is known about the cytoarchitecture of the human insula and findings from rodent research. The anteroventral portion of the insula is the location of the insula's agranular cortex in humans. Thus, the anteroventral insula results from our study recapitulate rodent studies demonstrating the agranular portion of the insula has the greatest structural connection to the BNST (Reynolds & Zahm, 2005). Functionally, the anteroventral insula specifically has been implicated in emotional regulation and processing (for review see Klein et al., 2013) and the BNST is associated with anxiety and threat processing (for review see Davis et al., 2010). Thus, structural connectivity between the BNST and anteroventral insula is also supported by a strong functional homology. Other studies specifically comparing ventral and dorsal anterior insula connectivity show selective connectivity between the ventral anterior insula and other limbic regions (e.g. Ghaziri et al., 2018; Nomi et al., 2017, 2016). This is also supported by studies in non-human primates where the agranular insula is structurally connected to other limbic regions (Augustine, 1996; Carmichael & Price, 1995). Thus, our results of structural connectivity between the BNST and agranular insula in humans emphasize how comparative anatomy can be used to drive findings in humans.

In this sample, men demonstrated greater BNST structural connectivity with the anterior compared to posterior insula but there were no differences in women. When comparing

regions between sexes, women had greater posterior insula connectivity but similar anterior insula connectivity compared to men. Although structural connectivity between the BNST and insula has not been examined previously, sex differences in both the BNST and insula have been demonstrated. One previous study in humans found that over 70% of brain regions examined had greater BNST structural connectivity in women compared to men (Avery et al., 2014), suggesting the findings of the current study have a more typical pattern in the posterior insula and less common findings with the anterior insula. To our knowledge, no other studies have examined sex differences in structural connectivity of specific insula subregions. Important next steps will be to replicate and expand on our sex differences in future studies. When evaluated in the context of these previous studies, the results of the current study emphasize the importance of considering sex differences in studies involving the BNST or insula.

Several limitations should be noted. First, voxel-based results suggest that the anterior insula connectivity is likely driven by the ventral portion of the anterior insula, indicating that subdivisions beyond the anterior and posterior insula could be necessary to better reflect insula heterogeneity. Second, the observed BNST-insula white matter connections were modest in strength. The BNST-insula connectivity likely represents a smaller white matter tract compared to the major BNST white matter pathways previously found in humans: the stria terminalis, anterior pathway, and posterior pathway (Krüger et al., 2015). Therefore, replicating and extending our findings in other samples will be critical next steps. Finally, DTI findings are not directional, meaning we are only able to hypothesize the directionality of the BNST-insula connection from rodent studies.

Conclusions and future direction

Structural connectivity analysis demonstrated connectivity between the BNST and anterior insula, specifically the anteroventral insula. These findings are concordant with results from rodent and nonhuman primate studies, suggesting convergence between species. Given recent rodent data identifying anterior insula projections to the BNST as critical for negative affect during abstinence (Centanni, Morris, et al., 2019), these findings of normative BNST-insula structural connectivity provide an important foundation for future translational studies.

The results of this study prompt interesting future directions. First, these findings suggest that future studies of the BNST network should investigate the anterior insula, in addition to other key brain regions like the amygdala and hippocampus. Second, these results add to a growing literature illustrating the need to divide the insula into smaller subregions to better reflect the insula's structural and functional heterogeneity. Research in other brain regions has benefitted from subdividing large areas of cortex, such as the prefrontal cortex and cingulate cortex. However, more work is needed to reach a consensus on the most appropriate way to divide the insula. Third, as the first study to demonstrate BNST-insula connectivity in humans, validation in future studies will be critical. Finally, important next steps will be to examine BNST-insula connectivity alterations in clinical populations, including anxiety disorders and substance use disorders.

CHAPTER III

BNST RESTING STATE FUNCTIONAL CONNECTIVITY WITH THE ANTERIOR INSULA AND POSTERIOR INSULA IN HUMANS

Introduction

The bed nucleus of the stria terminalis (BNST) is emerging into prominence as a brain region associated with a wide variety of functions, including a role in multiple psychiatric disorders. A critical component of the advancement in our understanding of the BNST has been the investigation of normative BNST connectivity and how the BNST interacts with other brain regions. Results demonstrated in Chapter II provide evidence for structural connectivity in humans; however, resting state connectivity between the BNST and insula remains poorly understood. Resting state connectivity allows for the investigation of how brain regions interact in the absence of any directed external stimuli and can provide important insight into how regions interaction, informing our understanding of the brain's organization and important pathways of communication between regions.

Due to its small size and deep-brain location, the BNST has only recently been accessible for *in vivo* investigation in the human brain. Early studies of the BNST in humans took an exploratory approach and evaluated BNST resting state connectivity throughout the brain. The first *in vivo* study of the human BNST described robust functional connections between the BNST and the amygdala, caudate, putamen, thalamus, nucleus accumbens, hippocampus, paracingulate cortex, and pallidum (Avery et al., 2014). Those findings converged with known

rodent BNST pathways and have since been replicated and expanded by other human neuroimaging studies (Gorka et al., 2017; Tillman et al., 2018; Torrisi et al., 2015). These early studies establish which regions have the most substantial resting state connectivity with the BNST. Building on this work, studies can use a hypothesis-driven approach to investigate specific brain regions that are known to be important drivers of BNST function in rodents, such as the insula. Establishing normative resting state connectivity between the BNST and insula will provide important information regarding the intrinsic communication between the two regions and can be used to compare to connectivity seen in patient populations.

Literature reporting the pattern of BNST resting state functional connectivity with the insula has demonstrated mixed results. Using a whole-brain, exploratory approach, Avery and colleagues reported BNST resting state connectivity in the insula, with significant voxels in both the anterior and posterior insula (Avery et al., 2014). Using a similar method, three other studies identified BNST connectivity in the posterior insula only (Gorka et al., 2017; Tillman et al., 2018; Torrisi et al., 2015). While this unbiased, voxel-based approach is critical for identifying the strongest areas of connectivity, it is not designed to examine specific a priori connections. Thus, the pattern of resting state connectivity between the insula and BNST has not been systematically investigated and remains an open question.

Resting state analysis is commonly performed using either single site data sets or, more recently, large, public access data sets. These public data sets have the benefits of a large sample size, cutting-edge equipment and protocols, and expert consensus on image acquisition methods. Importantly, many of these data sets, such as the Philadelphia Neurodevelopmental Cohort (PNC), have an emphasis on racial and ethnic diversity which is often overlooked in

smaller neuroimaging studies, skewing results (LeWinn, Sheridan, Keyes, Hamilton, & McLaughlin, 2017). Thus, combining data from these larger data sets with smaller, single-site neuroimaging studies can help untangle discrepancies in neuroimaging findings.

The aim of this study is to investigate resting state functional connectivity between the BNST and insula. Given the paucity of findings from previous BNST resting state studies, this study will compare anterior and posterior insula resting state connectivity with the BNST to directly test the hypothesis that the BNST has more connectivity with the anterior insula than the posterior insula at rest. The anterior insula is hypothesized to have greater BNST resting state connectivity because of findings from tract tracing studies in rodents (Reynolds & Zahm, 2005) and the involvement of both regions in emotional processing and motivation (for reviews see Craig, 2009, 2010a; Lebow & Chen, 2016). This hypothesis will be tested with healthy controls from two samples: 1) a Vanderbilt neuroimaging cohort and 2) the PNC data set.

Methods: Sample 1 (Vanderbilt cohort)

Participants:

The study used resting state functional MRI scans from the same group of individuals for the previous study (Chapter II). Of these initial 81 individuals, 78 had high quality resting state scans (described below). Participants were ages 18 - 57 years (mean ± SD = 30 ± 12 years), 47% women, and 85% right-handed. The participants were: 73% White/Caucasian, 21% Black/African-American, and 8% Asian. The original scans were collected as part of two ongoing studies. The Vanderbilt University Institutional Review Board approved the studies, and written informed consent was obtained for each participant. Participants included in this study met the

following criteria: 1) no current or prior mental health disorders based on evaluation with the SCID IV (First, 1997); 2) no psychotropic medication use in the previous 6 months; and 3) high quality resting state data.

MRI data acquisition

Resting state data was collected for seven minutes on two identical 3 Tesla Philips Achieva MRI scanners (Philips Healthcare, Inc.). Participants were asked to relax, close their eyes, and not fall asleep. Functional images were acquired using an echo-planar imaging sequence with the following parameters: volumes = 203; TR = 2s; TE = 34 ms; SENSE = 1.8; FOV = 240 mm; and matrix = 80 × 80. Each volume contained 28 4 mm slices (acquisition voxels = 3mm × 3 mm × 4 mm) and provided whole brain coverage. Images were later resampled to 3mm x 3mm x 3mm.

MRI data processing

Data were preprocessed using the default preprocessing pipeline in the Conn toolbox (Whitfield-Gabrieli & Nieto-Castanon, 2012), implemented in SPM through Matlab (Version R2018a, The MathWorks, Inc, Natick, MA). As part of this pipeline, the functional and structural scans for each participant were segmented into grey matter, white matter, and CSF and normalized to the MNI standard brain. Functional scans also underwent realignment and unwarping, slice time correction, outlier detection (scrubbing), and smoothing with a 6mm kernel. Preprocessed data were then run through a denoising procedure using CompCor

(Behzadi, Restom, Liau, & Liu, 2007), which first identifies and then removes sources of noise including white matter and CSF, motion, outliers, and signal trends. Next, the BOLD signal data was temporal bandpass filtered, removing signal outside 0.008-0.09 Hz. The BOLD timeseries for the BNST and insula masks was extracted and then averaged for each mask. The BNST mask used in this study has been previously validated (Avery et al., 2014) and the insula masks were created by combining subregion masks (Farb et al., 2013), using the central sulcus to divide the anterior and posterior insula (Figure 4). The BOLD timeseries averages were used to determine the correlation coefficient between the BNST and the anterior and posterior insula of each hemisphere.

Resting state connectivity analysis

To directly test the hypothesis that the BNST has greater connectivity with the anterior insula compared to the posterior insula, the correlation coefficients for the BNST-anterior insula and BNST-posterior insula were compared using a linear mixed model. The model was run using the lme4 package (Bates et al., 2015) in RStudio (R Core Team, 2017) and included region (anterior/posterior) and sex (men/women). BNST hemisphere (left BNST/right BNST) and insula hemisphere (left insula/right insula) were included as covariates of no interest. Sex and hemisphere were included due to previous studies showing BNST and insula sex differences (for examples see Allen & Gorski, 1990; Avery et al., 2014; Chung et al., 2002; Lotze et al., 2019; Ruigrok et al., 2014) and laterality (for examples see Baur et al., 2013; Gorka et al., 2017; Moran-Santa Maria et al., 2015; Onay et al., 2017; Ray et al., 2010). Effect sizes (η^2) were computed for each main effect and interaction.

Methods: Sample 2 (PNC cohort)

Participants

Participants were recruited as part of the Philadelphia Neurodevelopmental Cohort (PNC) study, a cross-sectional study in children, teenagers, and young adults (Satterthwaite et al., 2016). The original PNC data set participants were randomly selected from a pool of approximately 50,000 participants between the ages of 8-21 (at screening) from the greater Philadelphia area that had been previously enrolled in a genotyping study through the Children's Hospital of Philadelphia. For information concerning recruitment, screening, and inclusion and exclusion criteria, see Satterthwaite and colleagues (Satterthwaite et al., 2016, 2014). To minimize the influence of neurodevelopment or atypical development, the subset of PNC participants included in the present study were over 18 at the time of their MRI scan and were not diagnosed with a psychiatric disorder (n = 146, age = 19.7 ± 1.2). The participants included were 32% African American, 58% European American, and 10% other or mixed race. The final sample included 81 women (55.4%).

Data preprocessing

Resting state fMRI was collected using a standard PNC data acquisition protocol (for full details see (Satterthwaite et al., 2014). Preprocessing was conducted using SPM12 with the addition of the Computational Anatomy Toolbox (Gaser et al in review) for the segmentation. The preprocessing steps included: T1 and mean fMRI segmentation, motion correction, realignment, and denoising. Denoising used a regression to remove confounds from white matter, CSF, and motion and applied a bandpass filter between 0.01-0.10 Hz (Behzadi et al.,

2007). The mean BOLD timeseries was then extracted from the BNST, anterior insula, and posterior insula. The regions were defined by a priori masks (see Figure 4). The BNST mask has been previously validated in both controls and patient populations (Avery et al., 2014; Clauss, Avery, Benningfield, & Blackford, 2019). The insula masks (Figure 4) were created by combining previously published insula subregions masks (Farb et al., 2013), with the anterior insula including the subregions that are anterior to the central insula sulcus and the posterior insula including the subregions posterior to the central insula sulcus. The timeseries averages for the masks were then used to determine the Fisher-transformed correlation coefficient between the BNST and the anterior and posterior insula of each hemisphere.

Resting state connectivity analysis

To determine whether BNST has greater connectivity with the anterior insula than the posterior insula, the correlation coefficient for the BNST-anterior insula and BNST-posterior insula were compared using a linear mixed model. The model was run using the lme4 package (Bates et al., 2015) in RStudio (R Core Team, 2017) and included region (anterior/posterior) and sex (men/women). BNST hemisphere (left BNST/right BNST) and insula hemisphere (left insula/right insula) were included as covariates of no interest. Sex and hemisphere were included due to previous studies showing BNST and insula sex differences (for examples see Allen & Gorski, 1990; Avery et al., 2014; Chung et al., 2002; Lotze et al., 2019; Ruigrok et al., 2014) and laterality (for examples see Baur et al., 2013; Gorka et al., 2017; Moran-Santa Maria et al., 2015; Onay et al., 2017; Ray et al., 2010). Effect sizes (η^2) were computed for each main effect and interaction.

Results

The final sample of Vanderbilt participants was an average age of 30 ± 12 years, 73% white, and 47% women. The final sample of PNC participants was an average age of 19.7 ± 1.2, 58% white, and 55.4% women. Participants included in the PNC and Vanderbilt samples differed by age (t = 10.30, p < 0.001, Vanderbilt > PNC) and race (Vanderbilt sample included greater proportion of white participants; white vs non-white, χ^2 = 4.84, p = 0.028) but not by sex (χ^2 = 1.54, p = 0.21).

Sample 1 (Vanderbilt) results demonstrated a main effect of region (F(1,542) = 106.69, p < 0.001, η^2 = 0.16), with greater BNST resting state connectivity with the anterior insula compared to the posterior insula (Figure 8). There was a trend towards greater connectivity in men compared to women (F(1,76) = 3.76, p = 0.056, η^2 = 0.05). Mean connectivity adjusted for hemisphere and region was 0.018 (standard error = 0.02) for women and 0.071 (standard error = 0.02) for men). There was no region x sex interaction (F(1, 542) = 1.79, p = 0.18, η^2 = 0.003).

Sample 2 (PNC) results demonstrated a main effect of region with greater BNST resting state connectivity with the anterior compared to the posterior insula (F(1,1018) = 82.28, p < 0.001, η^2 = 0.07; Figure 9). There was no main effect of sex (F(1, 144) = 0.10, p = 0.75, η^2 = 0.001) nor a sex x region interaction (F(1, 1018) = 0.54, p = 0.38, η^2 = 0.001).

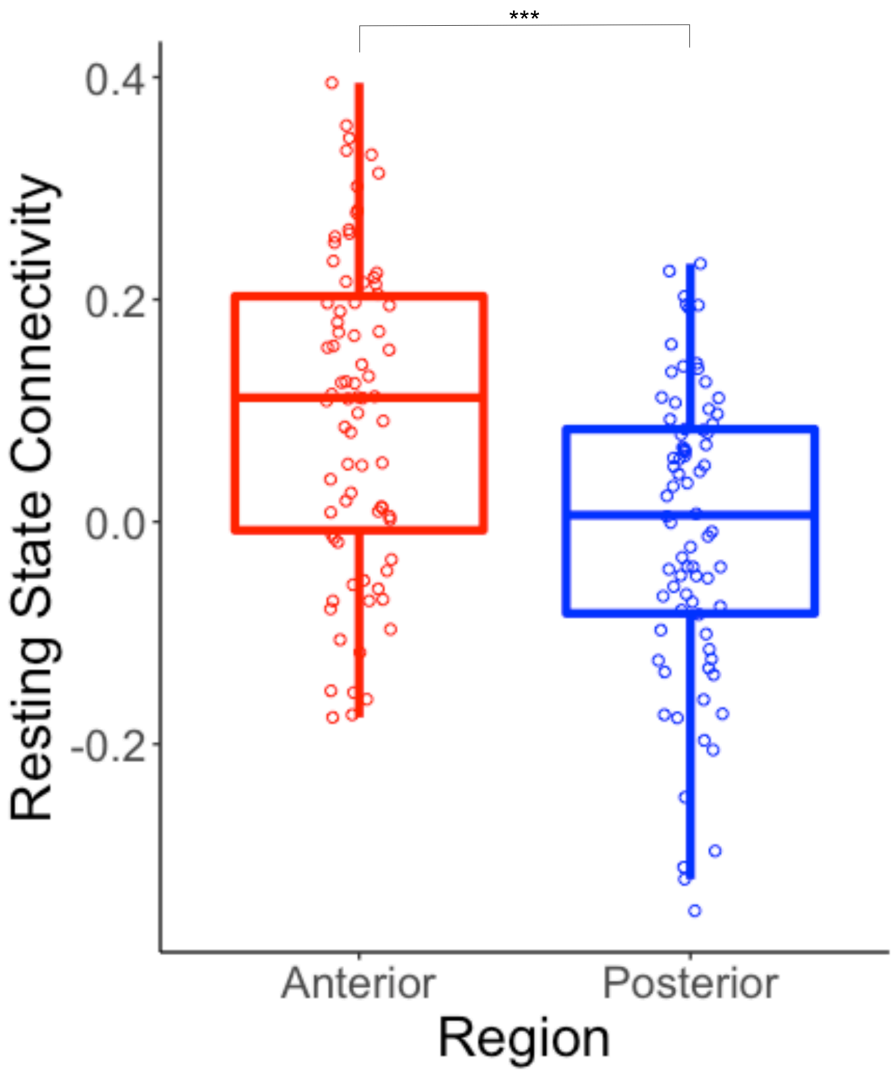

Figure 8. BNST resting state connectivity with the anterior and posterior insula

BNST resting state connectivity in the Vanderbilt cohort was greater with the anterior insula compared to the posterior insula. Data averaged by sex and hemisphere. Resting state connectivity values represent the correlation between BOLD signal of BNST and insula regions, averaged across hemispheres. *** $p < 0.001$

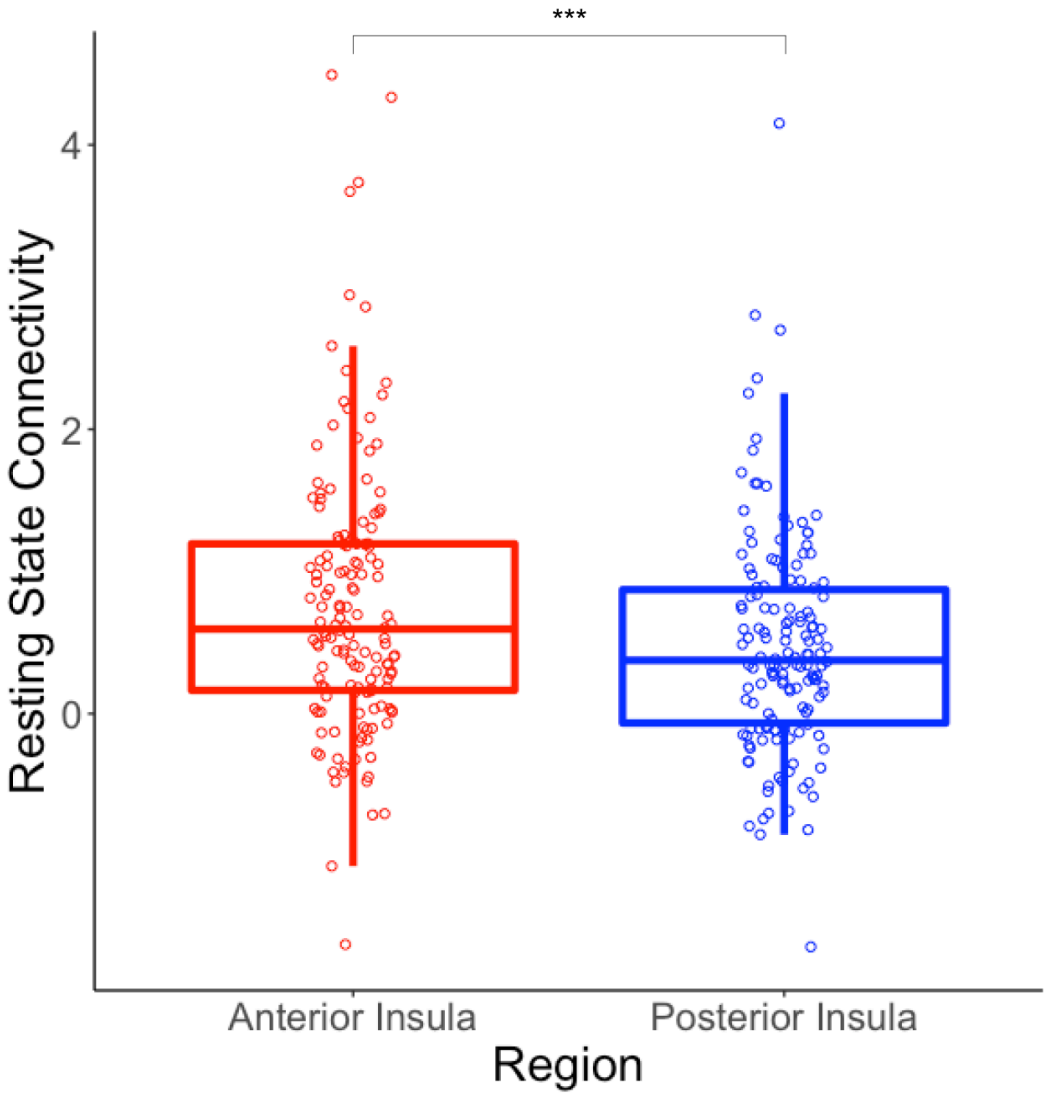

Figure 9. BNST resting state connectivity with the anterior and posterior insula.

In the PNC data set, BNST resting state connectivity with the anterior insula was greater than with the posterior insula. Resting state connectivity values represent the correlation between BOLD signals from the BNST and insula regions, averaged across hemispheres. Correlation values were Fisher r-to-z transformed and averaged across hemispheres. *** $p < 0.001$

Discussion

The results of this study demonstrate greater functional connectivity between the BNST and anterior insula compared to the posterior insula in two, discrete, healthy populations of adults. By directly comparing BNST connectivity with the anterior and posterior insula, this finding helps provide clarity to the existing literature regarding BNST resting state connectivity with the insula.

Findings suggest the BNST has greater resting state functional connectivity with the anterior insula compared to the posterior insula. Previous literature has not directly compared BNST connectivity between the anterior and posterior insula, but instead taken a whole brain approach and identified voxels within the insula. Using this method, BNST connectivity with the anterior insula has been seen in one previous study (Avery et al., 2014), but previously studies have predominantly identified posterior insula connectivity only (Gorka et al., 2017; Tillman et al., 2018). One possible reason for the discrepancy from the findings of the current study is the methodologies used in the previous studies. The previous studies of BNST resting state connectivity identified insula connectivity with a cluster-based whole brain approach as opposed to specifically targeting the insula. As a result, these approaches do not evaluate BNST connectivity with the insula, rather the cluster-based approach identifies clusters of voxels with significant connectivity across the whole brain and then reports a region based on coordinates of the cluster. A group of significant voxels, known as a cluster, often does not abide by the borders of a prescribed region, meaning that the voxels can be limited to a small part of region and even stretch through multiple regions. For example, the BNST resting state connectivity identified in the posterior insula by Tillman and colleagues also extended through central

operculum, parietal operculum, and transverse temporal gyrus (Tillman et al., 2018). These methods make it difficult to compare findings between certain regions, as the results could include more than just the anterior or posterior insula, potentially explaining the discrepancy between the findings presented in the present study and the mixed results of previous literature.

One major strength of this study is the converging evidence from two independent samples, which replicated greater BNST resting state connectivity with the anterior insula compared to the posterior insula. However, important differences did emerge between the two samples: one sample suggested greater resting state connectivity in men that was not seen in the other sample, prompting further questions about sex differences in BNST-insula resting state connectivity. Given that the sex differences did not replicate across samples, it is likely that the result was driven by a difference in the samples.

Conclusion and future directions

In two different samples, we have shown that the BNST has greater resting state connectivity with the anterior insula compared to the posterior insula. This study was the first to systematically compare BNST resting state connectivity with the anterior and posterior insula in humans.

Several limitations and future directions should be noted. First, this study is limited to healthy controls and does not examine connectivity differences in psychiatric populations. Future studies investigating BNST-insula connectivity in psychopathology will be important next

steps. Second, the study findings demonstrated greater BNST resting state connectivity with the anterior insula, though previous literature has more often reported BNST resting state connectivity with the posterior insula. The conflicting results of the present study with the previous literature suggest a cluster of voxels with BNST resting state connectivity within the posterior insula, but greater connectivity in the anterior insula on average. This could suggest that the current parcellation of the insula into anterior and posterior regions is not able to capture all areas with BNST resting state connectivity within the insula. Future studies could parcellate the insula into smaller regions to better uncover the precise pattern of BNST resting state connectivity. Third, our findings of BNST-insula resting state connectivity provide important insight into the intrinsic architecture of the brain but do not demonstrate the functional implications of the connection. Studies that examine connectivity during tasks, such as threat anticipation or anxiety inductions, will help clarify if the function of the BNST-insula connectivity is related to specific behaviors or cognitive processes such as those underlying anxiety. Finally, sex differences were shown in one sample but not both, suggesting a cohort effect. Future studies will be needed to determine the factors that influence BNST-insula resting state connectivity in men compared to women, as these factors could be important for understanding sex differences in normative connectivity and psychopathologies.

CHAPTER IV

BNST-INSULA RESTING STATE AND STRUCTURAL CONNECTIVITY IN ABSTINENCE[4]

Introduction

In the previous two chapters normative structural connectivity and resting state connectivity between the BNST and insula was established in humans. The findings showed that the BNST has strong structural connectivity and resting state connectivity with the anterior insula relative to the posterior insula. The next two chapters will now explore how normative BNST connectivity with the anterior and posterior insula differs during abstinence from AUD and whether there is a relationship between this BNST-insula connectivity and common symptoms of abstinence.

BNST-insula connectivity differences in abstinence could help illuminate the mechanism underlying the symptoms of abstinence that lead to relapse. Rodent studies have demonstrated that the BNST-insula pathway alters negative affect behaviors specific to abstinence, providing evidence for the potential of this pathway in understanding and treating AUD. Translating these studies into humans is the next step towards investigating the biological basis of abstinence symptoms. Although BNST-insula connectivity has not yet been evaluated in abstinence, a growing number of human studies show structural and functional differences in AUD and

[4] Parts of this chapter have been adapted from "Alterations in connectivity of the bed nucleus of the stria terminalis (BNST) during early abstinence in individuals with alcohol use disorder", published in *Alcoholism: Clinical and Experimental Research* and has been reproduced with the permission of the publisher and my co-authors: B Feola, MM Silveri, DG Winder, MM Benningfield, and JU Blackford

individuals in abstinence from AUD (for reviews see Ibrahim et al., 2019; Naqvi & Bechara, 2009).

Structural connectivity studies typically identify alterations in the integrity of the major white matter tracts in the brain, with less information regarding tracts between specific regions. Studies examining the major white matter tracts during abstinence have typically shown that individuals with AUD have less structural integrity in several tracts including the corpus callosum, cingulate gyrus, and fornix compared to controls (e.g. Monnig et al., 2015; Pfefferbaum et al., 2006; Pfefferbaum & Sullivan, 2002; Yeh et al., 2009; Zou et al., 2017). Although these studies suggest widespread white matter integrity decreases in abstinence from AUD, evidence from longitudinal data suggests that the decreased integrity is related to drinking and starts to recover during abstinence (Pfefferbaum et al., 2014). Importantly, these widespread white matter changes might not extend to findings of structural connectivity between two specific regions, for example, the BNST-insula pathway.

A handful of functional MRI studies of individuals abstinent from alcohol have examined the insula, particularly studies examining alcohol cue reactivity. While many studies have examined insula activation in response to alcohol cues (for review see Schacht et al., 2013), less has been shown regarding insula connectivity. One functional connectivity study demonstrated that, in response to alcohol cues, abstinent individuals with AUD show increased anterior insula connectivity with the basal ganglia and anterior prefrontal cortex but decreased anterior insula connectivity throughout the parietal and temporal cortices compared to non-abstinent dependent drinkers (Strosche et al., 2021). This study goes on to demonstrate that among both current drinkers and abstinent individuals, craving for alcohol was associated with greater

functional connectivity between the posterior insula and medial orbitofrontal cortex. Resting state connectivity studies have also shown differences in insula connectivity during abstinence, with greater connectivity between the anterior insula and striatum (Kohno, Morales, Guttman, & London, 2017) and less connectivity between the anterior insula and basolateral amygdala (Orban et al., 2013). Although the previous abstinence literature suggests greater insula connectivity in response to alcohol cues and altered resting state connectivity during abstinence, to date, alterations in BNST-insula resting state connectivity during abstinence remains an open question.

The purpose of this study is to test the hypothesis that BNST-insula connectivity differs between abstinent individuals with AUD compared to controls. Specifically, we hypothesize that the anterior insula will have greater structural and resting state connectivity with the BNST in the abstinence group compared to the controls. The hypothesis reflects rodent findings demonstrating that anterior insula input to the BNST contributes to negative affect during abstinence (Centanni, Morris, et al., 2019) and previous human studies demonstrating altered anterior insula connectivity with limbic regions in abstinence (Kohno et al., 2017; Orban et al., 2013). BNST connectivity with posterior insula will be tested as a more exploratory analysis, given the limited evidence for the posterior insula in abstinence.

Methods

Participants

Study participants were 20 individuals during abstinence (30-180 days) from an AUD and 20 light social drinkers (controls). Participants in the abstinence group were recruited using

advertisements, an email distribution list, and referrals from a local rehabilitation center in Nashville, TN. Participants for the control group were recruited using an email distribution list. Participants were included if they were between 21-40 years of age, had no major medical illness or history of traumatic brain injury, passed an MRI safety screen, and had no current or lifetime psychotic disorder. Additionally, the abstinence group participants were required to meet criteria for an AUD within the past year, as determined SCID-RV (First et al., 2015), and be 30-180 days sober at the time of the initial study visit. Exclusion criteria for the controls included: use of psychoactive medication (last 6 months), lifetime history of alcohol or substance abuse, or current psychiatric disorder as determined by the SCID-RV (for AUD diagnosis; First et al., 2015) and the Mini International Neuropsychiatric Interview for DSM-IV (for other psychiatric diagnoses; MINI, Sheehan et al., 1998). In addition, controls were light social drinkers and were excluded if they reported either binge drinking or no alcohol use for the year prior to enrolling in the study. Abstinence participants were excluded for current drug or alcohol use (except nicotine), current psychiatric disorder other than AUD, depression, or anxiety (as determined by the MINI, Sheehan et al., 1998), or use of psychoactive medication use other than a stable dose of SSRI/SNRIs. Abstinence from drugs of abuse was self-reported and confirmed at the initial and MRI study visits using a breathalyzer, urine drug screen, and urine ethyl glucuronide (ETG) test. The Vanderbilt University Institutional Review Board approved the study and written informed consent was obtained after providing subjects with a complete description of the study.

Alcohol questionnaires

Alcohol use was assessed using the Alcohol Use Disorders Identification Test (AUDIT; Babor, de la Fuente, Saunders, & Grant, 1989) and the Lifetime Drinking History (LDH; Koenig, Jacob, & Haber, 2009). The AUDIT was used to assess alcohol use severity. The LDH was used to quantify alcohol consumption; a quantity frequency index (QFI) was calculated for each participant's most recent drinking history. The QFI is the daily average consumption of standard drinks per month. For the abstinence group, the QFI was calculated for the drinking period immediately prior to their current abstinence period. For the control group, the QFI reflected the current drinking period. One participant from the abstinent group did not correctly complete the LDH questionnaire and did not have a QFI.

Data acquisition

Neuroimaging data were acquired on a 3T Philips Intera Achieva scanner (32-channel receive head coil, single-band imaging; Philips Healthcare, Andover, MA) located at the Vanderbilt University Institute for Imaging Sciences. Standard T1-weighted structural scans were collected for anatomical information (voxel size = 0.9mm^3, echo time = 4.6ms, TR = 9.1ms). For structural connectivity, high-angular radial diffusion-weighted imaging (HARDI) scans (2.5-mm isotropic resolution, 60 directions, b value = 2000 s/mm, 5 b0 images) were collected with a SENSE factor of 2.2 to reduce echo time and echo-planar image distortions. Resting state connectivity was collected over a seven-minute scan with the parameters: volumes = 203, TR = 2s, TE = 35ms, slice thickness = 4mm, flip angle = 79, acquisition matrix = 80 x 80, and voxels = 3 x 3 x 4mm.

Data Processing

Structural connectivity

Structural connectivity analysis was performed using FMRIB Software Library (FSL, version 5.0; Oxford Centre for Functional MRI of the Brain (FMRIB), UK; http://www.fmrib.ox.ac.uk/fsl/). Diffusion data were eddy current corrected, skull-stripped, and visually inspected for significant artifacts (Andersson et al., 2003; Smith, 2002). Diffusion tensors were then fitted at each voxel and probabilistic fiber tractography was performed to evaluate structural connectivity for each hemisphere between the ipsilateral BNST and insula subregions. In accordance with standard FSL analysis procedures, the subject weighted diffusion images were preprocessed in subject space. All masks were transformed from MNI space to native subject space using FLIRT, an FSL linear registration tool (Jenkinson, Bannister, Brady, & Smith, 2002; Jenkinson & Smith, 2001). A previously published mask was used for the BNST (Avery et al., 2014, Figure 4). For the anterior insula and posterior insula, subregion masks from a previous study (Farb et al., 2013) were combined to create a mask anterior to the central insula sulcus (anterior insula) and a mask posterior to the central insula sulcus (posterior insula, Figure 4). Fiber tracking was initiated from every voxel within the BNST (samples = 5000) and the number of streamlines connecting to each insula mask was recorded. Due to a technical failure at this step, data from 1 abstinence participant was excluded from all connectivity analysis. The average number of streamlines per voxel in each ROI was log transformed for analysis; this method inherently adjusts for positive skew and variance in BNST and insula volumes between subjects. The transformed data were normally distributed as evidenced by non-significant Kolmogorov-Smirnoff tests ($p > 0.05$).

Resting state connectivity

Resting state connectivity data were preprocessed using the default preprocessing pipeline in the Conn toolbox (Whitfield-Gabrieli & Nieto-Castanon, 2012), implemented in SPM through Matlab (Version R2018a, The MathWorks, Inc, Natick, MA). As part of this pipeline, the functional scans for each participant were segmented into grey matter, white matter, and CSF and normalized to the MNI standard brain. Functional scans also underwent realignment and unwarping, slice time correction, outlier detection (scrubbing), and smoothing with a 6mm kernel. Preprocessed data were then run through a denoising procedure, which first identifies and removes sources of noise including white matter and CSF, motion, outliers, and signal trends (Behzadi et al., 2007). Next, the BOLD signal data was temporal bandpass filtered, removing signal outside 0.008-0.09 Hz. The BOLD timeseries for the BNST and insula masks was then averaged for each mask. The BOLD timeseries averages were used to determine the correlation coefficient between the BNST and the anterior and posterior insula of each hemisphere.

Statistical analysis

Both the structural and functional data for the anterior and posterior insula were analyzed using linear mixed models with group (abstinence/control) as the fixed factor. Hemisphere was included as a covariate of no interest to control for laterality. Subject was included as a random effect variable. Effect sizes were calculated for group connectivity results using partial eta-squared (η^2). Effect size interpretation was informed by *Statistical Power*

Analysis for the Behavioral Sciences (Cohen, 1988). All analyses were conducted in R (R Core Team, 2017) using the *lme4* (Bates et al., 2015) packages for linear mixed models.

Exploratory Sex Analysis

Given findings of sex differences in Chapters II and III, an exploratory investigation into the effect of sex was also conducted. Sex was added as an additional fixed factor in the linear mixed models for both the structural and functional connectivity, and a sex x group interaction was included. A post-hoc analysis ($\alpha = 0.05$) was conducted of any significant interaction findings using the the emmeans (Lenth, 2019) package in R.

Results

Participant Characteristics

Participant characteristics are shown in Table 3. The groups did not significantly differ by age or race/ethnicity. For all participants, reported gender identity was consistent with sex assigned at birth. The abstinence group had higher AUDIT and QFI scores compared to the control group. The abstinence group was an average of 127 days sober on the day of the MRI scan.

Structural connectivity

Anterior insula: There were no group differences in BNST structural connectivity with the anterior insula ($F(1,36) = 0.23$, $p = 0.64$, $\eta^2 = 0.006$). The exploratory sex analysis (Table 4) revealed a group x sex interaction in the anterior insula ($F(1,34) = 5.84$, $p = 0.02$, $\eta^2 = 0.15$). Post-hoc analysis showed a trend towards greater connectivity in women in the abstinent group

compared to controls (t = 2.01, p = 0.054) but no differences in men between groups (t = 1.43, p = 0.16). There was no main effect of sex (F(1,34) = 0.59, p = 0.45, η^2 = 0.02) in the anterior insula.

Posterior insula: The posterior insula findings also did not show a significant group effect (F(1,36) = 2.69, p = 0.11, η^2 = 0.07, Figure 10); however, the results demonstrated a medium effect size of group, with greater BNST-posterior insula structural connectivity in the abstinence group. The exploratory sex analysis (Table 4) showed a BNST-posterior insula sex by group interaction (F(1,34) = 9.05, p = 0.005, η^2 = 0.21). Post-hoc analysis showed greater connectivity in women in the abstinent group compared to controls (t = 3.38, p = 0.002) but no differences in men between groups (t = 0.94, p = 0.36). There was no main effect of sex (F(1,34) = 0.05, p = 0.82, η^2 = 0.001).

Resting state connectivity

Anterior insula: There was a no significant effect of group in the anterior insula; however, the power analysis demonstrated a medium effect of group with positive resting state connectivity seen in the controls that was absent in the abstinence group (F(1, 37) = 3.18, p = 0.08, η^2 = 0.08). The exploratory sex analysis (Table 4) demonstrated no main effect of sex (F(1, 35) = 0.92, p = 0.34, η^2 = 0.03) or group x sex interaction F(1,35) = 0.08, p = 0.78, η^2 = 0.002) in the anterior insula.

Posterior insula: The posterior insula showed similar BNST resting state connectivity between the control and abstinence groups (F(1,37) = 1.16, p = 0.29, η^2 = 0.03, Figure 11). The

exploratory sex analysis (Table 4) showed no main effect of sex ($F(1, 35) = 0.09$, $p = 0.77$, $\eta^2 = 0.002$) or group x sex interaction ($F(1,35) = 0.33$, $p = 0.57$, $\eta^2 = 0.01$) in the posterior insula.

Table 3. *Study participant characteristics*

	Controls	Abstinence	Group comparison
	N (%)		χ^2 ***(p)***
Women	11 (55%)	9 (47%)	0.23 (0.63)
White	13 (65%)	15 (79%)	0.936 (0.33)
	Mean (SD)		***t (p)***
Age, years	29.0 (4.4)	31 (5.8)	1.22, (0.23)
AUDIT score	2.8 (1.8)	25.4 (9.2)	10.78 (< 0.001)
Days abstinent at scan	N/A	127.4 (47.8)	
QFI	3.76 (3.01)	206.65 (297.23)	3.06 (0.004)

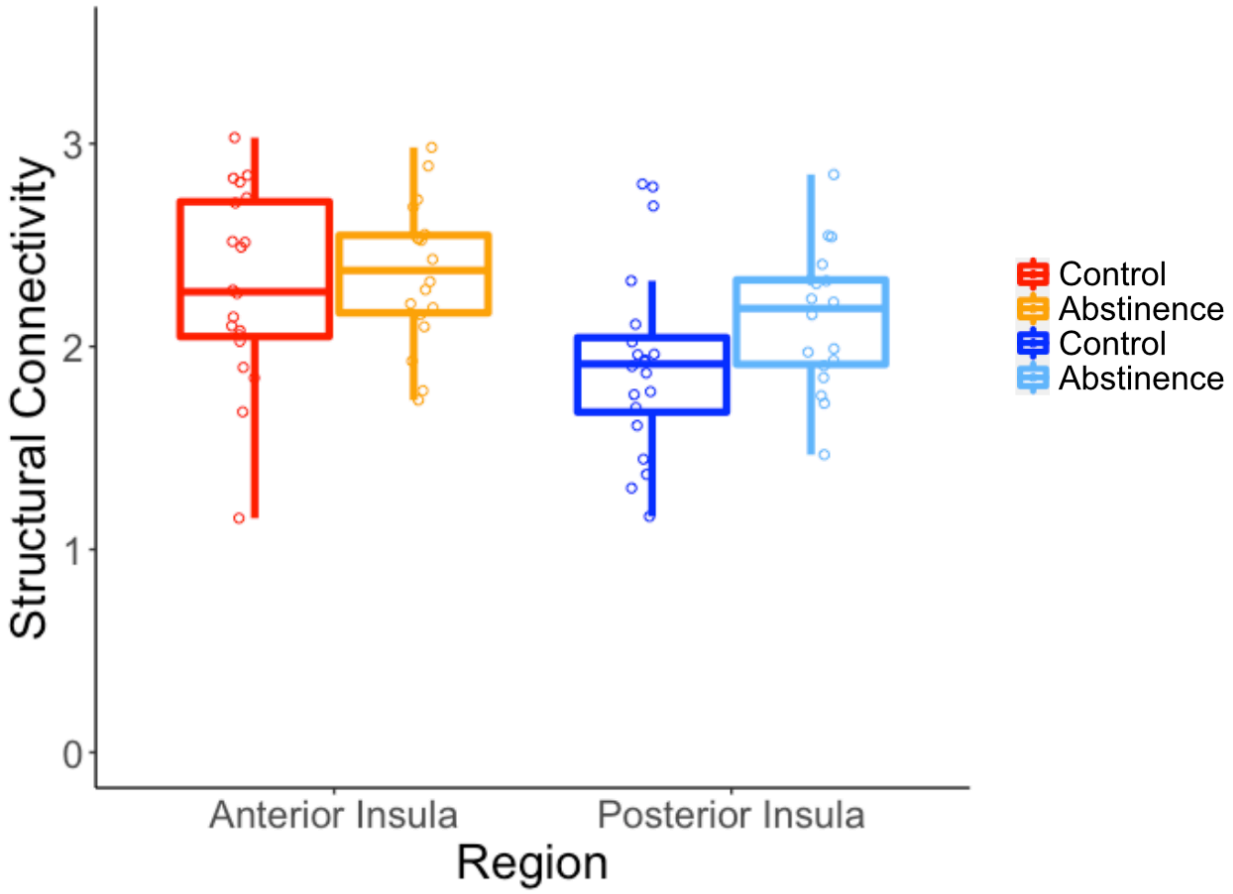

Figure 10. BNST structural connectivity with the anterior and posterior insula by group

The posterior insula demonstrated greater BNST structural connectivity in the abstinence group compared to controls (medium effect size, $\eta^2 = 0.07$); there was no effect in the anterior insula. Structural connectivity values are the log-transformed averaged number of tracts per voxel between the BNST and insula regions, averaged across hemispheres.

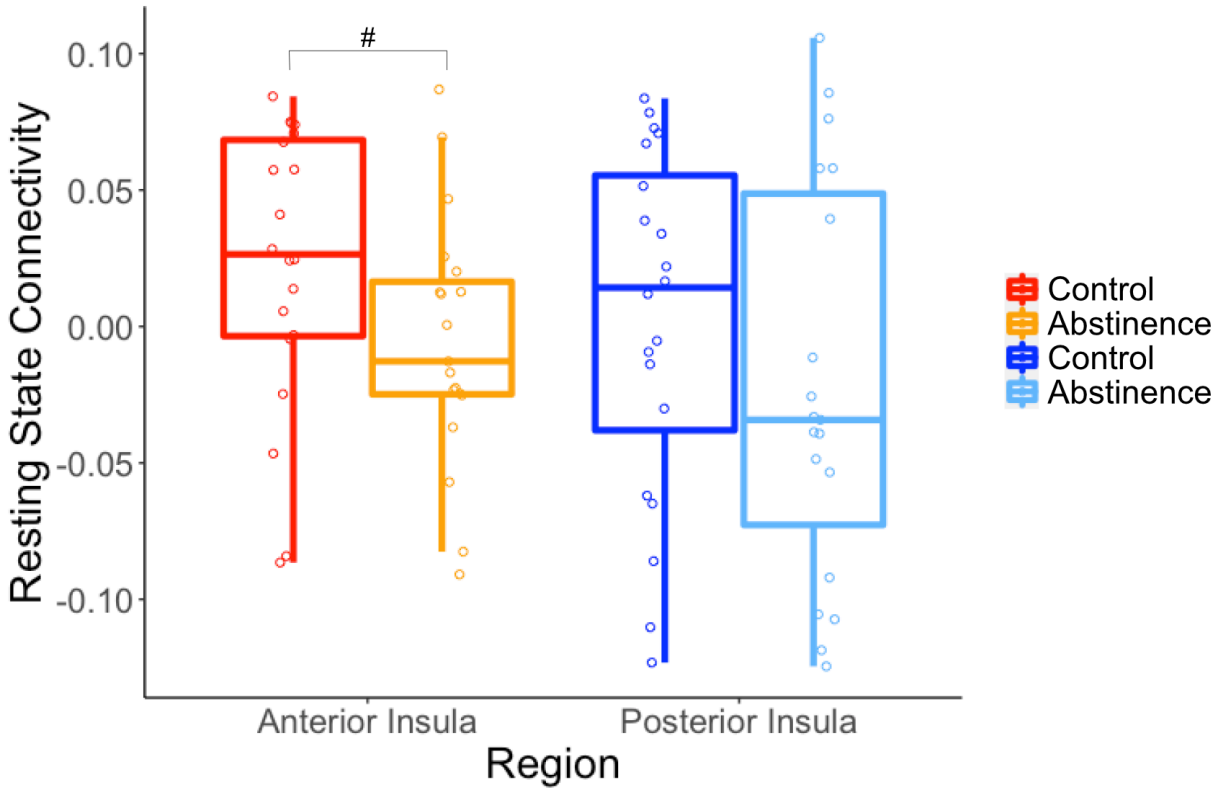

Figure 11. BNST resting state connectivity with the anterior and posterior insula by group

The anterior insula had greater BNST resting state connectivity in the control group compared to the abstinence group (medium effect size, $\eta^2 = 0.08$). This was not seen in the posterior insula. Resting state connectivity values represent the correlation between BOLD signal of BNST and insula regions, averaged across hemispheres. # $p < 0.1$

Table 4. *Adjusted BNST-insula structural connectivity means by sex*

	Women Mean *(95% CI)*	Men Mean *(95% CI)*
Structural connectivity		
Anterior insula		
Abstinence	**2.47 *(2.20-2.74)***	2.26 (*1.99-2.53*)
Control	**2.12 *(1.88-2.36)***	2.52 *(2.26-2.79)*
Posterior insula		
Abstinence	**2.34 (2.08-2.59)**	1.94 *(1.69-2.20)*
Control	**1.77 (1.54-2.00)**	2.11 (1.85-2.36)
Resting state connectivity		
Anterior insula		
Abstinence	-0.02 *(-0.05-0.02)*	0.004 *(-0.03-0.04)*
Control	0.02 *(-0.01-0.05)*	0.03 *(-0.01-0.06)*
Posterior insula		
Abstinence	-0.03 *(-0.08-0.02)*	-0.01 *(-0.06-0.03)*
Control	0.004 *(-0.04-0.05)*	-0.001 *(-0.05-0.05)*

Bold = significant difference between groups

Discussion

The results of this study demonstrated two moderate effects of group: greater BNST structural connectivity with the posterior insula but less resting state connectivity between the BNST and anterior insula in abstinence compared to controls. There were no effects of group on anterior insula structural connectivity or posterior insula resting state connectivity. Exploratory analysis revealed that both the anterior and posterior insula have greater structural connectivity with the BNST in abstinent women compared to controls, but there were no group differences in men.

The finding of greater BNST-posterior insula structural connectivity in abstinence differs from the large number of studies primarily showing less white matter integrity in abstinent individuals. Studies have identified less white matter integrity in abstinence across a number of large white matter tracts, including the corpus callosum, cingulate gyrus, and fornix (e.g. Monnig et al., 2015; Pfefferbaum et al., 2006; Pfefferbaum & Sullivan, 2002; Yeh et al., 2009; Zou et al., 2017). However, the methodology of these previous studies differs from the current study, as the previous studies examine the white matter integrity of large white matter pathways whereas the current study is examining the strength of a specific white matter connection between two a priori defined regions. Interestingly, the group differences in structural connectivity seem to be specific to women; both the anterior and posterior insula show greater BNST structural connectivity in abstinent women compared to control women. A few studies have demonstrated greater white matter integrity in women during abstinence (Rivas-Grajales et al., 2018; Sawyer et al., 2018, 2016), but the finding is not consistent (e.g. Pfefferbaum et al., 2006; Pfefferbaum & Sullivan, 2002). These mixed findings could indicate

that specific pathways, such as the BNST-insula, are greater in abstinent women, but this does not extend to all individuals during abstinence nor all white matter pathways. The stronger structural connectivity found in this study could represent a unique effect of alcohol on white matter in women. Alternatively, the findings could reflect premorbid differences in structural connectivity that is more likely to be seen in women, possibly related to risk of AUD development or relapse.

The overall structural connectivity reflects greater posterior insula connectivity in abstinence compared to controls, but this difference was not seen in the anterior insula as hypothesized. The posterior insula is critical for sensory integration, particularly of external stimuli and internal sensations, known as interoception. Craving is associated with autonomic changes and often occurs in response to external stimuli such as alcohol or context cues. Therefore, greater structural connectivity between the posterior insula and BNST could underlie the transition from craving to drug seeking, given the BNST's role in relapse (for examples see Buffalari & See, 2011; Pina, Young, Ryabinin, & Cunningham, 2015).

The results demonstrate less BNST-anterior insula resting state connectivity, despite rodent findings that excitatory inputs from the anterior insula trigger negative affect during abstinence. One possible explanation lies in the nature of the BNST, which is a region with substantial internal feedback loops and substantial inhibitory signaling (Thomas Louis Kash, 2012). While the anterior insula projections to the BNST are excitatory, inhibitory local processing within the BNST might result in an overall *decrease* in signaling, resulting in less resting state connectivity between BNST and anterior insula. The small size of the BNST precludes the investigation of these microcircuits in human neuroimaging studies; rodent

studies, however, have the tools to investigate both the microcircuits of the BNST neurons associated with abstinence-induced negative affect and how anterior insula excitatory signaling in abstinence impacts this signaling.

Conclusion, limitations, and future directions

The anterior insula and posterior insula have BNST connectivity differences in abstinence. The pattern suggests that individuals in abstinence have greater BNST-posterior insula structural connectivity and less BNST-anterior insula resting state connectivity. To our knowledge these are the first results of BNST-insula connectivity during abstinence in humans and represent intriguing insight into the pathway associated with abstinence-induced negative affect.

Several limitations and future directions should be discussed. First, this study does not answer the question of whether structural or resting state connectivity differences in the abstinence group is a result of a predisposition towards developing an AUD, is a consequence of an AUD, or is only seen in abstinence. Future longitudinal studies should address whether these findings demonstrate a premorbid difference in connectivity or not. Second, the study has a small sample size for the group x sex interaction and results should be verified in a larger study designed to test for sex differences. In addition to sex differences, there are likely other subgroups within AUD that have important neural and clinical differences. Factors such as length of abstinence, history of trauma, and history of smoking or other SUD should be systematically investigated in a larger study. Third, structural connectivity analysis does not provide insight into the functional ramifications of the group differences shown in this study.

Similarly, the resting state connectivity findings are not always indicative of task-based connectivity. Future task-based functional connectivity studies could be used to address the functional significance of the connectivity findings of the present study. Fourth, the group effects in structural and functional connectivity results are seen in different insula subregions. Functional connectivity does not always replicate structural connectivity, but often the two methods are complementary (further discussed in Chapter VI). Future rodent studies will be needed to investigate what changes in signaling occurred between the anterior insula and BNST such that the structural connections did not change, or minimally changed, but the functional connectivity differed. Similarly, rodent studies can investigate why BNST structural connectivity with the posterior insula did not coincide with a difference in resting state. Finally, this study provides initial insight into differences in BNST-insula connectivity during abstinence, but it is likely that BNST-insula connectivity during abstinence is influenced by additional brain regions that are not captured in these analyses.

CHAPTER V

RELATIONSHIP BETWEEN BNST-INSULA CONNECTIVITY AND SYMPTOMS OF ANXIETY AND DEPRESSION IN ABSTINENCE

Introduction

Symptoms of anxiety and depression are prominent during abstinence from alcohol use disorder (AUD) and common triggers of relapse, making these symptoms critical towards understanding and preventing relapse. Identifying the neural basis for these symptoms during abstinence could provide important treatment targets for reducing relapse risk. Although progress has been made in rodent models of AUD, little is known about negative affect during abstinence in humans.

Rodent studies have identified a BNST-insula pathway as having a key role in negative affect behaviors during abstinence. In a recent study of abstinence following chronic ethanol exposure, inhibiting insula neurons projecting to the BNST resulted in less negative affect behaviors (Centanni, Morris, et al., 2019). Importantly, these same insula neurons projecting to the BNST did not impact behavior in alcohol-naïve mice, suggesting that the impact is specific to abstinence. Centanni and colleagues also demonstrated that the insula projections targeted corticotropin releasing hormones (CRH) BNST neurons (Centanni, Morris, et al., 2019). CRH is a hormone involved in the stress response, and CRH neurons in the BNST regulate negative affect during abstinence and alcohol seeking (Le et al., 2000; Lowery et al., 2010; Olive, Koenig, Nannini, & Hodge, 2002). CRH-expressing neurons in the BNST have also been implicated in

stress-induced relapse to other drugs of abuse (e.g. Erb & Stewart, 1999; Vranjkovic, Gasser, Gerndt, Baker, & Mantsch, 2014). In combination, rodent studies demonstrate that CRH cells in the BNST mediate negative affect and stress-induced relapse, and the insula plays a critical role in BNST-mediated expression of negative affect specific to abstinence. The rodent literature illustrates a promising role for the BNST-insula pathway in regulating negative affect during abstinence, which ultimately could further our understanding of stress- and negative affect-induced relapse.

One way to investigate the role of the BNST-insula pathway in abstinence-induced negative affect in humans is to test whether BNST-insula connectivity is correlated with negative affect in abstinent individuals. Negative affect in rodents can be translationally approximated to symptoms of anxiety and depression in humans. Although human studies have not specifically examined the role of the BNST-insula pathway in abstinence-induced anxiety and depressive symptoms, the BNST and insula have been identified for critical roles in anxiety and depression (Avery et al., 2016; Paulus & Stein, 2010; O. J. Robinson, Pike, Cornwell, & Grillon, 2019). For example, the BNST has greater activity in patients with anxiety disorders and during anxiety-provoking tasks (for review see Avery et al., 2016). Although depression has not been examined as extensively, preliminary evidence from individuals with obsessive compulsive disorder (OCD) has linked the BNST to a role in depressive symptoms; studies in individuals with OCD showed that deep brain stimulation of the BNST can alleviate depressive symptoms (Luyten, Hendrickx, Raymaekers, Gabriëls, & Nuttin, 2016; Raymaekers et al., 2017).

The insula has also been shown to have activation and connectivity differences in anxiety and depression, especially for the anterior insula. In research investigating anxiety

symptoms and disorders, the anterior insula demonstrates increased activation during the anticipation of a threatening stimuli in healthy controls (Alvarez et al., 2011; Sarinopoulos et al., 2010; Shankman et al., 2014; Simmons et al., 2011; Somerville et al., 2013), and this activation is even greater in patients with anxiety disorders (Fonzo et al., 2014; Simmons et al., 2011). Although not as commonly demonstrated, the posterior insula has also been implicated in anxiety. In a task of social exclusion, individuals with high social anxiety exhibit greater posterior insula activation compared to individuals with low social anxiety (Wang et al., 2019). In research investigating depression and depressive symptoms, patients with major depressive disorder (MDD) show less anterior insula resting state connectivity with the amygdala (Veer et al., 2010). Another study showed greater anterior insula activation was associated with depressive symptoms when control and MDD participants were anticipating unknown or negative images (Herwig et al., 2010). In summary, these human studies provide some initial evidence for BNST and insula alterations in anxiety and depression, although no studies have investigated the relationship between these symptoms BNST-insula connectivity during abstinence.

The purpose of this study is to investigate the relationship between BNST-insula structural and resting state connectivity with symptoms of anxiety and depression in adults with AUD in abstinence. We hypothesize that, based on a more established role of the anterior insula in emotional processing, BNST connectivity with the anterior insula will have a positive relationship with anxiety and depressive symptoms. As less is known about the posterior insula, we will conduct an exploratory analysis of the relationship between BNST-posterior insula connectivity and anxiety and depressive symptoms.

Methods

Participants

Study participants included were 19 abstinent individuals (30-180 days) from an AUD. Information about the participants and study methods are described in Chapter IV. Individuals in the abstinence group were permitted to have comorbid anxiety or depression; five individuals met criteria for social anxiety disorder (SAD) or generalized anxiety disorder (GAD), three individuals met criteria for dysthymia, and one met criteria for MDD. Two of these individuals had comorbid diagnosis (1: GAD and agoraphobia, 2: dysthymia and GAD).

Anxiety and depression measures

Anxiety symptoms

Participants completed several self-report measures to provide a comprehensive assessment across multiple domains of anxiety symptoms. Participants completed the State-Trait Anxiety Inventory (Spielberger, Gorsuch, Lushene, Vagg, & Jacobs, 1983), Brief Fear of Negative Evaluation (Leary, 1983), Intolerance of Uncertainty Scale (Carleton et al., 2007), Liebowitz Social Anxiety Scale (Liebowitz, 1987), the Beck Anxiety Index (Beck, Epstein, Brown, & Steer, 1988), and Penn State Worry Questionnaire (Meyer et al., 1990). The anxiety measures were moderately correlated for both the control and abstinence groups (average r = 0.50); therefore, a composite anxiety score was created by averaging standardized scores (M = 0, SD = 1) for each questionnaire. Questionnaire and composite scores for the abstinence group are included in Table 5. Suggested interpretations and clinical cutoffs are provided for context.

Depressive symptoms

Depressive symptoms scores (Table 5) were obtained from the Beck Depression Index (BDI, A T Beck, Ward, Mendelson, Mock, & Erbaugh, 1961). The depressive score for 1 participant was 32, which was found to be a significant outlier (based on the function dixon.test from the RStudio package “outliers”) and excluded from analysis. This participant was also excluded in the previous chapter due to technical issues in their structural connectivity data processing.

Table 5. ***Abstinence group anxiety and depressive symptom scores***

Questionnaire	Mean (SD)	Possible Score Range	Participant Score Range	Suggested score interpretation	Participants with clinical scores (%)
State-Trait Anxiety Index (Trait)	39.99 (8.61)	20 – 80	20 – 56	> 39 clinically significant	52%
Brief Fear of Negative Evaluation	37.58 (11.45)	12 – 60	20 – 58	> 37 clinically significant	47%
Intolerance of Uncertainty	61.68 (21.75)	27 – 135	35 – 111	None specified	N/A
Liebowitz Social Anxiety Scale	41.58 (24.92)	0 – 144	2 – 97	>59 SAD is probably	15%
Beck Anxiety Index	15.39 (9.61)	0 – 63	1 – 37	>15 moderate or severe symptoms	47%
Penn State Worry Questionnaire	52.74 (14.46)	16 – 80	32 – 58	>39 moderate or high symptoms	79%
Anxiety composite	0.55 (0.72)	N/A	-0.85 – 1.8	N/A	N/A
Beck Depression Index	10.47 (3.78)	0 – 63	1 – 17	>19 moderate or severe symptoms	0%

Data acquisition and processing

Structural connectivity and resting state connectivity were acquired, preprocessed, and analyzed as described in Chapter IV. The final sample size was 18 for the structural connectivity analyses and 19 for the functional connectivity analyses.

Statistical analysis

The relationship between anxiety and depressive symptoms and BNST-insula connectivity in abstinence was investigated using linear mixed models (LMM). The LMM analyses were performed separately for structural and functional connectivity. The effects of anxiety and depressive symptoms were separately assessed as a fixed factor in the LMMs for both the anterior insula and posterior insula (α = 0.05). Subject was included as a random effects variable. To account for hemispheric differences, hemisphere was included as a covariate of no interest. Due to the small sample sizes, partial eta squared (η^2) are provided. All analyses were conducted in R (R Core Team, 2017) using the *lme4* (Bates et al., 2015) packages.

Results

Structural connectivity

Anxiety scores were not significantly associated with BNST structural connectivity with the anterior insula (F(1,16) = 0.56, p = 0.46, η^2 = 0.03) or the posterior insula (F(1,16) = 0.95, p = 0.34, η^2 = 0.06, Figure 12). Depressive symptoms were significantly related to BNST structural connectivity with both the anterior insula (F(1,16) = 7.82, p = 0.01, η^2 = 0.33) and posterior

insula ($F(1,16) = 28.46$, $p < 0.001$, $\eta^2 = 0.64$, Figure 13). Individuals with higher depression symptoms had stronger BNST-insula connectivity.

Resting state connectivity

There was no relationship between anxiety and BNST resting state connectivity with the anterior insula ($F(1,17) = 0.05$, $p = 0.82$, $\eta^2 = 0.003$) or posterior insula ($F(1, 17) = 0.20$, $p = 0.66$, $\eta^2 = 0.01$, Figure 14). In addition, there was no relationship between depressive symptoms and BNST connectivity with the anterior insula ($F(1,17) = 0.07$, $p = 0.78$, $\eta^2 = 0.004$) or posterior insula ($F(1,17) = 0.30$, $p = 0.53$, $\eta^2 = 0.02$, Figure 15).

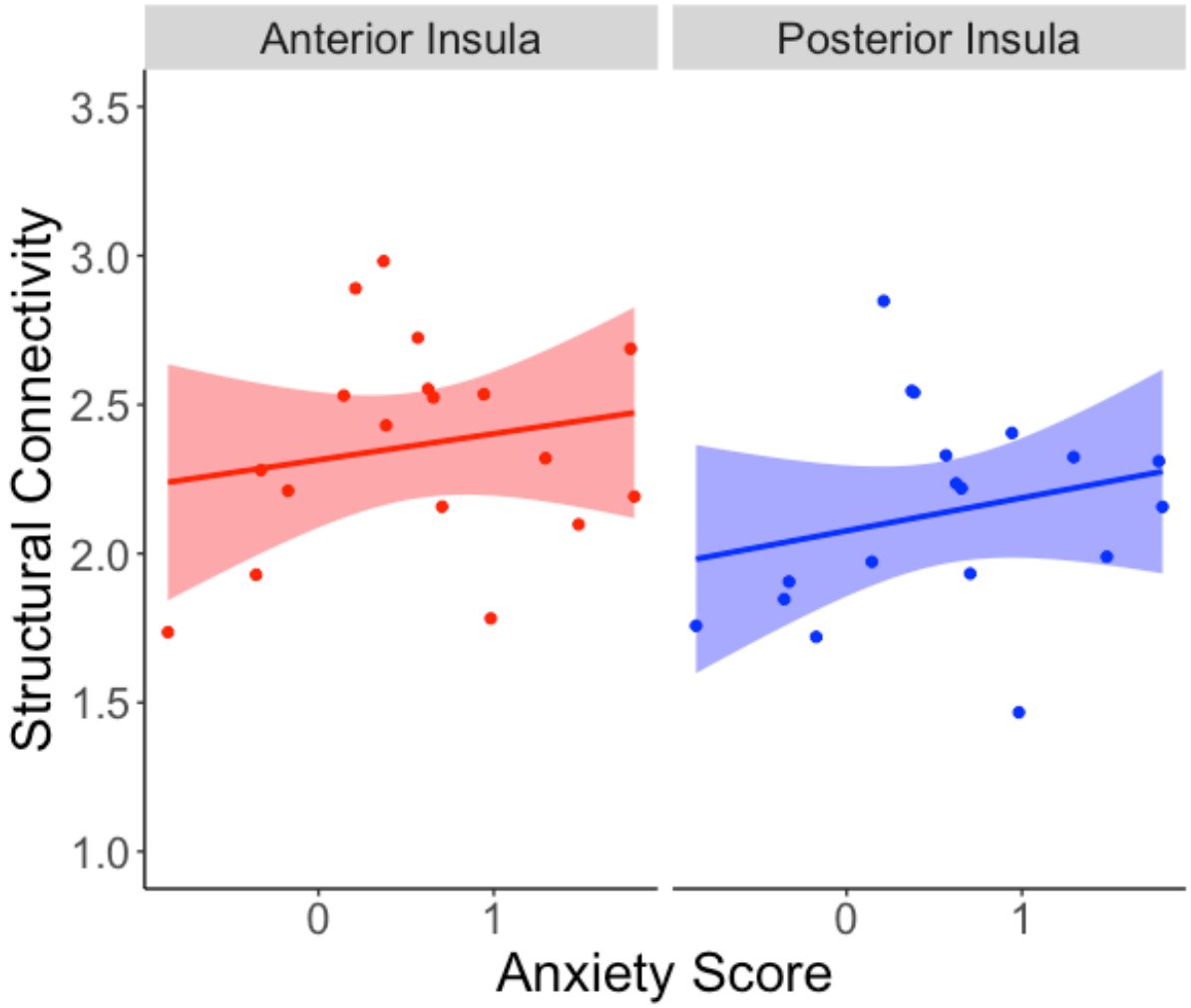

Figure 12. Relationship between structural connectivity and anxiety symptoms

There was no relationship between anxiety scores and BNST structural connectivity with the anterior or posterior insula. Structural connectivity values are the log-transformed, averaged number of tracts between the BNST and insula regions, averaged across hemispheres.

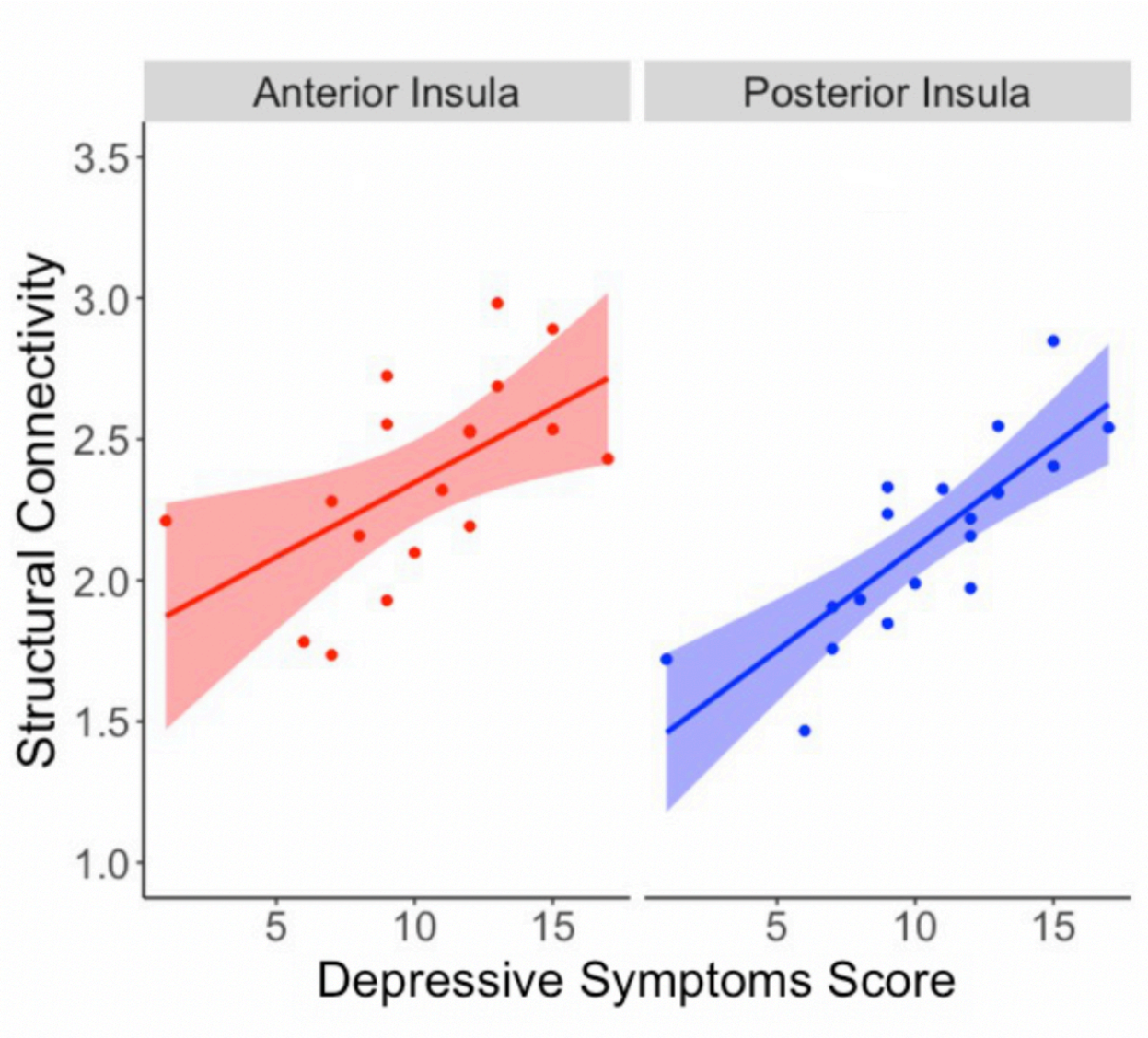

Figure 13. Relationship between structural connectivity and depressive symptoms

Greater BNST structural connectivity with both the anterior and posterior insula was associated with higher depressive scores. Structural connectivity values are the log-transformed, averaged number of tracts between the BNST and insula regions, averaged across hemispheres.

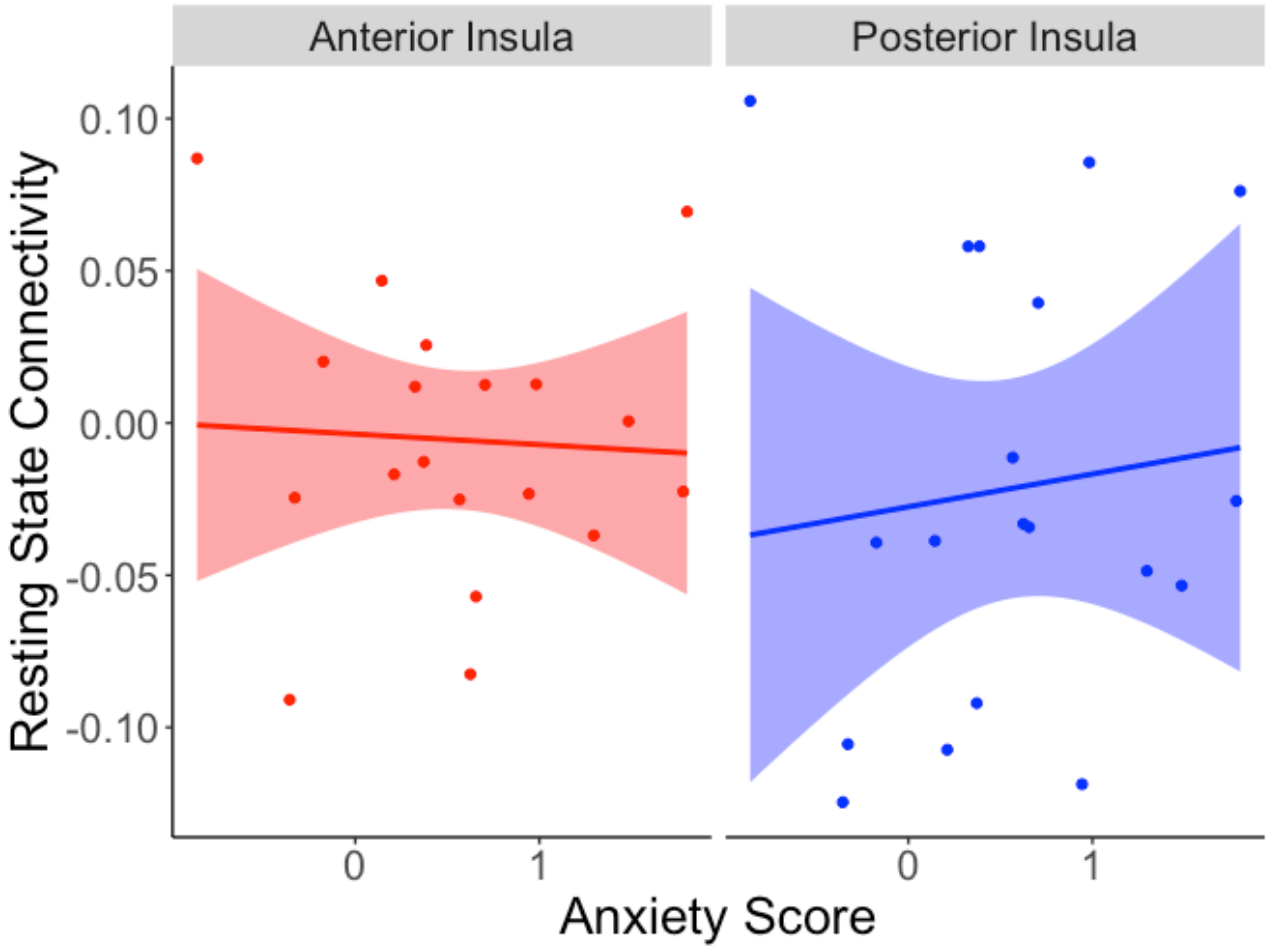

Figure 14. Relationship between resting state connectivity and anxiety symptoms

There was no relationship between BNST-insula resting state connectivity and anxiety symptoms. Resting state connectivity values represent the correlation between BOLD signal of BNST and insula regions, averaged across hemisphere.

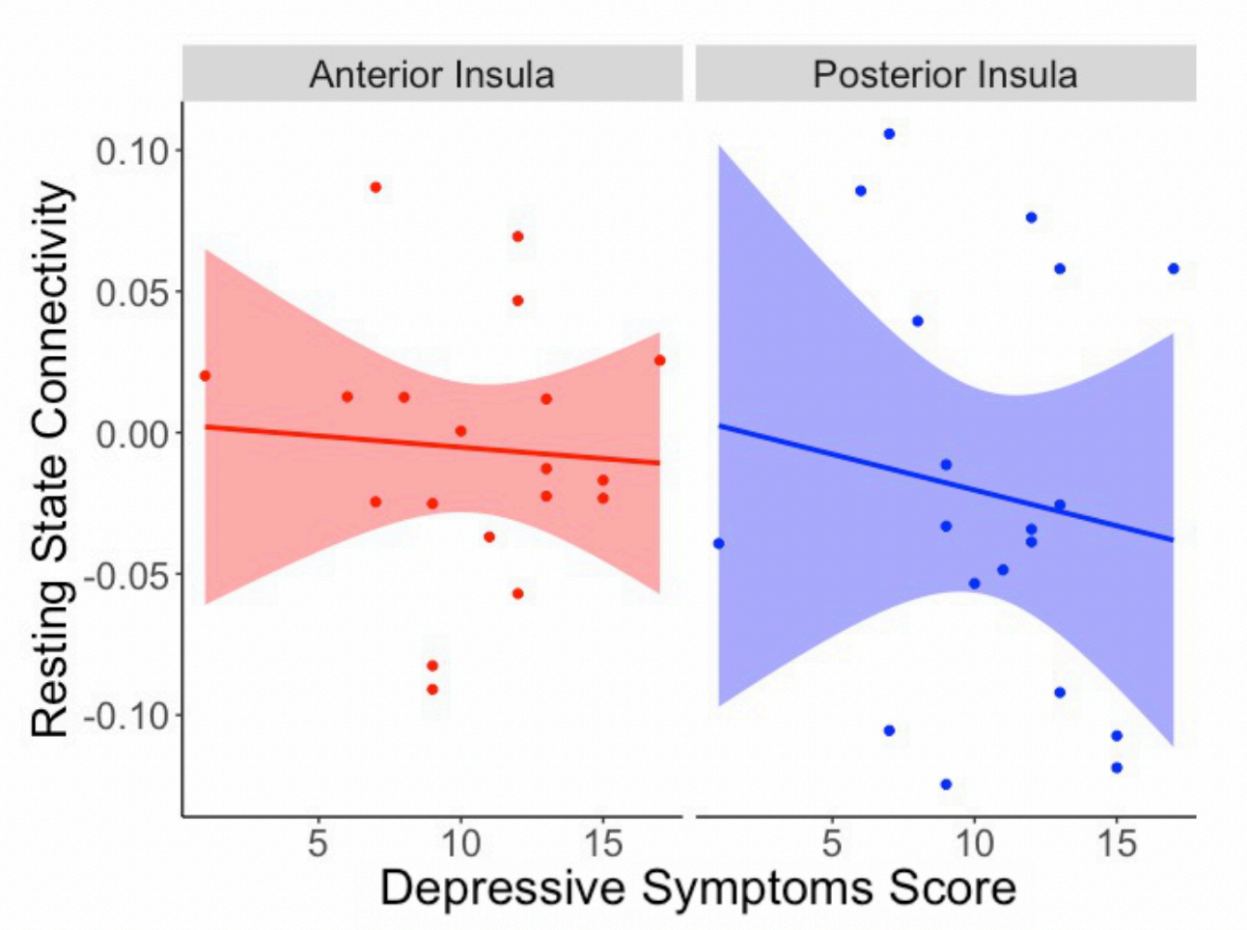

Figure 15. Relationship between resting state connectivity and depressive symptoms

There was no relationship between BNST-insula resting state connectivity and depressive symptoms. Resting state connectivity values represent the correlation between BOLD signal of BNST and insula regions, averaged across hemisphere.

Discussion

In abstinent individuals with AUD, higher depressive symptoms were associated with greater BNST structural connectivity with both the anterior and posterior insula. This relationship was not seen for anxiety and structural connectivity. BNST resting state connectivity was not associated with anxiety or depressive symptoms in either the anterior or posterior insula. The results suggest that depressive symptoms during abstinence are associated with individual differences in connectivity and should not be combined with anxiety symptoms.

Although this is the first demonstration of a relationship between depression and BNST-insula structural connectivity in abstinence, rodent studies and other areas of human literature can help to provide context for the findings. In rodent studies, depressive-like behavior is associated with prolonged abstinence from chronic alcohol use (Dao et al., 2020; Holleran et al., 2016). The administration of ketamine, which alters BNST neural activity, can block this depressive phenotype (Vranjkovic, Winkler, & Winder, 2018), linking the depressive-like behaviors during abstinence with BNST activity. In human studies, depressive symptoms are associated with cortical and limbic structural connectivity differences in major depression disorder (MDD). For example, adolescents with MDD have less white matter integrity in the pathway between the anterior cingulate cortex (ACC) and the amygdala (Cullen et al., 2010). A recent longitudinal study has even shown that white matter integrity of the ACC-amygdala pathway predicts the development of MDD in adolescence (Jin et al., 2021), suggesting a causal link between structural connectivity and symptoms. Specific to the insula, adolescents with MDD have a greater number of tracts between the insula and the hippocampus compared to

controls (Chu et al., 2018). In summary, the rodent research links the BNST and depressive-symptoms during abstinence, and the human literature shows structural connectivity differences related to MDD and depressive symptoms. The findings of the current study provide a bridge between these two literatures, in which structural connectivity between the BNST and insula in abstinent humans is associated with depressive symptoms.

Individual differences in BNST-insula connectivity were not related to anxiety symptoms during abstinence in this sample. Although BNST-insula connectivity has not been previously evaluated in abstinence, a growing number of studies have shown an associated between anxiety and BNST activation and connectivity (for review Avery et al., 2016; O. J. Robinson et al., 2019). The discrepancies between the findings presented in this study and the previous literature of anxiety and connectivity could have a number of explanations. It is possible that the anxiety experienced by the abstinent individuals fundamentally differs when compared to state or trait anxiety in controls or individuals with an anxiety disorder. Alternatively, BNST-insula connectivity during abstinence might not be associated with anxiety during resting state but, instead, requires an anxiety-inducing task to be discernible.

Conclusions and Future Directions

To our knowledge, this is the first study demonstrating a relationship between BNST-insula structural connectivity and depressive symptoms in abstinent individuals with AUD. The relationship with BNST-insula connectivity was specific to depressive symptoms and structural connectivity, as there was no relationship between depressive symptoms and functional connectivity nor evidence for a relationship between anxiety symptoms and BNST-insula

connectivity. This study represents an important first step in our understanding of neural mechanisms underlying negative affect symptoms in abstinent individuals. Furthermore, the findings suggest that depression and anxiety symptoms may have distinct associations with connectivity and, in the case of this abstinence cohort, should not be combined into a single construct of negative affect.

Important limitations and future directions for this study should be noted. First, the sample size for this study is relatively small and the findings should be replicated in independent samples. Second, recovery from AUD is remarkably heterogeneous. In addition to anxiety and depressive symptoms, many other individual differences likely contribute to differences in BNST-insula connectivity in abstinence. Future studies with larger sample sizes will need to explore the impact of factors such length of abstinence, trauma history, and medication. Finally, as a cross sectional study, it is not possible to determine if individual differences in BNST-insula connectivity are causing the depressive symptoms, reflecting preexisting depressive symptoms, or the relationship is the result of a confounding variable. Complicating matters further, it will be critical for future studies to examine if this relationship exists prior to AUD onset, results from brain changes associated with AUD, or is in response to abstinence from AUD.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Conclusion

Alcohol use disorder (AUD) affects millions of individuals and contributes substantial burden to the individual, the families of those affected, the health care system, and the economy (Centers for Disease Control and Prevention, 2020). In part, AUD has such devastating consequences because of the difficulty for individuals to maintain sobriety. Even following treatment, the majority of individuals will relapse within a year of abstinence (Bradizza et al., 2006b; Sinha, 2011; Zywiak et al., 1996). A common trigger of relapse is increased stress and symptoms of anxiety and depression that individuals experience during abstinence (G. F. Koob, 2008, 2009). Little is known, however, regarding the neural mechanisms in humans underlying these symptoms in periods of abstinence. Understanding the brain regions contributing to negative affect during abstinence could help to identify treatment targets that may prevent relapse.

Recent work in rodent models of addiction has identified a key neural pathway, from the insula to the BNST that drives negative affect behaviors during abstinence (Centanni, Morris, et al., 2019). This finding is intriguing given the roles for both the BNST and insula in emotional processing and addiction (Centanni, Bedse, et al., 2019; Davis et al., 2010; Ibrahim et al., 2019; Paulus & Stein, 2006). This pathway, however, has not been systematically investigated in humans.

For the first time in humans, the results presented here describe normative structural and functional connectivity between the BNST and insula and investigate differences in BNST-insula connectivity in individuals with abstinence. When comparing BNST connectivity with the anterior and posterior insula, the findings support the hypothesis that the BNST has greater normative structural (Chapter II) and resting-state functional connectivity (Chapter III) with the anterior insula compared to the posterior insula. The structural connectivity findings also demonstrated a sex x region interaction. Post-hoc analysis revealed that men but not women had greater anterior than posterior structural connectivity; furthermore, men and women had similar anterior insula structural connectivity with the BNST but women had greater BNST-posterior insula structural connectivity compared to men (Chapter II).

In addition to demonstrating BNST-insula normative connectivity, this project also investigated region specific differences between individuals with and without AUD. In the abstinence group, there were no significant differences in structural connectivity compared to the control group (Chapter IV). However, because the sample size was relatively small, which could result in Type II errors, effect sizes were computed to assess the strength of the group difference. For both the structural connectivity and resting state connectivity analyses, the effect sizes were of medium strength. For structural connectivity, there was greater posterior insula structural connectivity with the BNST in the abstinence group compared to controls. For resting state connectivity, the anterior insula had greater BNST resting state connectivity in the control group compared to the abstinence group. When investigating negative affect symptoms within the abstinence group, BNST structural connectivity with both the anterior and the posterior insula was associated with depression but not anxiety scores (Chapter V). The resting

state analysis did not show a relationship between BNST-insula connectivity and anxiety or depressive symptoms (Chapter V). In summary this project led to the discovery of a translational BNST-insula pathway. In abstinent individuals with AUD, BNST-insula connectivity differs and is associated with depressive but not anxiety symptoms.

Clinical implications and future directions

Below is a review of both the clinical implications of this work and future directions for research. First, I consider explanations for divergent functional and structural connectivity findings. Then, I evaluate the implications of the robust, normative BNST connectivity with the anterior insula. Third, I explore a possible explanation for the relationship between BNST-insula structural connectivity and depressive symptoms. Fourth, I discuss the importance of understanding sex differences in connectivity among abstinent individuals with AUD. Next, I examine how the findings might impact the current abstinence literature. Finally, I consider how the translational approach used in this project could benefit other translational work.

<u>What does it mean when structural and functional findings diverge?</u>

One intriguing discovery from this work is the discrepant functional and structural connectivity findings; specifically, group differences were demonstrated in the *anterior insula* for resting state connectivity but the *posterior insula* for structural connectivity.

In general, functional connectivity follows known structural connectivity patterns (Damoiseaux et al., 2006; Rykhlevskaia, Gratton, & Fabiani, 2008), although there are

circumstances where this is not true (Damoiseaux & Greicius, 2009; Honey et al., 2009). In a limited sample, a comparison of resting state MRI and DTI scans showed that strong structural connectivity was associated with strong functional connectivity, but strong functional connectivity was observed between brain regions that were not structurally connected (Honey et al., 2009). Resting state connectivity relies on the time-related correlations of blood flow to brain regions as an approximation for brain activity (referred to as the blood-oxygenation-level-dependent, or BOLD, signal). While it is likely that brain regions with highly correlated BOLD signals (i.e. strong functional connectivity) are directly communicating through axons that link the two regions (i.e. strong structural connectivity), there are circumstances where the BOLD signal of two regions might be correlated for a different reason. For example, two unconnected regions might both have strong coherence with a third region, with which they both share a structural connection. With an understanding of why the structural and functional connectivity findings might differ, we can now investigate what underlying biology might led to the different structural vs resting state findings during abstinence demonstrated in this project.

For the anterior insula, the findings suggest that individuals in abstinence have a lack of normative BNST-anterior insula resting state connectivity, but preserved structural connectivity. This discrepancy could have a number of explanations. First, the change in resting state connectivity could be due to a third brain region that is disrupting the signal between the BNST and anterior insula without altering the direct BNST-anterior insula structural connection. Second, changes specific to the synapse, such as altered receptor expression, could alter communication between regions without changing the white matter connecting them. Supporting this possibility, work in rodents has shown that chronic exposure to alcohol is

associated with changes in neurotransmission in BNST (for review see Harris & Winder, 2018). Thus, the lack of BNST-anterior insula resting state connectivity during abstinence could be due to microstructural white matter differences or from alcohol-related and/or stress-related alterations in synaptic plasticity not detected by structural connectivity studies.

For the posterior insula, individuals in abstinence had greater structural connectivity with the BNST, with similar levels of resting state connectivity. Structural connectivity differences might not create resting state connectivity differences in situations where the projections are highly specialized for a specific function or task. For example, structural connectivity between the posterior insula and BNST might be associated with task-based connectivity instead of resting state connectivity. An alternative explanation is the time frames which these data are collected. Resting state connectivity detects dynamic, millisecond changes in neural activity by measuring alterations in blood flow known as the BOLD signal; however, the timeframe of the BOLD signal is 2-7 seconds. As a result, rapid changes in neural activity can be averaged over during data collection. A recent study compared resting state BOLD signal with local field potentials (LFPs) and determined that the BOLD signal was more similar to low frequency LFPs as opposed to high frequency LFPs (Shi et al., 2019), suggesting that different types of neural activity are not equally represented by the BOLD signal. Therefore, it is possible that the communication between the BNST and posterior insula is not captured by resting state connectivity, either because it is more associated with a task or because the signal generated is difficult to detect using functional MRI.

BNST- anterior insula connectivity: no relationship with anxiety symptoms?

The findings of these studies provided evidence that the BNST has both structural and functional connections with the anterior insula and that BNST-anterior insula connectivity is altered during abstinence. Both the BNST and anterior insula have key roles in normative anxiety, anxiety disorders, and negative affect during abstinence, which led to the hypothesis that variability in BNST-anterior insula connectivity would be associated with anxiety and depressive symptoms. However, this hypothesis was only partially supported, as there was no relationship between connectivity and anxiety.

There are a number of explanations for why BNST connectivity with the anterior insula was not associated with anxiety. One possible explanation is that the connectivity is not associated with anxiety during rest, as assessed in this study, but may be engaged during a task, for example, an unpredictable threat task, in which individuals associate certain cues with a neutral stimulus, an unpleasant stimulus, or an unknown neutral or unpleasant stimulus (Schmitz & Grillon, 2012). A second possibility is that the anxiety associated with abstinence has a different neurobiological profile than anxiety in controls or individuals with an anxiety diagnosis, and is not associated with the anterior insula or BNST. This explanation seems less likely as rodent findings have demonstrated that the BNST mediates anxiety-like behaviors in a number of models of psychopathology (e.g. Avery et al., 2016; Flook et al., 2020; Verbitsky et al., 2020), although there is less data regarding the anterior insula across psychopathologies in rodents. A third explanation is that the anxiety-related signal between the insula and BNST is associated with a specific part of the anterior insula that would require more specific

parcellations to uncover. Future studies should investigate functional connectivity of the BNST and insula in response to an anxiety-inducing task to determine if individual differences in anxiety symptoms during abstinence relate to a BNST-insula connectivity during the task. In addition, different parcellations of the insula could be used to determine if a more specific area within the anterior insula is associated with anxiety.

Based on the current literature, the explanation that BNST-anterior insula connectivity would be sensitive to a task is the most plausible. An important and well-characterized role of the anterior insula is being a central hub in the salience network. The salience network serves to filter through and detect important stimuli and recruit appropriate functional networks, which helps to explain studies demonstrating anterior insula cue reactivity to alcohol in recovering individuals with AUD (for review see Schacht, Anton, & Myrick, 2013). The anterior insula receives input from a diverse collection of brain regions including the posterior insula, prefrontal regions, the thalamus, the amygdala, and the entorhinal cortex (Augustine, 1996). The anterior insula is proposed to integrate information from these regions with sensory information to form emotions and self-awareness and assign saliency (Craig, 2010b; Seeley et al., 2007). During a resting state scan, there are minimal incoming stimuli to process, potentially indicating a reduced need for communication between the anterior insula and BNST and explaining why a relationship between BNST-insula connectivity and anxiety was not detected during resting state.

Examining the anterior insula as a hub of the salience network could also provide insight into how the posterior insula, anterior insula, and BNST all work in concert. Although most rodent evidence suggests the BNST does not project to the anterior insula, the anterior insula

receives interoceptive information from the posterior insula that could reflect a BNST-mediated state of negative arousal. For example, the salience network might increase activity in response to cues from the posterior insula, such as rapid breathing or increased heart rate, indicating that the body is undergoing a stress response (Chong, Ng, Lee, & Zhou, 2017; Kim et al., 2019). After receiving input from the posterior insula and integrating it with information from other regions, the anterior insula projections to the BNST might then alter the BNST's stress response depending on if there are salient anxiety-related cues. Given the BNST's central role in coordinating the stress and anxiety response, this somatic feedback loop could be an indirect way for the BNST to engage the salience network. If this posterior insula -> anterior insula -> BNST pathway is important for anxiety, it could help to explain the efficacy of mindfulness and meditation exercises, such as deep breathing and somatic awareness that have been associated insula activity, in reducing anxiety (e.g. Zeidan et al., 2014).

What explains the relationship between BNST-insula structural connectivity and depressive symptoms?

Our findings illustrated an interesting relationship, where greater depressive symptoms were associated with greater BNST-insula structural connectivity during abstinence. These findings are intriguing for a number of reasons. First, the results suggest that depressive symptoms and anxiety symptoms are distinguishable constructs and should be evaluated separately. Second, the BNST is much more commonly studied for its role in anxiety symptoms and anxiety disorders. Perhaps research investigating the BNST in MDD or other similar disorders would also prove fruitful. Third, both the anterior and posterior insula showed a

relationship between BNST structural connectivity and depressive scores, suggesting a role for both parts of the insula in depressive symptoms associated with abstinence.

Anxiety and depression are highly comorbid and often combined, yet there are some distinguishing features between the two constructs. Anxiety is commonly characterized by symptoms of hyperactivity and worry (American Psychiatric Association, 2013), which are associated with alterations in threat processing. On the other hand, individuals with MDD more often suffer from anhedonia and decreased motivation (American Psychiatric Association, 2013), which indicate the prominent involvement of reward system dysfunction in depressive symptoms. Addiction is also a disorder characterized by reward system dysfunction, and the insula's role in craving suggests reward-related alterations during abstinence (Naqvi, Gaznick, Tranel, & Bechara, 2014). Thus, the relationship between insula connectivity and depressive symptoms during abstinence could reflect reward-related processes. Greater BNST-insula connectivity might indicate a greater disruption in reward signaling during abstinence, which contributes to the experience of more depressive symptoms.

BNST structural connectivity with both the anterior and posterior insula was also associated with depressive symptoms in abstinent individuals with AUD. The insula is the major brain region for interoception, which refers to the physiological condition of the internal state, including temperature, pain, touch, and visceral discomfort (for review see Paulus and Stein 2010). Interoceptive processing involves both the anterior and posterior insula. In general, the posterior insula receives sensory information about the internal state of the body in addition to external sensory information. The posterior insula then sends the interoceptive and other sensory information to the anterior insula, where it is integrated and combined with other

limbic and cortical information to create a subjective feeling state (Craig et al 2003). As mentioned previously, AUD, depression, and pain processing are closely linked. Perhaps BNST structural connectivity with the posterior insula provides information about pain information and the anterior insula connectivity reflects more about the interpretation of the pain as depressive symptoms. As this relationship was only seen in structural connectivity, a first and important step will be to determine if BNST-insula functional connectivity differs in response to a task. In addition, if the relationship between depressive symptoms and BNST-insula structural connectivity is related to interoception and pain processing, future studies in other psychopathologies will be important to determine the specificity of these findings in AUD and possibly uncover treatments from other disorders that could be beneficial in AUD.

What is the relevance of the sex differences?

One notable finding that needs to be evaluated more closely is the finding of sex differences in both normative BNST-insula connectivity and abstinence. Chapter II describes greater anterior than posterior insula structural connectivity in men but not women, associated with greater posterior insula structural connectivity in women. These results were not seen in normative resting state connectivity (Chapter III), which showed a trend towards greater overall resting state connectivity in men compared to women but only in one sample. In the abstinence study, women had greater anterior and posterior structural connectivity with the BNST in the abstinence group compared to controls, which was not seen in men. These results will need to be replicated in studies specifically designed to evaluate the effect of sex during abstinence.

Greater structural connectivity in women during abstinence is interesting because rodent studies also demonstrate findings specific to females and clinical data suggest sex differences. The findings of many rodent studies examining abstinence-related negative affect and BNST alterations, including the study that inspired this project, are limited to female rodents (Centanni, Morris, et al., 2019; Holleran et al., 2016; Vranjkovic et al., 2018). The clinical presentation of AUD also demonstrates sex differences, in which women experience more anxiety and depressive symptoms during abstinence than men (Becker & Koob, 2016; Peltier et al., 2019). Furthermore, these sex differences have important prognostic and treatment implications, as women are more likely to relapse in response to stress and negative affect (for review see "2017 National Conference on Alcohol and Opioid Use in Women and Girls: Advances in Prevention, Treatment and Recovery," 2017). In summary, the effect of sex during abstinence is a critical component for understanding the neurobiological basis for abstinence symptoms and clinical outcomes. Including sex as a factor in future abstinence studies could help provide a biological foundation for sex-specific treatments of AUD.

Uncovering a novel connection using translational tools: what is the relevance for future translational research?

One primary motivation behind this work was a recent rodent study demonstrating that insula projections to the BNST drive abstinence-induced negative affect. This finding indicated an area for important translational work, but BNST connectivity with the insula was still relatively unknown in humans. Using comparative anatomy, we determined that the insula findings from the rodent study were more analogous to a subregion within the human insula

that was confined to the anterior insula. For this reason, we opted to conduct this translational project using anterior and posterior divisions of the insula as opposed to the whole insula.

Using the anterior and posterior insula parcellation of the insula, we discovered that the BNST and insula are structurally and functionally connected in humans and that this connectivity is altered in abstinence. This translational approach highlights three important points: 1) rodent literature can be used to develop novel, hypothesis driven work in humans; 2) using a translational approach can inspire future directions for both rodent and human research, 3) discrepancies between rodent and human findings directly inform the limitations in translating findings between rodent and humans.

First, the findings of this study provide a powerful example of how rodent research can be translated into hypothesis-driven research questions in humans. In this case, the rodent literature was critical for guiding the approach to insula parcellation, particularly as previous human studies had not reported BNST-insula structural connectivity, and there were inconsistent resting state connectivity findings. Comparing the cytoarchitecture of the rodent and human insula illustrated that the agranular insula of the rodent insula, which is most connected with the BNST in rodents (Reynolds & Zahm, 2005), was more closely analogous with the anterior insula in humans. As shown in Chapter II and III, dividing the insula into an anterior and posterior division demonstrated that the anterior insula has BNST structural connectivity that was significantly greater than the negative control region whereas the posterior insula has similar connectivity with the negative control region. Thus, as seen in rodents, BNST-insula structural connectivity in humans is also more concentrated to the region of the insula that contains agranular cortex. The parcellation of the anterior and posterior insula does not exactly

reflect cytoarchitecture, and future studies aimed at creating more specific human parcellations will be important. Nonetheless, the findings suggest that comparing cytoarchitecture across species can be a powerful tool for developing translational hypothesis in regions with substantial heterogeneity.

Second, in addition to studies of the insula, cytoarchitecture could be used to guide research in other brain regions. Like the insula, the cingulate cortex has diverse cytoarchitecture with an anterior-posterior gradient associated with differences in function such that the anterior cingulate cortex (ACC) is associated with emotion and the posterior cingulate cortex (PCC) is more associated with memory and visuospatial orientation (Jumah & Dossani, 2021). One comparative anatomy study used rodent and human anatomy, histology, and function to identify analogous trans-species subregions within the cingulate as well as subregions that are unique to non-human primates and humans (Vogt & Paxinos, 2014). Future translational studies could use rodent-based segmentation to identify analogous regions of interest to investigate in humans. The use of these a priori regions can be critical in human studies, where findings can be overlooked due to the stringent correction for multiple comparisons needed for a whole brain approach (Poldrack et al., 2017). Using regions selected from a cross-species analysis of the cingulate, as was used in this study, could assist the translational potential of studies in the cingulate.

Third, cytoarchitecture can also help to determine when analogous structures do not exist between rodents and humans. For example, the orbitofrontal cortex (OFC) is another region with substantial translational potential in addiction for its role in decision making and reward. Like the insula, the OFC includes regions of granular, dysgranular, and agranular cortex.

There has long been debate about an analogous region in rodents; the rodent frontal cortex does not contain granular cells which can be found in the majority of the human orbitofrontal cortex (for review see Wise, 2008). Determining the cytoarchitecture of the human OFC could guide translational research teams' decision making with regard to whether it is most appropriate to conduct follow up studies in rodents, when findings are from the agranular portion of the OFC, versus a non-human primate model.

Future directions for this research include studies that help us better understand the different contributions of the insula's cytoarchitectural subregions to the BNST and other regions. Although the anterior insula has greater connectivity with the BNST than the posterior insula, both appear to differ in abstinence. Investigating the granular insula's connection with the BNST in rodent could reveal a different or similar pattern as the agranular insula. Several questions remain: *Do the agranular and granular cortices project to the same BNST neurons? Does granular cortex input to the agranular cortex influence the output to the BNST?* In human literature, studies investigating specific anterior or posterior insula connectivity will be critical for translational work with other key addiction-related regions. For example, rodent work has demonstrated that agranular insula projections to the central amygdala is associated with relapse to methamphetamine following voluntary abstinence in a rodent model (Venniro et al., 2017). Using cytoarchitecture as a guide for translating rodent insula findings into humans allows for more selective, a priori hypotheses with powerful cross-species results.

Can this research be used to develop additional targets for addiction therapy?

The results from this work provide important feedback on the current neural model of abstinence-induced negative affect. As described in Chapter I, the current model of the abstinence circuit only includes the extended amygdala. This study, however, translates a recent rodent finding that the insula projections to the BNST regulate abstinence-induced negative affect (Centanni et al., 2019), together providing both rodent and human evidence for a possible expanded model that includes the insula. This expanded model is particularly intriguing, as substantial human evidence supports a role for the insula in craving and relapse (e.g. J. Liu et al., 2014; Naqvi et al., 2007), provide a biological basis for the link between negative affect, craving, and relapse.

Results from this work also suggest the insula may be an important target for treatment to reduce abstinence-related negative affect, specifically depressive symptoms. Targeting the insula has practical implications for treatment for a number of reasons. First, a compound targeting CRH receptors that are abundant in the BNST did not show efficacy in a clinical trial of anxiety (Grillon et al., 2015). A potential explanation is the complex neurocircuitry within the BNST, making it a difficult target for a treatment that might have opposing effects in different parts of the BNST. A possible solution is to identify structures, like the anterior insula, that influence the BNST, can indirectly alter BNST-mediated behaviors, and be investigated as novel treatment targets.

Second, the insula is more accessible compared to the BNST for newer treatment techniques such as transcranial magnetic stimulation (TMS) or transcranial direct current stimulation (tDCS). TMS is a non-invasive procedure in which individuals receive magnetic

pulses with a device placed on the skull. tDCS is also a non-invasive procedure in which individuals have two electrodes placed on the scalp which emit a weak current. With the transcranial placement of TMS and tDCS, brain regions more proximal to the surface of the brain (e.g. the insula) are more attainable treatment targets. Relatedly, the insula has previously been targeted with these non-invasive techniques (for examples see Malik et al., 2018; Spagnolo et al., 2019) with variable success, suggesting that more work will be needed to determine protocols for engaging the insula with TMS and tDCS. These protocols could then be tested in treatment for individuals in AUD as targets for relapse prevention (for review see Ibrahim et al., 2019).

Third, there is a critical need for personalized treatment approaches in psychiatry. Evaluating the effect of current treatments on the insula could provide critical insight into how treatments impact the brain in recovery and, potentially, predict which patients will respond to which treatments. For example, in non-treatment seeking individuals with AUD, a greater decrease in drinking following naltrexone administration was associated with less pre-administration kappa opioid receptor availability in the insula (de Laat et al., 2019). Thus, there is great translational potential for this work to directly inform personalized relapse prevention approaches in adults. Finally, uncovering a role for the insula in abstinence-induced negative affect, particularly depressive symptoms, highlights the opportunity to uncover more, previously overlooked brain regions that can serve as potential treatment targets.

Summary of the future directions

The results of this study provide exciting avenues for future directions. First, while the anterior and posterior insula have separable functions, the insula is highly interconnected and studies would benefit from examining both subregions. Both the anterior and posterior insula seem to interact with the BNST during abstinence in humans, and rodent studies will be important for delineating if the anterior and posterior insula communicate with the BNST in tandem or discretely. Furthermore, rodent models can investigate if the posterior insula alters the function of anterior insula projections to the BNST. Second, the unexpected finding of less resting state connectivity between the BNST and anterior insula in abstinence will need to be investigated. Task-based studies in humans could detect if the anterior insula-BNST connectivity is specifically activated in response to certain tasks. In rodents, studies could investigate the basal firing rates of insula neurons targeting the BNST and subsequent local processing in the BNST to provide translational insight into the resting state findings. Third, the findings highlight the importance of individual differences and the distinction between anxiety and depressive symptoms. Future studies of sex differences and symptom-specific evaluations will be critical. Finally, the BNST-insula connectivity represents an important opportunity for treatment, as the insula could be investigated as an additional treatment target for addiction and, specifically, relapse prevention.

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