RELATIONSHIP BETWEEN TEMPERAMENT, RESTING STATE FUNCTIONAL CONNECTIVITY, AND HEAVY DRINKING IN CYNOMOLGOUS AND RHESUS MACAQUES

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CERTIFICATE OF APPROVAL

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LIST OF ABBREVIATIONS

ACC – Anterior Cingulate Cortex

ASPD – Antisocial Personality Disorder

AUD – Alcohol Use Disorder

BA – Basal Amygdalar Nucleus

BOLD – Blood Oxygen Level-Dependent

CeA – Central Amygdalar Nucleus

d – day

dACC– Dorsal Anterior Cingulate Cortex

dlPFC – Dorsolateral Prefrontal Cortex

DMN – Default Mode Network

fMRI – Functional Magnetic Resonance Imaging

FSL – Functional Magnetic Resonance Imaging of the Brain Software Library

g/kg – gram per kilogram

GABA – Gamma-Amino Butyric Acid

GAD – Generalized Anxiety Disorder

h – hour

h/d – hour/day

HIT – Human Intruder Test

L – Left

LA – Lateral Amygdalar Nucleus

mg/dl – milligram/deciliter

mPFC – Medial Prefrontal Cortex

R – Right

ROI – Region of Interest

rsFC – Resting State Functional Connectivity

rs-fcMRI – Resting State Functional Connectivity Magnetic Resonance Imaging

rsFC – Resting State Functional Connectivity

SAD – Social Anxiety Disorder

TE – Time to Echo

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ABSTRACT

Alcohol consumption in the United States is unevenly distributed, with 64% of adults consuming alcohol but 8% meeting criteria for alcohol abuse. Many risk factors leading to excessive alcohol drinking have been identified, including aggressive and anxious temperaments, which can be studied in both human and non-human primate populations. However, aggression and anxiety have also been identified as consequences of heavy alcohol use in both human and animal populations. The functional neural correlates of aggressive and anxious temperaments have not been identified, although a network encompassing the amygdala and prefrontal cortical areas (see **Figure 1**) has been associated with anxious and aggressive behavior and alcohol dependence in humans. Longitudinal studies examining relationships between emotionally dysregulated behaviors and their associated neural correlates have not been performed, and can further understanding of the emergence and progression of alcohol dependence.

Thus, the goal of these studies was to explore associations between temperament, connectivity at rest between the amygdala and prefrontal cortex, and heavy ethanol self-administration and intoxication. Specifically, this dissertation aimed to identify the functional brain network modulating aggressive, anxious, and inhibited temperaments at rest, to determine in-vivo neural correlates of risk for progression to heavy drinking, and to assess changes in aggressive behavior and functional connectivity following chronic ethanol consumption in rhesus and cynomolgus

macaques using resting state functional connectivity magnetic resonance imaging (rs-fcMRI).

The results of these studies suggest that highly aggressive rhesus macaques are uniquely at risk for becoming heavy ethanol drinkers, but that chronic ethanol access decreases aggression and anxiety (observed during the descending limb of intoxication) in both species. While extreme reactivity to threat and heavy ethanol drinking were both associated with alterations in intrinsic amygdalocortical connectivity prior to ethanol exposure in rhesus macaques, the neural correlates of aggression and future heavy drinking at baseline differed. Aggression was associated with anticorrelated connectivity between the basal amygdalar nucleus and dorsolateral prefrontal cortex, whereas future heavy drinking was associated with higher positive connectivity between the basal amygdalar nucleus and orbitofrontal cortex and central amygdalar nucleus and dorsolateral prefrontal cortex. Importantly, alterations in the intrinsic amygdalocortical connectivity associated with risk for heavy drinking were observed after chronic heavy ethanol self-administration.

These results suggest that although a single specific alteration in amygdalocortical connectivity at rest does not mediate the relationship between aggression and heavy ethanol intake, significant associations between amygdalocortical connectivity, temperament, and ethanol intake can be found in macaques. These studies provide insight into behavior and brain connectivity as risk factors versus consequences of heavy ethanol use to inform future research to improve identification and treatment of at-risk individuals. .

CHAPTER 1: General Introduction

1.1 **Risk for versus consequence of alcohol abuse**

In the United States, 64% of adults consume alcohol, but only 8% meet criteria for alcohol abuse (Grant et al., 2004). Alcohol use disorders constitute the third major preventable cause of death in the USA behind smoking and obesity (Mokdad et al., 2004) and are one of the most preventable mental disorders globally, at approximately 7% of the population (Rehm et al., 2009). Alcohol consumption is a major risk factor for the burden of disease, and understanding individual risk for alcohol abuse is crucial. Given the current lack of effective treatments for alcohol use disorders (AUDs) and alcohol dependence, the study of risk factors represents an important opportunity to discover new behavioral and pharmacological targets for treatments. Existing treatments have only shown modest efficacy, and very few approved medical treatments are available (as reviewed in Franck & Jayaram-Linstrom, 2013).

Although many risk factors have been identified in human and monkey populations, including sex, availability, age of onset of drinking, anxiety and stress, atypical brain function and structure, and temperament (Barr et al., 2003; Barr & Goldman, 2006; Gordon , 2002; Grant et al., 2008a; Conner et al., 2010), these factors also act as consequences of acute and/or chronic alcohol use. Understanding the causal relationship between alcohol abuse and these factors is crucial for developing effective interventions and treatments.

1.2 **Non-human primate models of alcohol abuse and dependence**

A significant limitation of many studies assessing correlates of alcohol use in human subjects is that a single time point, well after establishment of alcohol dependence, is used to identify factors associated with alcohol use. The lack of longitudinal data and inconstant life histories of ethanol consumption and stress further complicate the results. In contrast, controlled animal models, and non-human primates in particular, can play a key role in understanding the risk factors contributing to heavy drinking. Non-human primates absorb and metabolize ethanol at similar rates as humans, possess large cortical volumes similar to humans, exhibit dimensions of temperament, experience complex social and affective processes, and chronically self-administer ethanol resulting in intoxication and physical dependence (Barr & Goldman, 2006; Grant & Bennett, 2003; Grant et al.,2008a). While many different species and models have been used to study the effects of alcohol and alcohol-seeking behavior, many animal models are limited by the inability to fully assess behavioral, neural, and cognitive factors in populations with limited ranges of behavior, smaller and dissimilarly organized brain structures, and diminished cognitive function (Barr & Goldman, 2006). Relatedly, both humans and non-human primates are highly social and develop complex social hierarchies that can both influence alcohol consumption and be altered by consumption of alcohol (e.g. Helms et al., 2012; McKenzie-Quirk & Miczek, 2008).

Although non-human primates provide ideal subjects for the study of alcohol, their use is relatively recent. Early studies generally attempted to either (1) model human AUD outcomes by forcibly exposing monkeys to alcohol or (2) behaviorally model human alcohol consumption and induce self-administration but ignore

consequences of alcohol exposure. Current models study sustained alcohol self-administration following induction, allowing for the study of risk factors rather than only outcomes. Various routes of administration, dosing schedules, experimental procedures, and environmental manipulations have been used to induce oral ethanol self-administration in non-human primates including sweetening vehicles, food and water deprivation, schedule-induced polydipsia (Mello & Mendelson, 1971; Grant & Johnson, 1988), and exposure to stressors (reviewed in Barr & Goldman, 2006; Grant & Bennett, 2003). One unique opportunity of non-human primate models of ethanol self-administration as compared to other animal models is the ability to study risk for abusive ethanol use, as discussed above. Non-human primates exhibit large individual differences in ethanol intake and intoxication that exceed those observed in rats in mice (Meisch et al, 1975, Henningfield & Meisch, 1978; Meisch & Lamaire, 1990; Henningfield et al., 1981; Vivian et al., 2001). These individual differences allow for the longitudinal study of individuals that drink excessively as compared to those that drink in moderation to determine possible mediating variables in a controlled setting.

1.3 **Temperament and measurements of anxiety and aggression**

Temperament, defined as a collection of individually variable emotional and behavioral reactions with temporal and situational stability (Kagan 1994), is an endophenotype for drinking to dependence that can be measured in both human and monkey subjects. While temperament and personality are terms that are frequently used interchangeably in modern research, past research suggested an earlier emergence and stronger biological basis of temperament (Buss, 1987). However, both

concepts commonly refer to aspects of individual responses to stressors and other environmental stimuli, and a genetic component of both has been proposed (Bouchard & Loehlin, 2001). Temperament has been related to immune functioning and disease susceptibility (Capitanio et al., 1999; Sih et al., 2004), including the development of alcoholism. An individual's genetic propensity toward the development of alcoholism may be partially expressed via temperament (Chartier et al., 2010, review), and in human subjects, temperament can predict adolescent alcohol use (Dick et al., 2013). Although temperament was treated as a single dimension in early animal research (Wilson et al., 1993), current views suggest that temperament varies across multiple dimensions. Several independent dimensions that have emerged include traits related to fearfulness/anxiety, excitability, aggression, confidence, and sociability (Stevenson-Hinde et al., 1980; Capitanio 1999; Weiss et al., 2006). Within these dimensions, the majority of research has focused on emotional reactivity, novelty seeking, and impulsivity (as reviewed in Fairbanks & Jorgenson, 2011).

The Human Intruder Test (HIT) assesses adaptive responses to stress with two specific stimuli labeled the Profile and Stare conditions. During the HIT, the monkey is transported to a novel testing room and allowed to acclimate to a novel testing cage, which induces a mild degree of stress. The first highly stress inducing condition is the Profile, during which a human intruder (unfamiliar to the monkey) enters the testing room and stands in profile to the monkey, which elicits freezing behaviors (behavioral inhibition). The duration (magnitude) of these freezing behaviors is considered to be reflective of the degree of anxious temperament, and individual differences in the duration of these freezing responses is highly stable (Kalin & Shelton, 1989; Kalin &

Shelton, 2000). Following a control reacclimatizing period during which the human intruder exits the testing room, the intrude re-enters and makes direct eye contact with the monkey. This is labeled the Stare condition, and can elicit similar freezing behavior as observed during the Profile, as well as a wide variety of defensive and hostile behaviors. These two conditions utilize different aspects of social threats, with the Profile condition reflecting a threat in the environment that has not yet detected the (monkey) subject, and the Stare condition reflecting an active threat that has detected the subject that is inescapable (Kalin & Shelton, 1989). Importantly, these adaptive responses provide face validity with measures of temperament reflecting different ecologically relevant responses to threat. While the responses described above are adaptive, extreme durations of inhibited and defensive responding can be maladaptive and are the basis of anxious and aggressive temperaments measured by the HIT.

As described by Hirshfeld et al., “behavioral inhibition to the unfamiliar” is a type of temperament characterized by shy, timid, and cautious behavior in novel situations in human children and infants (1992). Additionally, children that exhibited inhibition throughout childhood were more likely to also develop anxiety disorders (particularly in response to a major life stressor), suggesting that there is an association between behaviorally inhibited behavior and anxiety (Hirshfeld et al., 1992; Biederman et al., 2001; Schwartz et al., 1999). Based on studies of behavioral inhibition in young children (Kagan et al., 1998), the HIT was designed to measure similar defensive behaviors in non-human primates to improve understanding of the cues eliciting these expressions of fear and individual differences in response to these cues (Kalin & Shelton, 1989; Kalin, Shelton & Takahashi, 1991). The freezing behaviors elicited during the HIT serve as a

potent measure of behavioral inhibition in non-human primates in a behavioral test that has been similarly performed in infant children (Garcia Coll et al., 1984). However, these inhibited freezing behaviors differ from other defensive responses also linked to anxiety, such as teethgrinding or yawning elicited during the Stare condition. Although the HIT uses both the Profile and Stare conditions to assess temperament, the focus of past research has been on behavioral inhibition (freezing) primarily during the Profile as related to anxious temperament. The defensive behaviors characterized by reactive anxiety and/or reactive aggression in response to the direct stare from the human intruder in the form of behaviors such as teeth grinds, yawns, and threats, and appear to represent a different phenotype of emotionality than the freezing behaviors (behavioral inhibition). Assessment of both inhibited and defensive responses to the HIT provides an opportunity to evaluate differences in behavioral styles in response to active social threats, which have not been well characterized, despite evidence of differing neuroanatomical and physiological systems controlling inhibited behaviors and defensive response (Kalin et al., 1998; Kalin et al., 2005; Kalin et al., 2007).

Although the HIT is a well standardized method for assessing temperament, the behaviors elicited by the HIT can by influenced by the physiological and emotional state of the animal during testing, which can be a detriment. However, the high degree of stability and test-retest reliability of responses to the test indicate that the HIT is a useful measure of temperament (Kalin & Shelton, 1989). Other techniques to assess anxiety and aggression in non-human primates are available, including simple non-invasive behavioral observations, novel objects tests either in the home cage (novelty seeking) or in a novel testing cage (novelty induced stress), and the Intruder Challenge Test

(impulsive aggression) (reviewed in Fairbanks & Jorgensen, 2011). Each has significant advantages and disadvantages, with one primary advantage of the HIT above others being its strong test-retest reliability, establishing its ability to measure trait-like individual differences in temperament.

1.3.1 *Advantages of a non-human primate model of temperament*

Non-human primates, and macaques in particular, are an ideal species to study mechanisms underlying emotional reactivity as it relates to human psychopathology. Rhesus macaques and humans exhibit similarities in brain structure and function, endocrine reactions in response to stress, social behavior, social hierarchy, and psychopathology (Kalin & Shelton, 2000; Bakshi & Kalin, 2002; Harlow, 1959; Reite, 1977; Suomi, 1983). Non-human primates present a unique opportunity to explore the neural correlates and consequences of anxious and aggressive behaviors in a controlled setting, given that macaques possess well-developed bidirectional amygdala and prefrontal cortical connections beyond those observed in rodents (Goldman-Rakic, 1987; Amaral, 1992; Carmichael & Price, 1995; see **Figure 1**), which is crucial for the study of emotional regulation. These common behavioral and neural characteristics separate non-human primates and humans from other species and underlie key components of emotional regulation (Davidson; 2000).

1.4 **Anxiety and aggression and alcohol abuse**

In human subjects, externalizing disorders with aggressive components such as conduct disorder and oppositional defiant disorder have been found to predict or

associate with substance use in adolescence (Disney et al., 1999; Boyle et al., 1999; Pardini et al., 2007; Fergusson et al., 2007), even when controlling for anxiety/depression and a family history of alcoholism (Jester et al., 2008). In addition, aggression outside of the diagnosis of a specific disorder may also relate to future alcohol abuse, with aggression at ages 5–10 increasing the odds of adolescent alcohol abuse (Brook et al., 1992) and aggression at first grade indirectly associating with substance use problems (Fothergill and Ensminger 2005). There are well-known associations between difficult temperament, antisocial personality, unstable temperament, and alcohol abuse (DeJong et al., 1993; Kessler 2004; Skodol et al., 1999). However, the direction of the association between aggression and alcohol consumption is unclear, with chronic alcohol problems associating with violence empirically in many populations and alcohol acting as a factor in 57–79 % of violent crimes (Mayfield 1976; Virkkunen 1974). Alcohol has frequently been associated with violent behavior (reviewed in Charmack & Giancola, 1997), though many factors (such as personality and environment) influence the likelihood of alcohol inducing aggression. While acute ethanol intoxication can increase aggression, the effects of chronic alcohol use on aggression are less well understood (Collins & Schlenger, 1988; reviewed in Heinz et al., 2011). Finally, past studies examining the association between alcohol and aggression have not specified a relationship with reactive versus controlled-instrumental aggression, with reactive aggression being more impulsive and controlled-instrumental aggression being more goal-oriented (Vitiello and Stoff 1997).

Similarly, the high degree of comorbidity between anxiety disorders and alcoholism in humans (Kushner et al., 2000) suggests an association, though the

direction of the association is again unclear. Alcohol has anxiolytic potential, and higher levels of anxiety have been proposed to lead to higher levels of alcohol intake. In human children, behavioral inhibition is characterized by extreme shyness and fearfulness and is predictive of future anxiety disorder development (Biederman et al., 2001; Caspi and Silva 1995; Svihra and Katzman 2004, review). Conversely, behavioral undercontrol is characterized by aggression, impulsivity, irritability, difficulty in state control, and a lack of persistence. A study by Caspi et al., (1996) following 1000 New Zealand children found both behavioral inhibition and behavioral undercontrol to be predictive of future alcohol problems in the male but not female subjects. Conversely, studies with human subjects show conflicting results regarding the behavioral consequences of alcohol use, with alcoholics reporting worsened symptoms of anxiety following alcohol consumption as well as improved capability to cope with anxiety (Kusher et al., 1990). The complicated relationship between anxiety and alcohol consumption is not limited to human research. For example, peer-reared rhesus monkeys that were separated from their mothers early in life and raised in a nursery environment displayed more anxiety-related behaviors and drank more flavored alcohol solution than monkeys raised by their mothers (Higley et al., 1991). However, rather than anxiety-like and aggressive behavior predicting alcohol intake, chronic daily exposure to a low dose of ethanol (0.5 g/kg/day) increased anxiety and aggression in socially housed female cynomolgus monkeys (Shively et al., 2002).

To disentangle the role of temperament as a risk factor for or a consequence of heavy ethanol intake, Aim 1 used late adolescent male and female monkeys to measure associations between baseline measures of anxiety and aggression and future self-

administration of ethanol. Behavioral testing performed shortly after one year of ethanol intake (in monkeys with access to ethanol) and/or water intake (in monkeys with access to only water) was used to assess the possibility of temperament acting as a consequence of heavy drinking. Based on previous studies, I hypothesized that monkeys with higher levels of anxiety or aggression at baseline would self-administer higher levels of ethanol when compared to monkeys with lower levels of anxiety or aggression. Additionally, I expected monkeys with higher levels of ethanol intake to exhibit increased aggressive-like behavior and decreased anxiety-like behavior compared to monkeys with lower levels of ethanol intake and controls (monkeys with no ethanol access).

1.5 **Amygdalocortical circuitry and anxiety and aggression**

Individual differences in temperament are associated with differences in brain and peripheral physiological functioning (Davidson & Tomarken, 1989; Kagan, Reznick, & Snidman, 1988). As described above, non-human primates exhibit similar dimensions of temperament as human subjects, and like humans, they experience complex social and affective processes (Grant & Bennett, 2003). Measurements of temperament use analogous behavioral tests in humans and monkeys, and the use of non-human primates allows for standardization of many factors that may influence threat-related responses, such as environmental factors (food, housing, lighting, health care, etc.). The HIT is most commonly used in monkeys to assess behavioral inhibition and defensive behaviors in response a social (human) threat. Monkeys that react to the HIT chronically with these fearful or anxious responses exhibit exaggerated defensive and

fear-like responses, which are in turn associated with alterations in patterns of brain activity in regions associated with anxiety and negative affect in humans, such as the prefrontal cortex (PFC) (Kalin et al., 1998; Davidson and Irwin, 1999). Non-human primates with anxious temperament exhibit extreme asymmetric right frontal electroencephalographic activity and increased levels of glucocorticoids (plasma cortisol, CSF corticotrophin releasing factor) (Kalin et al., 1999; Kalin et al., 1998). Additionally, anxious and fearful monkeys exhibit characteristics reflective of those observed in children with anxious temperaments, who react in behaviorally inhibited and shy patterns in response to novelty and strangers (Kalin & Shelton; 1989), and are at higher risk for the development of anxiety and depressive disorders (Biederman et al., 2001; Caspi et al., 1995; Fox et al., 2005). As described above, a subset of monkeys will respond to the HIT by freezing excessively in the presence of the human intruder, which is similar to responses of anxious children in the presence of a stranger (Kalin & Shelton; 1989). Increased amygdalar activity has been hypothesized to mediate behavioral inhibition (Kagan et al., 1988), but little research has been performed on the construct in recent years (Shackman et al., 2009).

Importantly, most studies utilizing the HIT to assess temperament have focused on behavioral inhibition to characterize anxiety as described above (Kalin et al., 2001; Fox et al., 2008; Kalin et al., 2007). However, the individual variability in types of responses to the HIT allows the opportunity to assess differences in subjects responding with behaviorally inhibited (freezing) versus actively anxious (defensive behaviors such as teethgrinding and yawning) or aggressive (defensive behaviors such as threats) styles. While research on behavioral inhibition has been quite limited,

research on defensive hostile responses to the human intruder is even less well-characterized, particularly with regard to underlying brain function. Nevertheless, the mechanisms underlying the formation of individual differences in temperament can be informed by characterizing the interaction of temperament-associated behavioral and physiological states.

Anxious and aggressive temperament in human and monkey subjects have been associated with altered brain function, particularly in systems involved in processing of stress and emotion. Several cortical regions of the brain including the orbitofrontal (OFC), anterior cingulate cortex (ACC), and more recently, the dorsolateral prefrontal (dlPFC) cortex have been suggested to play a role in processing of stress and emotion, particularly in a modulatory role (Diorio et al., 1993; Sullivan & Gratton, 2002; Cerqueira et al., 2008; reviewed in Davidson 2002). These cortical regions are frequently implicated in neuroimaging studies examining anxiety, emotional regulation, and threat response and detection in human subjects (Bechara et al., 1997; Bishop et al., 2004), as well as animal models of reward and punishment prediction, habitual responding, fear extinction, and anxiety (reviewed in Kalin & Shelton 2003). Prefrontal-limbic connections in particular have often been implicated in studies of emotional processing. The bidirectional connections between the prefrontal cortex and amygdala observed in both human and non-human subjects represent a significant focus of study. Both the basolateral amygdala (BLA) regions and the central amygdalar nucleus (CeA) have been assessed (reviewed in Bishop 2007). However, the BLA is rarely examined with regard to the specific subnuclei (basal amygdalar nucleus [BA] and lateral amygdalar nucleus [LA]). In the rat, each individual amygdala subnuclei is associated with a distinct

mechanism underlying conditioned fear responses (Killcross et al., 1997). While the BLA has historically been considered the primary informational input and the CeA the primary output of the amygdalar complex, current research also suggests direct PFC projections to the CeA as well as subcortical projections emerging from the BLA (Carmichael & Price, 1995; Price, 2003; see **Figure 1**).

Recent developments in neuroimaging have allowed for improved translational non-invasive assessment of anxiety and other disorders. Resting state functional connectivity magnetic resonance imaging (rs-fcMRI) is a non-invasive and translational tool to measure spontaneous fluctuations of the blood oxygen level-dependent (BOLD) signal time course at rest (Biswal et al., 1995). rs-fcMRI utilizes correlations between a particular seed region and other regions of interest (ROIs) or all other regions in the brain to compare BOLD signal fluctuations. Regions with correlated temporal BOLD fluctuations are considered “functionally connected” and reflect underlying neuroanatomical circuitry (Heuvel et al., 2009). Prior research has indicated a high degree of similarity in structural and functional connectivity matrices in anesthetized macaque and awake human subjects (Miranda-Dominguez et al., 2014; reviewed in Smucny et al., 2014; Hutchison et al., 2013; Vincent et al., 2007). rs-fcMRI, like anxious temperament, has high intra-subject reproducibility (Shehzad et al., 2009; Zuo et al., 2010) and individual variability in rs-fcMRI and behavior are associated (Kelly et al., 2008). While anxiety has been associated with altered resting state functional connectivity (rsFC) in humans between the amygdala and insula (Baur et al., 2013) and OFC (Hahn et al., 2011), and aggression has been associated with altered rsFC in humans between the amygdala and OFC (Fulwiler et al., 2012), the relationship

between the specific subnuclei of the amygdala and PFC in association with anxiety and aggression have not been examined. Similarly, a single study has examined associations between anxious temperament in macaque subjects and rsFC between the amygdala and PFC (Birn et al., 2014), while no studies have examined associations between aggressive behaviors in macaques and rsfc-MRI in any regions.

To better understand the potential physiological mechanisms underlying anxious temperament, Aim 2 used late adoFlescent male and female monkeys to assess the relationship between temperament and rsFC between the amygdala and prefrontal cortex. In particular, I was interested in elucidating the specific roles of the right and left BA, LA, and CeA subnuclei in connection with the right and left dACC, OFC, and dlPFC, which has been studied less frequently with regard to emotional processing. Given the inconsistent associations between aggression and different types of anxiety (social, general, trait) with connectivity between the amygdala and prefrontal cortex, my goal was to assess the specific roles of each amygdalar subnuclei across two different presentations of anxious behavior and one presentation of aggressive behavior. Based on evidence from studies using human and animal subjects, I hypothesized that amygdalocortical connectivity would be dysregulated in subjects with higher levels of anxiety and aggression. Specifically, I hypothesized that connectivity between the amygdala and OFC would be more strongly anticorrelated in subjects with greater reactive aggression in response to threat, while connectivity between the amygdala and dACC and dlPFC would be more strongly anticorrelated in subjects with greater defensive anxious responses but more strongly positive correlated in subjects with greater inhibited responses to threat.

1.6 **Amygdalocortical circuitry and alcohol abuse**

Alcohol addiction is characterized by a loss of control over alcohol intake which has frequently been attributed to dysregulated function of subcortical reward circuitry (reviewed in Kalivas & Volkow, 2005). Increases in dopamine after drug exposure in midbrain regions including the substantia nigra and ventral tegmental area and their projections (the ventral striatum and dorsal striatum) resulted in a historical focus of research in these brain regions (Nestler, 2005). However, recent advances in neuroimaging research exploring regions outside of this circuitry have established a key role of the prefrontal cortex (PFC) and amygdala in drug and alcohol use (Everitt & Robbins, 2005; Kalivas, 2009; Abernathy, Chandler, & Woodward, 2013). Given the large body of research reporting strong associations between anxiety, aggression, and emotional reactivity and dysregulation with alcohol abuse (see Section 1.4), it is not surprising that the limbic circuitry associated with aggressive and anxious behaviors (see Section 1.5) would also be associated with alcohol abuse. Studies of the effects of chronic alcohol have found that the amygdala and cortex to be particularly vulnerable to alcohol-associated brain damage, with studies showing decreased gray and white matter volumes in the cortex (Harris et al., 2008; Sullivan & Pfefferbaum, 2005) and amygdala (Makris et al., 2008), which are in turn associated with impaired executive functioning and personality (Oscar-Berman & Hutner, 1993). Amygdalocortical circuitry is crucial for regulatory control of emotional-related behavior, as well as the perception and expression of emotion (Phelps & LeDoux; 2005). Based on these associations, research has suggested that dysfunctional amygdalocortical circuitry may underlie the behavioral and emotional impairments observed in chronic alcoholics. However, these

associations between dysregulated emotional circuitry and behavior and alcohol abuse produce an important question regarding causality. Much like behavioral risk factors such as anxiety and aggression, it is unclear whether abnormalities in limbic circuitry are produced by chronic alcohol abuse, or whether the abnormalities predate the development of alcohol abuse and contributed to its development. The existence of limbic dysregulation in individuals with a family history of alcoholism (who are also at a higher risk for developing alcohol dependence) (Dick & Foroud, 2003; Oscar-Berman & Bowirratm, 2005) supports the hypothesis that these brain abnormalities may serve as risk factors for rather than consequences of alcohol use.

The impaired emotional processing observed in humans with AUDs and alcohol dependence support a role for amygdalocortical circuitry dysfunction either prior to or following alcohol abuse. However, despite several studies observing atypical activation in amygdala and PFC regions in response to emotional stimuli alcohol addicted subjects (e.g. Heinz et al., 2007; Salloum et al., 2007; Marinkovic et al., 2009), very little research has focused on amygdala and PFC functional connectivity. As previously described (see Section 1.5), rs-fcMRI is a noninvasive technique to assess functional connectivity between brain regions, and can be performed in both human and non-human primate subjects. rs-fcMRI is considered particularly useful for the identification of neural circuitry underlying neuropsychiatric disorders (Fox & Greicius, 2010), in part due to the consistency of networks identified (both within and between individuals, (Chen et al., 2008). Despite the advantages associated with rs-fcMRI, few studies have examined rsFC correlates of alcohol addiction. A recent study utilized a functional connectivity density approach to assess whole brain resting state connectivity in

association with acute and chronic alcohol exposure, and found higher functional connectivity density in heavy drinkers as compared to normal controls, as well as significantly increased functional connectivity density within the thalamus following acute alcohol (Shokri-Kojori et al., 2016). Acute alcohol exposure has also been shown to influence connectivity from the posterior cingulate cortex (Zheng et al., 2015) and nucleus accumbens (Cservenka et al., 2014).

While no studies to date have found significant alterations in amygdalocortical circuitry associated with acute or chronic alcohol use, significant associations have been observed in rs-fcMRI studies of other drugs of abuse. Altered amygdala connectivity with the mPFC and ACC has been observed in subjects addicted to cocaine (Gu et al., 2010), heroin (Wang et al., 2010) and opioids (Upadhyay et al., 2010). All three studies reported decreased rsFC strength between the amygdala and PFC, suggesting commonalities in structural and functional abnormalities across varying substances. Altered connectivity within between dlPFC and ACC regions has also been observed in heroin (Ma et al., 2011; Yuan et al., 2009) and cocaine (Kelly et al., 2011) users. These studies, in combination with atypical amygdalocortical circuitry associated with alcohol abuse and behavioral risk factors for AUDs suggest that atypical intrinsic connectivity between the amygdala and prefrontal cortex may mediate the relationship between temperament and heavy alcohol consumption.

To assess the relationship between amygdalocortical connectivity and heavy ethanol self-administration as both a predictor and consequence of drinking, Aim 3 used late adolescent male and female monkeys to measure rsFC between the amygdala and prefrontal cortex in the same networks associated with temperament (see Aim 2) at

baseline prior to ethanol access and after chronic ethanol access. Specifically, the distinct roles of the left and right BA, LA, and CeA subnuclei as they functionally associate with the left and right PFC (OFC, dlPFC, and ACC) were assessed to compare to those connections associated with different temperament phenotypes serving as risk factors for heavy drinking (see Aim 1). Most rs-fcMRI studies focusing on alcohol or substance abuse have not included examinations of networks encompassing the amygdala or amygdala to cortical projections, despite overwhelming evidence of the involvement of the amygdala in alcohol abuse. Given the associations observed between temperament and heavy drinking in Aim 1 and temperament and amygdalocortical connectivity in Aim 2, my goal was to assess the specific associations of each amygdalar subnuclei in heavy ethanol self-administration longitudinally to compare risk versus consequence as well as look for overlapping relationships with the rsFC underlying aggressive temperament. Based on evidence from studies using human and animal subjects, I hypothesized that amygdalocortical connectivity would be dysregulated in subjects with future higher levels of alcohol intake and intoxication, and would also be further dysregulated after chronic heavy alcohol intake as compared to subjects without access to ethanol and subjects with non-heavy alcohol intake. Specifically, I hypothesized that connectivity between the amygdala and OFC/ACC would be anticorrelated in subjects with higher future ethanol intake, while connectivity between the amygdala and dlPFC would be more strongly positive correlated in subjects with higher future ethanol intake. Chronic heavy drinking was expected to further reduce connectivity from baseline between the amygdala and OFC/ACC and increase connectivity from baseline between the amygdala and dlPFC as compared to

controls and non-heavy drinkers. Finally, the changes in behavior expected with chronic ethanol access (see Aim 1) were expected to be mediated by the same connections associated with temperament at baseline (see Aim 2).

1.7 **Species differences**

1.7.1 *Behavior*

The final aim of these studies was to attempt to replicate the above concepts in another cohort of cynomolgus macaque monkeys. Rhesus and cynomolgus macaques are the two most commonly used species of monkey in biomedical research, but are infrequently directly compared. Although phylogenetically close, rhesus and cynomolgus macaques exhibit species specific social- and stress-related behaviors. While cynomolgus macaques and rhesus macaques exhibit similar behavioral responses and reactions to stress, the degree, duration, and type of behaviors frequently differ. Rhesus and cynomolgus macaques show distinct differences in patterns of aggression, reconciliation, dominance, and temperament (Clark & Mason, 1988; Theirry et al., 2000; Sussman et al., 2013). For example, the general temperament and behavior of rhesus and cynomolgus macaques appear to differ, with rhesus macaques exhibiting higher levels of aggression towards humans and cynomolgus macaques expressing higher levels of fearfulness (Clarke & Mason, 1988; Sussman et al., 2013). Other studies have described cynomolgus macaques as more passive or “reserved” (Clarke & Lindburg, 1993) in comparison to other macaque species. Stressful stimuli have been reported to influence cynomolgus and rhesus macaques differentially, with maternal separation

resulting in greater negative impacts in cynomolgus infants, despite similarities in the types of behaviors induced by the separation (Seay & Gottfriend, 1975). Social group behaviors also differ, with rhesus social hierarchies being more hierarchical and nepotistic than cynomolgus hierarchies (Thierry et al., 2000). Cynomolgus macaque social groups have been described as more highly affiliative with lower intensity of aggressive encounters (Theirry et al., 2000), which mirror the individual behavioral differences in response to stress observed. However, it is important to note that the magnitude of these species differences in generally small, with cynomolgus and rhesus macaques exhibiting more similar behavior and social patterns than other species of macaques.

1.7.2 *Ethanol intake and intoxication*

While both rhesus and cynomolgus macaques have been used to study patterns, predictors, and consequences of ethanol self-administration, average intakes and levels of intoxication have not been directly compared. Alcohol dependence in a self-administration model has been established in both species, and both have shown individual differences in ethanol intake and intoxication (as reviewed in Grant & Bennet, 2003). However, to date, no studies have included subjects of both species.

1.7.3 *Brain structure and function*

Brain structure and function in rhesus and cynomolgus macaques has also been infrequently compared. Many studies include both cynomolgus and rhesus macaques but do not directly compare their results, or include all subjects as a single sample

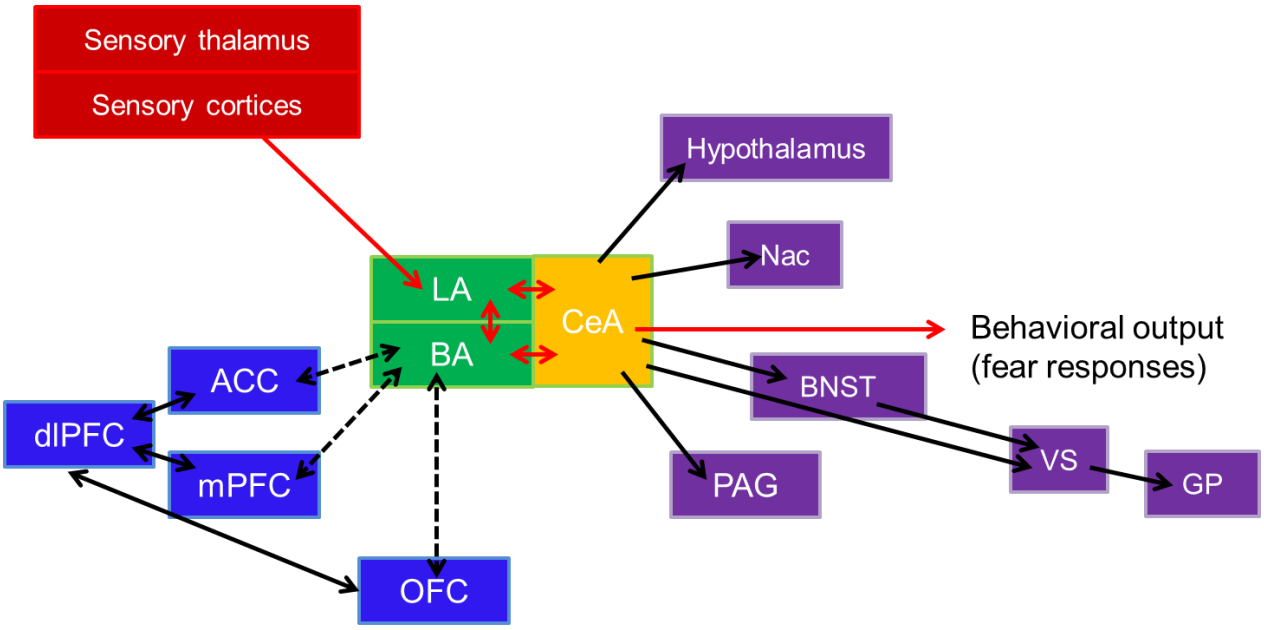
(Baleydier & Mauguiere, 1987). A single study examining intra-amygdaloid connections in both rhesus and cynomolgus macaques described the amygdaloid complex as very similar in appearance and suggested that no significant differences in anatomical measures were observed between the two (Aggleton, 1985). The study does, however, also mention a more prominent dorsolateral extension of the lateral basal nucleus in the cynomolgus macaque, suggesting that anatomical species differences within the amygdaloid complex and related circuitry are possible. The development of a stereotaxic atlas of the cynomologus macaque brain (Szabo & Cowan, 1984) also indicates slight morphological differences, with cynomolgus brains being generally smaller than rhesus brains, but no specific cytoarchitectonic differences described. Despite these potential differences in morphology, current research generally collapses across macaque subjects (e.g. Mandeville et al., 2011). This is supported by a recently developed MRI based monkey stereotaxic atlas developed using an average of cynomolgus and rhesus subjects (Frey et al., 2011). Species differences in rs-fcMRI have not been assessed directly or indirectly in any network in the brain.

To assess possible species differences and attempt to replicate Aims 1-3 as described above, Aim 4 used an additional cohort of cynomolgus macaques to repeat the behavioral and MRI experiments described in Aim 1-3. Based on the species differences in behavior, I expected to see significant differences in responses to the HIT as well as differing predictors of heavy ethanol intake. Based on the general similarities across rhesus and cynomolgus macaque brain structure, I expected amygdalocortical connectivity at rest to show a similar relationship with aggressive and anxious temperament and heavy drinking as predicted in Aim 3.

1.8 Summary

Temperament is a well-defined risk factor for alcohol abuse in human and non-human primate subjects (Chartier et al., 2010; Dick et al., 2015; Grant & Bennet, 2003, McClintick & Grant, 2016) associated with altered amygdala and prefrontal cortical function and structure (Davidson & Tomarken, 1989; Kagan, Reznick, & Snidman, 1988; Kalin et al., 1998; Davidson and Irwin, 1999; Kagan et al., 1988). Research identifying shared changes in dysregulation of limbic circuitry related to alcohol abuse and behavioral disinhibition and affective dysregulation (Virkunnen & Linnoila., 1993; Tessner et al., 2010) suggest that pre-existing atypical functional connectivity may be associated with anxious and aggressive temperaments and, in turn, lead to alcohol dependence. Additionally, the limbic system and frontal lobes are especially vulnerable to damage and dysfunction after chronic alcohol abuse (Harper et al., 1998; Oscar-Berman & Hutner, 1993; Dirksen et al., 2006; Pfefferbaum et al., 1997; Ratti et al., 2002; Tapert et al., 2001). Despite these associations, few studies have assessed intrinsic connectivity in affective regulation circuitry in relation to alcohol use or temperament. Therefore, my dissertation used the HIT, rs-fcMRI, and a unique model of chronic ethanol self-administration in non-human primates to examine the functional neural correlates of an established risk factor for progression to heavy drinking (temperament) and to assess changes in a functional network underlying temperament following chronic ethanol consumption. Improving understanding of the neurobiological basis of behavioral risks for and consequences of alcohol abuse could improve identification of subjects at risk for alcohol abuse and prevent transition to dependence.

Figure 1. The main subdivisions of the amygdala and their projections.



Adapted and modified from Roozendaal, McEwen, & Chattarji (2009)

The basolateral portion of the amygdaloid complex (primary input) receives sensory information via a rapid but basic input from the sensory thalamus and a slow but detailed input from the sensory cortex. This complex contains the lateral amygdalar nucleus (LA) and basal amygdalar nucleus (BA), which both receive direct cortical and thalamic projections and are richly connected with the central amygdalar nucleus (CeA) via the intercalated cells and serves as the primary output of the amygdaloid complex. Cortical inputs from the anterior cingulate cortex (ACC), medial prefrontal cortex (mPFC), and orbitofrontal cortex (OFC) to the amygdala reciprocally connect with the basolateral complex and play a central role in processing emotional stimuli. The dorsolateral prefrontal cortex (dlPFC) mediates emotional learning through its reciprocal

connections with the OFC, ACC, and mPFC. Finally, the CeA projects to forebrain structures and brainstem nuclei including the periaqueductal grey (PAG), bed nucleus of the stria terminalis (BNST), nucleus accumbens (Nac), ventral striatum (VS), and globus pallidus (GP) producing approach and avoidance behaviors related to fear and goal directed behavior. Red arrow shows processing of information through the amygdala to produce behavioral output. Dotted lines indicate inhibitory projections. Blue boxes indicate prefrontal cortical/cingulate cortex regions, red boxes indicate sensory cortex, green boxes indicate regions within the basolateral region of the amygdala, while the yellow box indicates the separate, central nucleus of the amygdala, and purple boxes indicate outputs of the central amygdalar nucleus.

CHAPTER 2: MATERIALS AND METHODS

2.1 **Animals**

2.1.1 *Rhesus monkeys (Macaca mulatta)*

The 32 subjects with ethanol access (drinkers) included two cohorts of female (cohort 6A n = 6, age 3 years 10 months–4 years 1 month, weight 4.0–6.3 kg; cohort 6B n = 5, age 5 years 7 months–6 years, weight 5.0–6.2 kg) and three cohorts of male (cohort 7A n = 8, age 3 years 11 months–4 years 7 months, weight 5.5–7.5 kg; cohort 7B n = 5, age 5 years 7 months–6 years 3 months, weight 7.0–11.7 kg; cohort 10 n = 8, age 4 years 7 months–6 years, weight 6.5–9.8 kg) rhesus monkeys (*Macaca mulatta*; born and raised at the Oregon National Primate Research Center, Beaverton, OR). Ages indicate age at first drink of ethanol (first day of ethanol induction—see Ethanol access section 2.3), with all animals aged 4 to 6 years. The 10 subjects without ethanol access (controls) included 1 cohort of female (cohort 6B, n = 2, age 5 years 6 months – 6 years 2 months, weight 5.0-6.0 kg) and 1 cohort of male (cohort 10, n = 8, age 4 years 8 months – 6 years, weight 6.2 – 9.1 kg) rhesus monkeys also born and raised at the Oregon National Primate Research Center, and housed in the same rooms as ethanol-drinking subjects in each respective cohort. A subset of these 42 animals were used to assess the neural correlates of heavy drinking, aggression, and anxiety. The MRI data included 12 subjects with ethanol access (drinkers) and 10 subjects without ethanol access (controls) were comprised of one cohort of female (cohort 6B) and one cohort of male (cohort 10) rhesus monkeys. All subjects were assessed with the HIT at baseline

and post-self-administration. See **Table 1** and **Figure 2** for timeline of scans, behavioral testing, and drinking.

Cohort nomenclature (6A, 6B, 7A, 7B, 10) is used to provide links to the drinking data through the website www.matrr.com. Monkeys were reared with their mothers in a troop until at least 2 years of age, after which they were moved to smaller group housing. All subjects were experimentally naïve at the onset of the study. Monkeys within each cohort had no common parents or grandparents. All monkeys were housed in individual cages with partitions allowing visual, auditory, and olfactory but non-physical contact to neighboring monkeys (0.8 × 0.8 × 0.9 m). Each individual cage contained an operant panel on one wall of the cage dispensing food and liquids. The housing room was maintained at a constant temperature (20–22 °C) and humidity (65 %) and a 12-h light cycle (lights on at 7:00 am). Body weights were taken weekly. Following acclimation to the laboratory, the monkeys were trained to participate in awake (non-anesthetized) venipuncture to obtain blood samples (Porcu et al., 2006) to assess blood ethanol concentration. All animal procedures were approved by the Oregon National Primate Research Center IACUC and were performed in accordance with the NIH and the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 2011).

2.1.2 *Cynomolgus monkeys (Macaca fasicularis)*

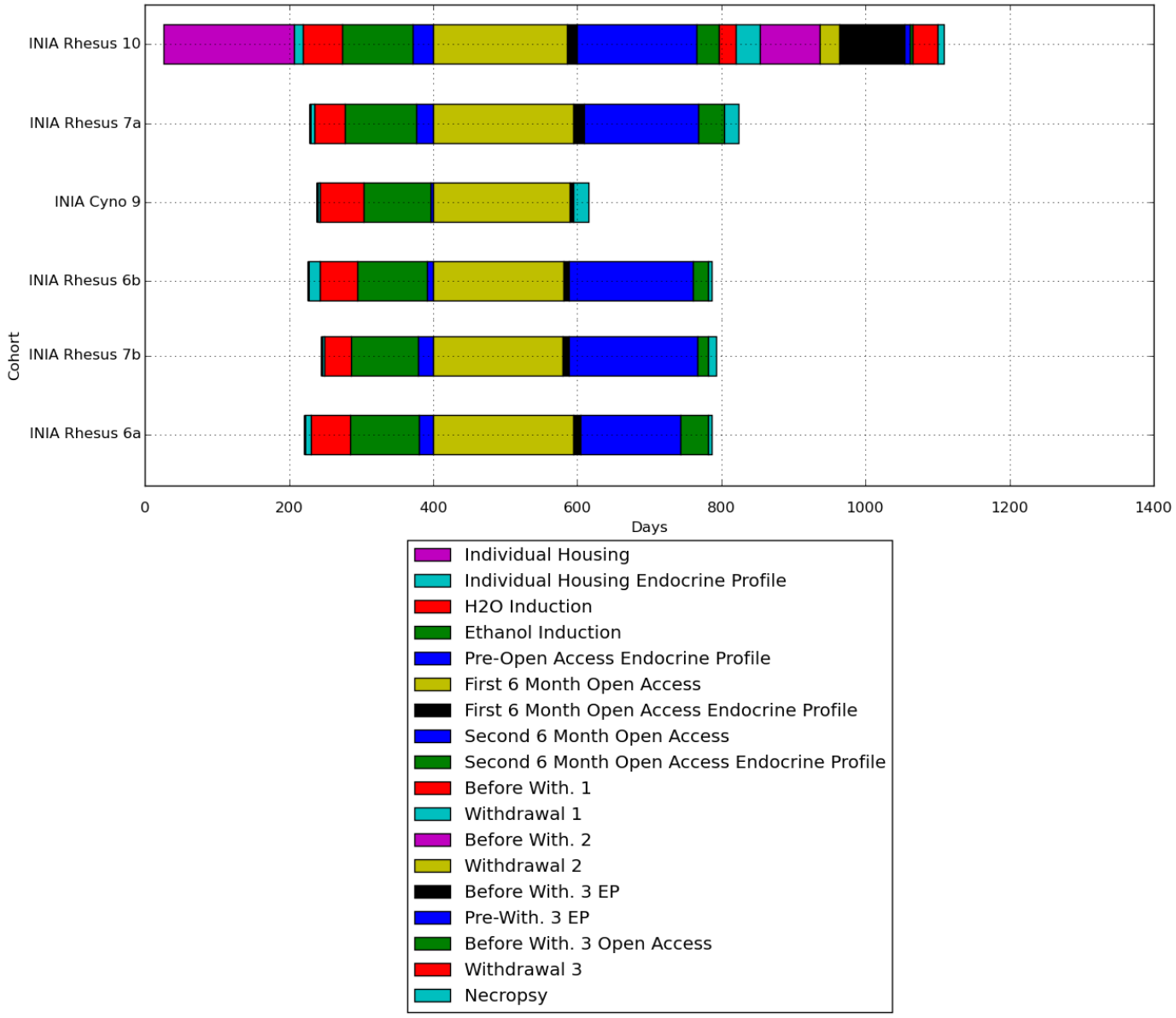
The 8 subjects with ethanol access (drinkers) included one cohort of male (cohort 9 n = 8, age 5 years 7 months–6 years 7 months) cynomolgus monkeys (*Macaca fasicularis*). Ages indicate age at first drink of ethanol (first day of ethanol induction—

see Ethanol access section 2.3), with all animals aged approximately 6 years. The subjects without ethanol access (controls) included 1 cohorts of male (cohort 9, n = 3, age 6 years 6 months – 6 years 7 months) cynomolgus monkeys housed in the same rooms as ethanol-drinking subjects in each respective cohort. See **Table 1** and **Figure 2** for timeline of scans, behavioral testing, and drinking. Cohort nomenclature (9) is used to provide links to the drinking data through www.matrr.com. Monkeys were bred at the Shin Nippon Biomedical Laboratories, Ltd. facilities in Alice, Texas or Everett, WA and were reared with their until weaning. Monkeys within each cohort had no common siblings. All subjects were experimentally naïve at the onset of the study. All monkeys were housed in individual cages with partitions allowing visual, auditory, and olfactory but non-physical contact to neighboring monkeys (0.8 × 0.8 × 0.9 m). Each individual cage contained an operant panel on one wall of the cage dispensing food and liquids. The housing room was maintained at a constant temperature (20–22 °C) and humidity (65 %) and a 12-h light cycle (lights on at 7:00 am). Body weights were taken weekly. Following acclimation to the laboratory, the monkeys were trained to participate in awake (non-anesthetized) venipuncture to obtain blood samples (Porcu et al., 2006) to assess blood ethanol concentration. All animal procedures were approved by the Oregon National Primate Research Center IACUC and were performed in accordance with the NIH and the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 2011).

Table 1. Experimental timeline (weeks) by cohort

Monkey Cohorts	Cohort 6A: Rhesus Females (n = 6)	Cohort 7A: Rhesus Males (n = 8)	Cohort 6B: Rhesus Females (n = 7)	Cohort 7B: Rhesus Males (n = 5)	Cohort 10: Rhesus Males (n = 16)	Cohort 9: Cynomolgus Males (n = 12)
Acclimation	1-8	1-8	1-5	1-8	1-5	1-11
Baseline temperament testing	14	23	6	27	6	16
Baseline MRI	-	-	105	-	16-17	30
Induction of self-administration (SIP)	41-52	24-38	103-120	40-58	23-43	64-85
22 h/d ethanol and/or water access	56-107	43-94	126-178	62-114	49-101	87-112
6 Month MRI	-	-	155	-	84	116
Post-22-h temperament testing	-	-	179	-	104	114
12 Month MRI	-	-	180	-	111	-

Figure 2. Experimental timeline (days) compared by cohort



Taken from www.mattr.com – demonstrates relative overlap of each cohort timeline for behavior each self-administration and induction period.

2.2 **Temperament testing**

2.2.1 *Temperament classification and behavioral testing*

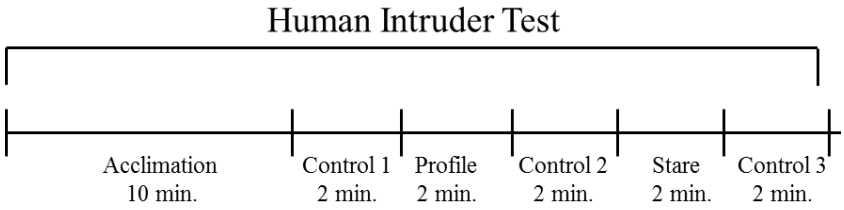
Anxious-like and aggressive-like behavior and temperament were measured via video recordings of the HIT (**Figure 3**). This test is commonly used to assess anxious, fearful, defensive aggressive, and inhibited behavior in human children and monkeys (Fairbanks and Jorgensen 2011). Temperament testing of all monkeys occurred within 7 months of entry to the laboratory between 9:00 am and 1:00 pm, and after 12 months of 22-h access to ethanol and/or water through an operant panel (**Table 1**, **Figure 2**). Post-22-h access testing occurred prior to availability of fluids, but access to ethanol was not restricted prior to the test, with access allowed through 8:00 am on testing day. Testing occurred in a novel individual testing cage in a behavioral suite physically separate from the laboratory and was video recorded from an anteroom through a one-way mirror. Test cages were cleaned after each monkey, and male and female subjects were never tested in the same cage or on the same day. Three rhesus cohorts (cohort 6A, 7A, and 7B) were not tested after 12 months of 22-h access. Video recordings of the HIT were scored by two observers unfamiliar with the monkeys and unaware of future alcohol consumption using the Observer XT software (Noldus Information Technology, Waegningen, Netherlands). Inter-rater reliability between the two observers was found to be very high ($\kappa = 0.81$, percentage of agreements = 84.5 %).

2.2.2 *Human Intruder Test*

The HIT reliably assesses individual differences in stress reactivity via three specific stimuli (Williamson et al., 2003). As shown in **Figure 3**, testing began with a 10-

min acclimation period and a 2-min control period during which the monkey was free to explore the testing cage in the absence of any other stimuli. During the 2-min profile period, the unfamiliar human “intruder” entered the testing room and stood 0.3 m away from the cage in profile to the monkey. A second 2-min control period followed the exit of the human intruder from the room and preceded the beginning of the stare phase. In this phase, the same human intruder entered the room and made continuous direct eye contact with the monkey for 2 min before exiting. Behaviors scored during this test include movements, vocalizations, exploration, and other reactions to the human intruder as listed in the ethogram (**Table 2**).

Figure 3. HIT Testing Protocol.



2.2.3 *Temperament variable organization*

Variables assessed during the HIT are found in **Table 2**. Durations of threat, partial threat, cage slap, and cage shake during the stare epoch were summed to create a new variable labeled “extreme threat.” Open mouth threat remained a separate measure of less extreme aggression. Durations of freeze non-vigilant and freeze vigilant during the profile epoch were summed to create a new variable “freeze profile,” and durations of freeze non-vigilant and freeze vigilant during the stare epoch were summed to create a new variable “freeze stare.” Durations of teeth grind and yawn during the stare were summed to create a new variable “active anxiety.” Grouping of these variables was based on single linkage cluster analysis (joining) measuring Euclidian distances (**Figure 4**), kappa statistic calculations (**Tables 3-4**), and Spearman’s correlations (**Table 5**), with higher degree of closeness (Euclidian Distances), concordance (Kappa values), and correlations between variables showing consistent relationships between the variables summed. Variables not observed (see variables with an * in **Table 2**) were not included in analyses. Cynomolgus macaques did not freeze during the Stare condition, resulting in these variables not being included in subsequent categorizations and analyses.

2.2.4 *Temperament categorization*

Responses to the human intruder were used to characterize monkeys as high and low aggression, high and low anxiety, and high and low inhibition. Groups were created with upper and lower quartiles taken from all subjects of each species (rhesus n = 48, cynomolgus n = 11) tested. Monkeys were characterized as high aggression,

anxiety, or inhibition if the duration of the sum of all behaviors in each category was at or above the upper quartile of all subjects, and at least one behavior was at or above the upper quartile of all subjects of their sex. Monkeys were characterized as low aggression, anxiety, or inhibition of the duration of behaviors in each category was at or below the lower quartile of all subjects. Behaviors used to characterize aggression included extreme aggression (threats, cage shakes, cage slaps) and open mouth threats. All defensive aggressive behaviors elicited by the HIT were reactive aggressive behaviors. Active anxiety (yawning and teethgrinding) was used to characterize anxiety. Freezing during the stare and profile was used to characterize inhibition. **Table 6** indicates temperament characterization for each individual rhesus subject and **Table 13** indicates temperament characterization for each individual cynomolgus subject. As stated in section 2.2.3, cynomolgus macaque subjects did not freeze during the stare period.

Table 2. Behavioral ethogram for temperament testing

Behavior[1]	Category[2]	Definition
Stationary	-	Subject is inactive without motile movement; may still involve head or arm movement
Locomote	-	Subject engages in movement from one location to another while using its entire body
Movement	-	Subject engages in movement of body but does not change position in cage
Freeze	Inhibition	Subject is not engaged in any movement of body or head
Explore	-	Subject inspects or manipulates cage
Sleep	-*	Subject is inactive with eyes closed
Cage bite	-	Subject uses mouth to grasp bars of cage
Self-groom	-*	Subject is picking through and/or slowly brushing aside own fur with hands and/or mouth
Abnormal	-*	Subject is engaged in atypical behavior; may include any of the following: *self-bite, copraphagy, floating limb*
Stereotypy	-	Subject paces back and forth or is engaged in other repetitive motion
Other	-	Subject is engaged in behavior not listed in Ethogram
HIT[3] Behavior		
Coo	Anxiety*	A short, high pitched soft vocalization
Shriek	Aggression*	A very high pitched loud vocalization
Grunt	-*	A short, low pitched vocalization
Bark	Aggression*	A very short, loud vocalization
Other vocal	-*	Any other vocalization.
Yawn	Anxiety	Subject opens mouth very wide, baring upper teeth
Scratching	Anxiety*	Subject uses fast movement of the hand or foot across the hair or skin
Cage slap	Aggression	Slapping the floor of the cage with hands
Teeth-grind	Anxiety	Subject engages in audible side to side movement of jaws with teeth rubbing together; usually directed at stranger
Lipsmack	-	Quick movement of jaw pressing lips together; usually directed at stranger
Threat	Aggression	Subject stares intensely with eyes wide open and/or ears pulled back; may contain facial, vocal, or physical components (e.g. *head thrusting, open mouth threat, scream, raised eyebrow, ground beating, lunge*); usually directed at stranger
Open mouth	Aggression	Subject opens mouth in “o” shape, may be accompanied with thrusting head; usually directed at stranger
Cage shake	Aggression	Subject uses hands and/or body to attempt to move cage back and forth
Partial threat	Aggression	Behavior that appears threatening, but does not fall into one of the other categories; may include slight lunge or charge directed at stranger
Fear grimace	Anxiety*	Subject has lips pulled back bearing teeth, in a “smile;” usually in response to stranger
No response	-	Subject is not engaged in any behavior directed towards stranger, or stranger is not present

[1] behaviors within a behavioral class are mutually exclusive and exhaustive

[2] behavior used to characterize temperament
– the behavior was not used to characterize temperament in response to the HIT

[3] Human Intruder Test (HIT)

* the behavior observed in ≤ 1 subjects during the HIT

Table 3. Kappa statistics describing the degree of association (concordance) between measurements of anxiety-like behavior from the HIT.

	Freeze Vigilant Profile	Freeze Non-Vigilant Profile	Freeze Vigilant	Freeze Non-Vigilant	Yawn	Teethgrind
Freeze Vigilant Profile	-	1	0.23	0.38	0.09	0.16
Freeze Non-Vigilant Profile	-	-	0.23	0.38	0.09	0.16
Freeze Vigilant	-	-	-	0.75	0	0
Freeze Non-Vigilant	-	-	-	-	0	0
Yawn	-	-	-	-	-	0.43
Teethgrind	-	-	-	-	-	-

All variables are from the Stare condition unless otherwise noted. Kappa values < 0.4 indicate poor to fair concordance, whereas values between 0.40 and 0.75 are moderate to good, and values > 0.75 are excellent (Landis & Koch, 1977; Fleiss, 1981).

Table 4. Kappa statistics describing the degree of association (concordance) between measurements of aggressive-like behavior from the HIT.

	Threat	Partial Threat	Open Mouth Threat	Cage Slap	Cage Shake	Ear Flap
Threat	-	0.48	0.07	1	0.48	0
Partial Threat	-	-	0.21	0.21	0.26	0
Open Mouth Threat	-	-	-	0.07	0.21	0.01
Cage Slap	-	-	-	-	0.48	0
Cage Shake	-	-	-	-	-	0.35
Ear Flap	-	-	-	-	-	-

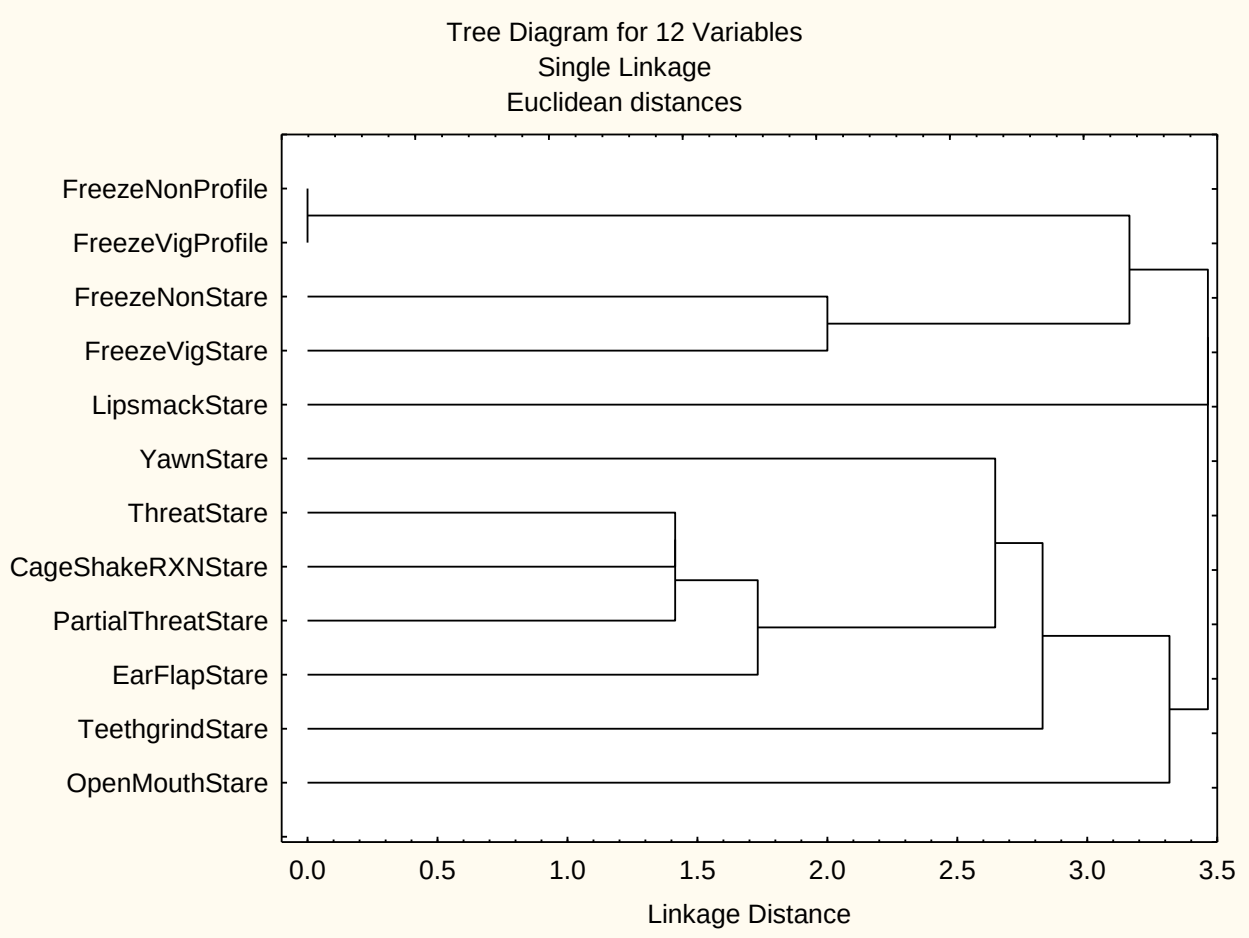
All variables are from the Stare condition. Kappa values < 0.4 indicate poor to fair concordance, whereas values between 0.40 and 0.75 are moderate to good, and values > 0.75 are excellent (Landis & Koch, 1977; Fleiss, 1981).

Table 5. Partial Spearman correlations between measurements of anxiety-like and aggressive-like behavior from the HIT.

	Freeze Vigilant Profile	Freeze Non-Vigilant Profile	Freeze Vigilant	Freeze Non-Vigilant	Yawn	Teeth-grind	Threat	Partial Threat	Open Mouth Threat	Cage Slap	Cage Shake
Freeze Vigilant Profile	-	0.25, 0.16	0.17, 0.35	-0.07, 0.68	0.34, 0.05	0.25. 0.17	-0.05, 0.79	0.14, 0.44	0.12, 0.53	-0.05, 0.79	-0.02, 0.90
Freeze Non-Vigilant Profile	-	-	0.14, 0.43	0.25, 0.17	0.10, 0.60	0.25, 0.17	0.03, 0.87	0.09, 0.61	0.34, 0.05	0.03, 0.87	0.04, 0.84
Freeze Vigilant	-	-	-	0.78, <0.0001	0.17, 0.34	-0.11, 0.55	-0.20, 0.27	-0.12, 0.52	-0.37, 0.04	-0.20, 0.27	-0.36, 0.04
Freeze Non-Vigilant	-	-	-	-	-0.03, 0.88	-0.30, 0.09	-0.17, 0.36	-0.18, 0.34	-0.39, 0.03	-0.17, 0.36	-0.30, 0.10
Yawn	-	-	-	-	-	0.37, 0.03	0.24, 0.18	0.02, 0.90	0.25, 0.18	0.24, 0.18	0.31, 0.08
Teeth-grind	-	-	-	-	-	-	0.21, 0.24	0.45, 0.01	0.40, 0.02	0.21, 0.24	0.47, 0.007
Threat	-	-	-	-	-	-	-	0.56, 0.0009	0.28, 0.11	1.0, <0.0001	0.60, 0.0003
Partial Threat	-	-	-	-	-	-	-	-	0.36, 0.04	0.28, 0.11	0.34, 0.06
Open Mouth Threat	-	-	-	-	-	-	-	-	-	0.28, 0.11	0.34, 0.06
Cage Slap	-	-	-	-	-	-	-	-	-	-	0.60, 0.0003
Cage Shake	-	-	-	-	-	-	-	-	-	-	-

All variables are from the Stare condition unless otherwise noted.

Figure 4. Single linkage cluster analysis (joining) measuring Euclidian distances to assess similarity between HIT variables.



Smaller numbers indicate higher degree of closeness, with Non-Vigilant Freeze and Vigilant Freeze during the Profile epoch showing the highest degree of closeness. 12 variables assessed.

2.3 **Ethanol access**

2.3.1 *Induction procedure*

As indicated in **Table 1** and **Figure 2**, a schedule-induced polydipsia (SIP) procedure was used to induce ethanol self-administration (Grant et al., 2008a). Timing of the onset of induction following temperament testing varied due to schedule constraints. Briefly, the scheduled delivery of 1-g food pellets every 5 min (fixed-time 5 min) was used to induce rapid intake of an available fluid. During SIP induction, the monkeys were subjected to the FT-5 min schedule of pellet delivery until a specified volume of water or 4 % ethanol (w/v, in water) was consumed. Every 30 days, the dose of ethanol consumed was increased from 0 g/kg/day (water volume equivalent to 1.5 g/kg ethanol) to 0.5, 1.0, and finally to 1.5 g/kg/day. The monkeys were allowed up to 16 h to drink the specified volume of water or ethanol but normally finished between 5 min and 3 h (see Grant et al., 2008a for additional details). Following the 120 sessions of induction, “open-access” self-administration began and ethanol (4 % w/v) and water were concurrently available for 22 h/day, 12 pm–10 am. Control monkeys without ethanol access performed the same induction procedure with water only, and were then given “open-access” self-administration to water available in two spouts concurrently for 22 h/day on the same schedule as the drinkers. Monkeys with at least 20% of their daily average ethanol intake greater than 3.0 g/kg were defined as heavy drinkers, and monkeys with intakes below this threshold were defined as non-heavy drinkers (Baker et al., 2014). Rhesus subjects self-administered ethanol for approximately 12 months of ethanol access and cynomolgus subjects self-administered ethanol for approximately 6 months.

2.3.2 *Self-administration equipment*

Within each monkey's home cage, a drinking panel on one wall permitted access to all fluid and food. Drinking panels were controlled via a computerized system (Macintosh G4, Apple Computer Inc., Cupertino, CA, with National Instruments hardware and programming environment, National Instruments Corporation, Austin, TX). Each panel contained two drinking spouts: a set of three lights (red, white, and green) located below one of the spouts and a centrally located opening containing a dowel with an associated stimulus light. Each spout was connected by tubing to a 1–1 fluid reservoir and placed on a digital scale (Ohaus Navigator Balances N1B110, Ohaus Corporation, Pine Brook, NJ) interfaced to the computer system. Drinking volumes and patterns were acquired by using serial communication to retrieve changes in the weight of the fluid reservoirs.

2.3.3 *Assays*

Blood draws (3 ml) provided the plasma to be used for future assays. Blood ethanol concentrations (BECs) were measured using 20 µl of whole blood and headspace gas chromatography approximately every fifth day 7 h into the 22 h/day drinking session, resulting in approximately 66 observations per monkey during 12 months of ethanol self-administration and 33 observations per monkey during 6 months of ethanol access. BECs were also measured within one hour prior to the post-22-h access temperament test.

2.4 **Magnetic Resonance Imaging (MRI)**

2.4.1 *MRI acquisition*

Imaging was performed on a subset of animals during a single session for each animal subject on a 3T Siemens Tim Trio scanner with a 15-channel knee coil adapted for monkey head scanning. **Table 1** and **Figure 2** indicate the timelines for scans, behavioral testing, and drinking procedures for rhesus (n = 22) and cynomolgus (n = 11) subjects. A baseline scan prior to ethanol exposure and scans after approximately 6 months (cynomolgus and rhesus) and 12 months (rhesus only) of 22-h ethanol/water access were performed to assess longitudinal changes in rsFC.

Subjects were sedated with an initial dose of ketamine (15 mg/kg), intubated, and maintained with <1% isoflurane anesthesia for the duration of MRI procedures. Physiological monitoring throughout anesthesia included heart rate, respiration, and peripheral oxygen saturation. Data acquisition included four high-resolution T1-weighted structural images (TR = 3200 ms, TE = 497 ms; 0.5 mm^2 in plane resolution, 1 mm slice thickness, 56 slices, FOV = 128×128 mm), which were averaged to improve the signal-to-noise ratio. A functional MRI scan lasting 30 min commenced exactly 45 min after the time of ketamine administration (delaying the beginning of the acquisition as necessary to maintain the time from ketamine induction across all animals), using a gradient echo EPI sequence sensitive to BOLD contrast (TR = 2070 ms, TE = 25 ms, FA = 90°, 1.5 mm3 voxels, 32 slices with interleaved acquisition, FOV = 96 × 96 mm). A field map scan was acquired (TR = 450 ms, TE = 5.19 ms/7.65 ms, FA = 60°, 1.25 × 1.25 × 2 mm3 voxels, 40 slices, FOV = 120 × 120 mm) to correct for image distortion.

2.4.2 *MRI general preprocessing*

Standard preprocessing steps included slice-timing correction, correction for odd versus even slice intensity differences attributable to interleaved acquisition without gaps, rigid-body correction for head motion, and rigid-body coregistration of the fMRI volumes with the high-resolution T1-weighted structural image. Intensity normalization was applied to each run to a whole-brain mode value gradient of 1000. All data were also transformed using 12-paramater affine registration to conform to a T1-weighted atlas image that was an average of 112 monkeys (http://brainmap.wisc.edu/monkey.html) in the widely used F99 space. The registration parameters obtained from each step allowed raw fMRI images to be transformed into atlas space, combining motion correction, field map unwarping, and atlas transformation in one interpolation step. T1, DFM, and EPI masks were visually inspected and manually corrected using FSLView, software version 3.1.8, for each individual subject to ensure accuracy. Both species were processed with the same general process, with cynomolgus subjects typically requiring manual corrections.

2.4.3 *Functional connectivity preprocessing*

Functional connectivity preprocessing prepared data for connectivity analyses (Fox et al., 2005). These steps included spatial smoothing (3 mm full width at half maximum), regression of 24 motion parameters obtained by rigid body head motion correction (Satterthwaite et al., 2013; Yan et al., 2013; Power et al., 2014), regression of nuisance signals (ventricular, white matter, and whole-brain signal) and their first order derivatives, and temporal band-pass filtering (0.009 Hz < f < 0.08 Hz). Ventricular and

white matter masks were based on their corresponding regions in the INIA19 atlas (Rohlfing et al., 2012). As per Hallquist et al., (2013), the frequencies of nuisance regressors and fMRI data matched before nuisance regression, which was conducted before band-pass filtering. In addition, frame-by-frame spatial deviations of the acquisition time series were assessed using the temporal derivative of the time courses (i.e., frame displacement [FD]; Fair et al., 2012; Power et al., 2012). All analyses were conducted after the removal of frames with displacement FD > 0.2 mm.

2.4.4 *Functional connectivity maps*

A seed-based connectivity analysis was used to examine functional connectivity of the basal (BA), central (CeA), and lateral amygdalar (LA) nuclei (see **Figure 5** for visual representation of ROIs) with the dlPFC, dACC, and OFC cortices. Each ROI was defined with the INIA 19 template (Rohlfing et al., 2012). ROI MNI coordinates based on this atlas are shown in **Table 6**. Rs-fcMRI data was analyzed by correlating time courses of the amgydalar subregion BOLD signals with PFC voxels, generating a resting state functional connectivity map for each subject. The time course of each BOLD signal was averaged across the voxels within each amygdalar subnuclei ROI seed, and then correlated with the time course all other PFC region voxels. Correlation coefficients (r values) were exported in Matlab and transformed into Fisher Z scores to improve normality. The general process and steps are shown in **Figure 6**.

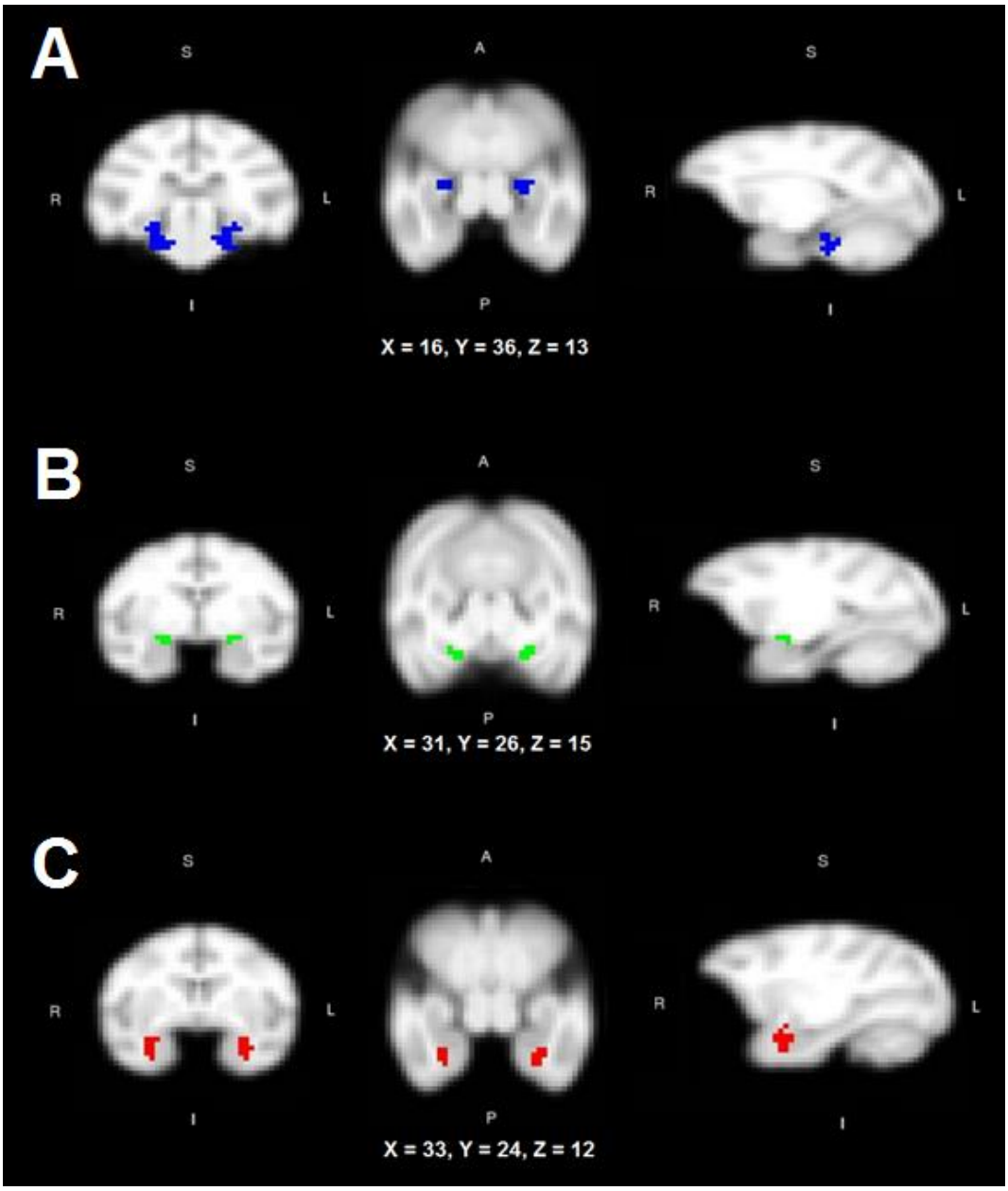
While the results of these studies report data taken from unilateral rsFC connections, a bilateral atlas collapsing across all four unilateral connections was also created and assessed in an attempt to reduce the likelihood of Type 1 Error by reducing

the number of comparisons performed in each seed analysis. However, further analysis revealed that bilateral amygdala-PFC connectivity did not reflect the underlying unilateral connectivity, and instead masked significant relationships. For this reason, results from the unilateral atlas and analyses are reported here. **Figure 7** shows all steps of data analysis and consolidation performed for both rs-fcMRI and behavioral data.

Table 6. Amygdala and prefrontal cortex ROIs and coordinates included in resting state functional connectivity analyses

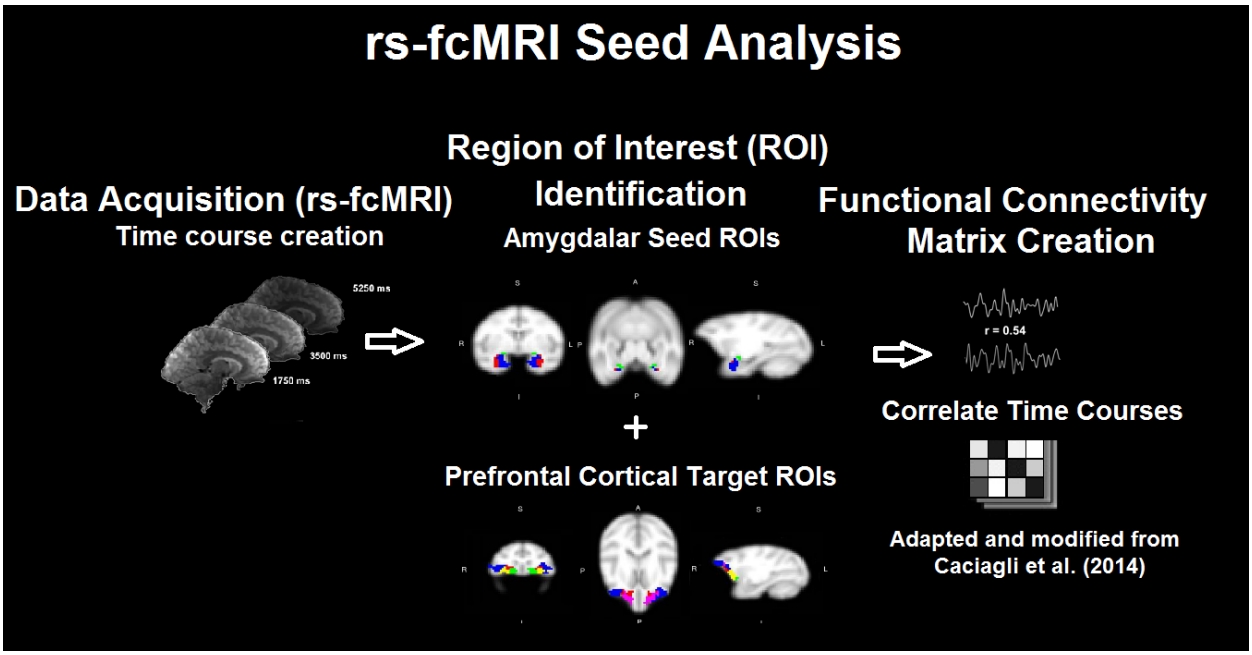
	ROI	MNI Coordinates (X, Y, Z)	Associated PFC Region
Left	11	11, 13, 7	Orbitofrontal Cortex (OFC)
	12	9, 13, 6	
	13	16, 10, 4	
	OPRO	16, -21, 6	
	OPAI	12, -21, 5	
	8(R)	17, 1, 7	Dorsolateral Prefrontal Cortex (dlPFC)
	9/46(V)	21, 0, 4	
	9	13, -23, 11	
	32	11, -5, -14	Dorsal Anterior Cingulate Cortex (dACC)
	Basal Amygdalar Nucleus (BA)	16, 10, 4	
	Central Amygdalar Nucleus (CeA)	14, 12, 4	
	Lateral Amygdalar Nucleus (LA)	3, -12, 9	
Right	11	-14, 11, 3	Orbitofrontal Cortex (OFC)
	12	-11, 10, 3	
	13	-11, -13, 14	
	OPRO	-3, -29, 7	
	OPAI	-6, -28, 5	
	8(R)	-19, -15, 10	Dorsolateral Prefrontal Cortex (dlPFC)
	9/46(V)	-18, -12, 10	
	9	-13, 3, 10	
	32	-8, -9, 16	Dorsal Anterior Cingulate Cortex (dACC)
	Basal Amygdalar Nucleus (BA)	4, -5, 12	
	Central Amygdalar Nucleus (CeA)	8, 2, 12	
	Lateral Amygdalar Nucleus (LA)	21, 0, 4	

Figure 5. Visual depiction of amygdalar subnuclei seed ROIs used for rsFC analyses.



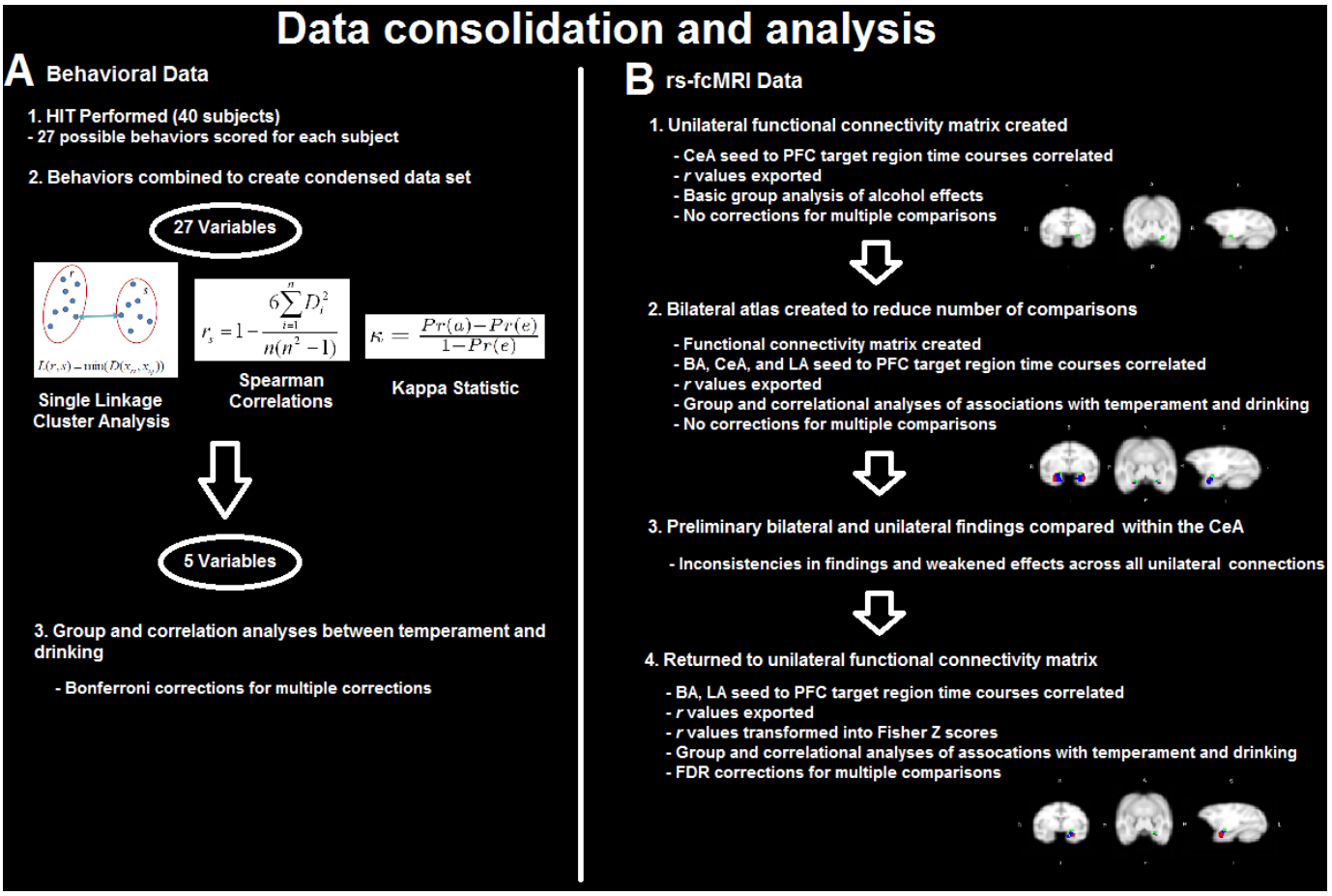
A basal lateral amygdalar nuclei (BA, blue), **B** central amygdalar nuclei (CeA, green) and **C** lateral amygdalar nuclei (LA, red) seeds. Shown bilaterally. Defined by the INIA19 atlas; as displayed in FSL with MNI space coordinates. S = Superior, I = Inferior, R = Right, L = Left, A = Anterior, P = Posterior.

Figure 6. Resting state functional connectivity MRI seed analysis steps.



Time courses acquired from the rs-fcMRI scans were correlated between the seed regions in the amygdala subnuclei (basal, blue; central, green; lateral, red) and the target regions in the prefrontal cortex (example shows orbital frontal cortex targets: areas 11, pink; 12, red; 13, blue; OPRO, yellow; OPAI, green). Functional connectivity matrices for each seed were created and the correlations between the time courses (*r* values) exported to Microsoft Excel from Matlab for data analysis.

Figure 7. Data consolidation and analysis steps.



A Behavioral data from the Human Intruder Test (HIT) was reduced via Spearman correlations, Kappa statistics, and Single Linkage Cluster Analyses and corrected for multiple comparisons with Bonferroni corrections. **B** Resting state functional connectivity (rs-fcMRI) data was first reduced with the creation of a bilateral atlas, but the unilateral functional connectivity matrix was used for the final results and corrected with FDR corrections.

2.5 **Statistical analysis**

2.5.1 *Aim 1: Behavioral predictors of heavy ethanol intake*

Prior to all analyses, distributional assumptions were tested. Durations of aggressive, anxious, and inhibited behavior were non-normally distributed and could not be transformed to meet assumptions, requiring the use of non-parametric statistical analyses. Independent variables included sex, temperament status at baseline (groups determined as described in Methods), and durations of anxious-like and aggressive-like behaviors at baseline. Dependent variables included daily ethanol intake (g/kg/day) and BEC (mg/dl) averaged across 12 months of 22-h access to ethanol, and behavioral change scores computed from post-22-h access temperament tests compared to baseline temperament tests.

Sex differences in the dependent variables were assessed with independent two-sample t tests, while sex differences in behavioral responses from the HIT were assessed with Mann-Whitney U tests. Group differences in ethanol self-administration (daily ethanol intake) and intoxication (BEC) based on baseline temperament (high aggression versus low aggression, etc.) were conducted with independent two-sample t tests. Multiple comparisons were corrected with the Bonferroni correction. Correlations between behavior and drinking variables were analyzed with partial Spearman's rank-order correlations controlling for sex. Correlations driven by a single data point were omitted. All analyses were conducted by using Statistica Academic with alpha values considered significant at $p \leq 0.05$.

2.5.2 *Aim 1: Behavioral consequences of heavy ethanol intake*

Distributional assumptions were tested for post-22-h durations of aggressive, anxious, and inhibited behaviors, which were again non-normally distributed and required the use of non-parametric statistical tests. Independent variables included sex, group (Control or Drinker), drinking status (Heavy or Non-Heavy drinker), and 12 month averages of ethanol intake and intoxication. Dependent variables included behavioral change scores computed from post-22-h access temperament tests compared to baseline temperament tests. Differences in the change in behavior from baseline were assessed with sign tests comparing baseline and post behavior durations separately in drinkers and controls, and Mann-Whitney U tests were used to compare behavioral change scores between ethanol and control subjects and heavy and non-heavy drinkers. Sex differences in the change in behavior were assessed with Mann-Whitney U tests. Multiple comparisons were corrected with the Bonferroni correction. Correlations driven by a single data point were omitted. All analyses were conducted by using Statistica Academic with alpha values considered significant at $p \leq 0.05$.

2.5.3 *Aim 2: Neural correlates of temperament*

Prior to all analyses, distributional assumptions were tested. Durations of aggressive, anxious, and inhibited behaviors were non-normal and could not be transformed to meet normality assumptions. Thus, non-parametric statistics were used in data analysis containing behavioral variables. Independent variables included temperament status, durations of anxious- and inhibited-like behaviors, and sex. Dependent variables included resting state functional connectivity (rsFC) between

amygdala (BA, CeA, and LA) and prefrontal cortex (dlPFC [9, 9/46, 8], dACC [32], and OFC [11, 12, 13, OPAI, OPRO]) for a total of 9 PFC ROIs assessed per amygdala sub nuclei seed.

Sex differences in the dependent variables and independent variables were assessed with independent Two Sample t-tests (for normally distributed variables) or Mann Whitney U-tests (for non-normally distributed variables). If group variances were significantly unequal (as assessed via an F-test), separate variance estimates were used. All other statistical analyses were performed across sex. Group differences in rsFC compared by temperament (high versus low anxiety, etc.) were conducted with independent Two Sample t-tests (for normally distributed variables) or Mann Whitney U-tests (for non-normally distributed variables). If group variances were significantly unequal (as assessed via an F-test), separate variance estimates were used. Correlations between rsFC data and temperament data were analyzed with partial Spearman's rank order (for non-normally distributed variables) correlations controlling for sex. Partial Spearman's correlations were assessed with SAS Studio (University Edition). All other analyses were conducted using Statistica Academic with alpha values considered significant at $p \leq 0.05$. Multiple comparisons were corrected using the false discovery rate (FDR) method (Bejamini et al., 1995). Corrections were performed to account for 36 total group comparisons or correlations per amygdalar seed analysis within each temperament category. Where appropriate, trends were reported at uncorrected $p < 0.003$.

2.5.4 *Aim 3: Neural correlates of future heavy ethanol intake*

Prior to all analyses, distributional assumptions were tested. All variables met assumptions, and parametric statistical analyses were used. Independent variables included resting state functional connectivity (rsFC) between amygdala (BA, CeA, and LA) and prefrontal cortex (dlPFC [9, 9/46, 8], dACC [32], and OFC [11, 12, 13, OPAI, OPRO]) for a total of 9 PFC ROIs assessed per amygdala sub nuclei. Dependent variables included average daily intake of ethanol (g/kg/d), average BEC (mg/dl), and drinking status (Heavy or Non-Heavy drinker).

Sex differences in the dependent variables and independent variables were assessed with independent Two Sample t-tests. If group variances were significantly unequal (as assessed via an F-test), separate variance estimates were used. All other statistical analyses were performed across sex. Group differences in rsFC compared by future drinking status (heavy v. non-heavy) were conducted with independent Two Sample t-tests. If group variances were significantly unequal (as assessed via an F-test), separate variance estimates were used. Correlations between rsFC data and drinking data were analyzed with partial linear Pearson's product-moment correlations controlling for sex. Correlations driven by a single data point were omitted. Partial Spearman's correlations were assessed with SAS Studio (University Edition). All other analyses were conducted using Statistica Academic with alpha values considered significant at FDR corrected $p \leq 0.05$. Where appropriate, trends were reported at uncorrected $p < 0.003$.

2.5.5 *Aim 3: Neural correlates of past heavy ethanol intake*

Prior to all analyses, distributional assumptions were tested. The change in duration of aggressive, anxious, and inhibited behaviors from baseline to post-22-h access were non-normal and could not be transformed to meet normality assumptions, thus requiring the use of non-parametric statistics. Independent variables included drinking status (Heavy or Non-Heavy drinker), group (Control or Drinker), 6 and 12 month averages of ethanol intake and intoxication, and behavioral change scores computed from post 22-h access temperament tests compared to baseline temperament tests. Dependent variables included the percent change in resting state functional connectivity (rsFC) between amygdala (BA, CeA, and LA) and prefrontal cortex (dlPFC [9, 9/46, 8], dACC [32], and OFC [11, 12, 13, OPAI, OPRO]) from baseline to post 6 months and 12 months of 22-h ethanol/water access. Correlations between rsFC change data and past drinking data were analyzed with partial linear Pearson's product-moment correlations controlling for sex. 6 and 12 month drinking and MRI data were compared in separate analyses. Finally, the change in behavior from baseline to post-12 months of 22-h access were correlated with the change in rsFC from baseline to post-12 months of 22-h access with partial linear Pearson's product-moment correlations controlling for sex and group (control or drinker). Correlations driven by a single data point were omitted. Partial spearman's correlations were assessed with SAS Studio (University Edition). All other analyses were conducted using Statistica Academic with alpha values considered significant at FDR corrected $p \leq 0.05$. Where appropriate, trends were reported at uncorrected $p < 0.003$.

2.5.6 *Aim 4: Species differences*

The analyses from Aim 1-3 were repeated in a cohort of cynomolgus macaques. This provided an opportunity to replicate the results of Aims 1-3 in an entirely new dataset. All analyses were as described above in each Aim. However, the rs-fcMRI data consolidation steps described in **Figure 7** did not include the preliminary attempts of Steps 1-3, and only involved the final consolidation process described in Step 4. Additionally, cynomolgus subjects did not freeze during the Stare period, and thus only freezing during the Profile was included in each analysis. Standard correlations were performed rather than partial correlations, given the inclusion of only male cynomolgus subjects. An additional analysis of species differences in behavior and amygdala-PFC rs-fcMRI was performed between Cohort 10 (male rhesus macaques, n = 16) and cohort 9 (male cynomolgus macaques, n = 11). Cohort 6B was not including in this analysis to remove the covariate of sex. Correlations between intoxication at the time of post-22-h HIT and changes in behavior from baseline were not assessed due to the lack of individual variability in intoxication.

CHAPTER 3: Results

3.1 Aim 1: Behavioral correlates heavy ethanol intake

3.1.1 *Temperament group characteristics*

Aggressive behavior was observed in 14 out of 16 female monkeys and 14 out of 32 male monkeys. A quartile approach was used to characterize subjects as high and low aggressive, anxiety, and inhibition, as described in the methods. Based on the aggressive behaviors exhibited during the HIT, 8 female and 3 male monkeys were characterized as high aggression and 2 female and 17 male monkeys were characterized as low aggression. Anxious behavior was observed in 9 female and 14 male monkeys. Based on the active anxious behaviors exhibited during the HIT, 4 female and 7 male monkeys were characterized as high anxiety and 7 female and 18 male monkeys were characterized as low anxiety. Freezing was observed in 25 male and 15 female monkeys. Based on the freezing behaviors exhibited during the HIT, 6 female and 5 male monkeys were characterized as high inhibition and 2 female and 10 male monkeys were characterized as low inhibition. The percentage of subjects falling into “low” categories exceeded 25% in cases with more than a quarter of the subjects eliciting no anxious, inhibited, or aggressive behaviors. **Table 7** indicates individual characterization of each monkey.

Table 7. Average daily intake of ethanol (4% w/v) and BEC (mg/dl) during 22-h ethanol access and anxious/aggressive status at baseline.

Sex	Cohort	Monkey	12 Month Intake (g/kg/d)	12 Month BEC(mg/dl)	Test BEC (mg/dl)*	HIT Aggression	HIT Active Anxiety	HIT Inhibition
Female	6A	97[a]	5.1	99.3	-	High	High	-
	6A	58[a]	4.9	80.7	-	-	-	High
	6A	35[a]	4.0	61.0	-	Low	Low	-
	6A	31[a]	3.9	66.5	-	-	-	High
	6A	85[a]	3.9	49.1	-	High	-	-
	6B	18[a]	3.9	111.2	-	High	High	High
	6A	34[a]	3.3	41.4	-	High	Low	-
	6B	46[a]	2.8	33.9	-	Low	Low	High
	6B	07	1.0	6.1	-	-	Low	-
	6B	26	1.7	7.7	-	High	-	High
	6B	39	1.3	11.1	-	-	Low	High
	6B	64	CONTROL	CONTROL	CONTROL	High	-	Low
	6B	79	CONTROL	CONTROL	CONTROL	-	Low	Low
Male	10	42[a]	4.2	167.3	80	High	-	-
	7A	68[a]	3.3	95.2	-	Low	Low	-
	10	82[a]	3.1	46.1	26	-	High	-
	7A	82[a]	3.1	72.7	-	Low	Low	High
	7A	48[a]	3.0	75.0	-	Low	Low	-
	7A	87[a]	2.8	67.4	-	Low	Low	-
	7B	54[a]	2.4	100.4	-	Low	-	-
	10	02[a]	2.3	41.3	0	Low	-	-
	10	41	2.4	60.6	29	High	High	-
	7B	25	2.3	68.7	-	-	Low	Low
	7A	42	2.3	33.9	-	Low	-	High
	10	56	2.3	52.9	26	Low	Low	High
	7B	40	2.1	60.4	-	-	Low	Low
	10	12	2.1	45.0	0	Low	Low	Low
	7A	90	2.0	32.7	-	Low	Low	-
	7B	84	1.8	56.8	-	-	Low	-
	7A	16	1.9	20.5	-	Low	Low	High
	7A	11	1.8	35.9	-	Low	Low	High
	10	58	1.5	17.7	0	Low	-	-
	7B	57	1.4	48.2	-	Low	High	-
	10	09	1.3	7.8	0	Low	Low	Low
	10	21	CONTROL	CONTROL	CONTROL	-	Low	Low
	10	46	CONTROL	CONTROL	CONTROL	High	High	Low
	10	19	CONTROL	CONTROL	CONTROL	-	High	Low
	10	19	CONTROL	CONTROL	CONTROL	-	High	Low
	10	68	CONTROL	CONTROL	CONTROL	-	Low	-
	10	34	CONTROL	CONTROL	CONTROL	-	-	-
	10	75	CONTROL	CONTROL	CONTROL	-	-	Low
	10	97	CONTROL	CONTROL	CONTROL	-	High	Low

[a] Indicates that the monkey was a heavy drinker, as defined by drinking >3.0 g/kg on at least 20% of days throughout 22-h access.

- Indicates that the monkey was neither high nor low and fell in the middle 50% of all subjects.

* Only 8 of the 32 drinkers were assessed for intoxication at time of second HIT

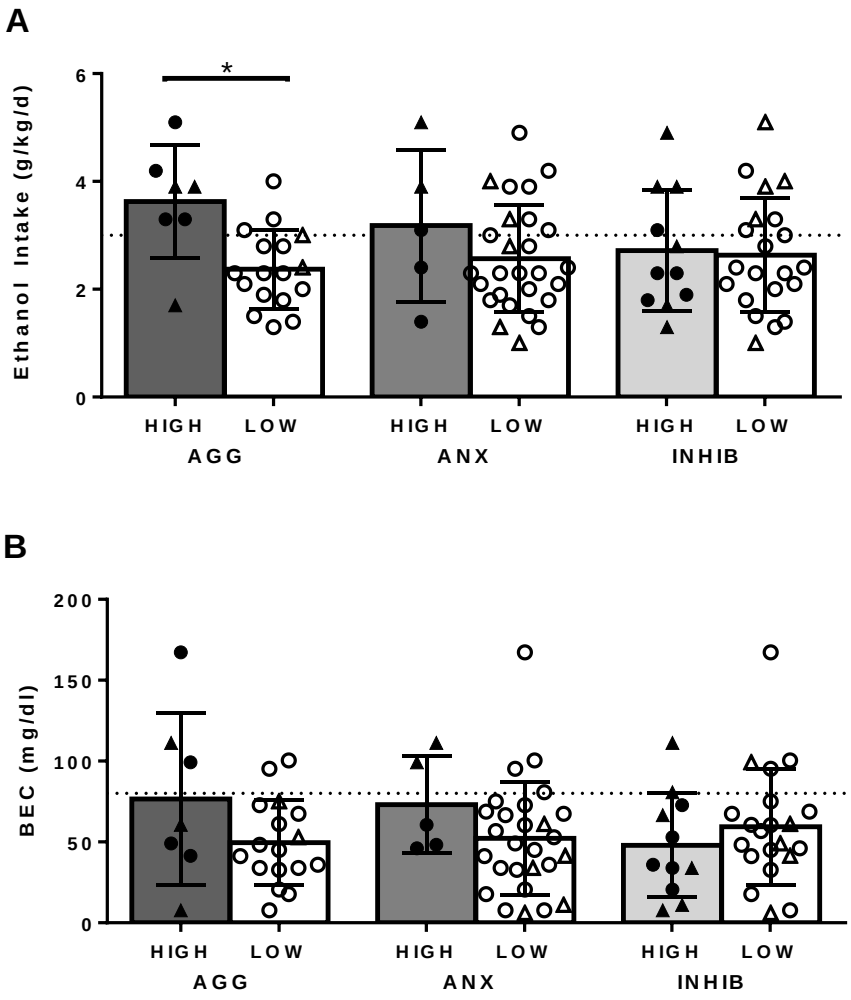
CONTROL indicates control monkeys without ethanol access.

3.1.2 *Baseline temperament and future heavy ethanol intake*

Higher aggression was significantly associated with future heavier ethanol self-administration. Specifically, high aggression monkeys (n = 11) self-administered significantly more ethanol (3.5 versus 2.4 g/kg/day, t_{22} = 2.9, *p* = 0.008, Cohen's *d* = 1.1, *r* = 0.5, **Figure 8A**) and achieved non-significantly higher BECs (77 versus 50 mg/dl, t_{22} = 1.7, *p* = 0.10, Cohen's *d* = 0.6, *r* = 0.3 Fig. 7B) compared to low aggression monkeys (n = 19). Conversely, no significant differences in ethanol intake or BECs were observed between high (n = 11) and low anxiety (n = 25) monkeys or between high inhibition (n = 11) and low inhibition (n = 12) monkeys (**Figure 8A-B**) (all *p*'s < 0.05/6, Bonferroni corrected for multiple comparisons).

Correlations between baseline behavior and future drinking measures showed a similar relationship, with aggression emerging as a distinct correlate of future heavy ethanol consumption. Across all drinkers (n = 32), baseline duration of extreme aggressive behavior positively correlated with future average daily ethanol intake (r_s = 0.49, *p* = 0.0047) and correlated at a trend level with future average BECs attained (r_s = 0.39, *p* = 0.032, **Table 8**). No significant correlations between durations of anxious or inhibited behavior and ethanol intake-related variables were observed (**Table 6**), and high active anxiety or behavioral inhibition status did not significantly increase risk for heavy drinking (**Table 9**) (all *p*'s < 0.05/8, Bonferroni corrected for multiple comparisons). Finally, the relative risk of heavy drinking was higher at a trend level (*p* = 0.06) in high aggression versus low/mid aggression monkeys, with high aggression monkeys at 100% more at risk for becoming heavy drinkers than low/mid aggression monkeys (**Table 9**).

Figure 8. Average daily intake and BEC by temperament group.



Mean ± SD of **A** daily ethanol intake and **B** BEC over 12 months of 22-h ethanol access plotted by temperament group. Mean daily ethanol intake (g/kg/ day) calculated from an average of 351 days of self-administration/monkey/cohort (range 336–384 days). Average BEC (mg/dl) calculated from 63 samples/monkey/cohort (range 59–67 samples). Individual monkeys within each group depicted with *circles* (males) or *triangles* (females). *$p \leq 0.05$ (Bonferroni corrected), statistically significant group difference.

Table 8. Spearman's partial rank-order correlations (r_s, p) describing the relationship between ethanol intake and intoxication during 22-h ethanol access and aggressive-like, anxious-like, and behaviorally inhibited behaviors during temperament testing.

HIT Variables	Ethanol Intake (g/kg/d)	BEC (mg/dl)
Freeze Profile	0.00, 0.98	0.03, 0.87
Freeze	0.16, 0.39	-0.05, 0.77
Active Anxiety	0.30, 0.11	0.27, 0.14
Open Mouth	0.12, 0.53	0.23, 0.22
Extreme Aggression	0.49, 0.005[a]	0.39, 0.032[b]

r_s values correspond to a Spearman partial rank-order correlation controlling for sex;

[a]significant at Bonferroni corrected $p < 0.05$, [b]trend at uncorrected $p < 0.05$

Behaviors observed during the Stare condition unless otherwise noted.

Table 9. Drinking outcomes compared by baseline temperament

Rhesus Monkey Cohorts	Heavy Drinkers (n = 16)	Non-Heavy Drinkers (n = 16)	Relative Risk	
Characteristic	Percent	Percent	Value (95% CI)	*p*
High Aggression (n = 7)	31.3	12.5	2.0 (1.0 – 4.0)	0.06
High Anxiety (n = 5)	18.8	12.5	1.4 (0.6 – 3.1)	0.48
High Inhibition (n = 16)	31.3	68.8	0.83 (0.3 – 2.2)	0.72

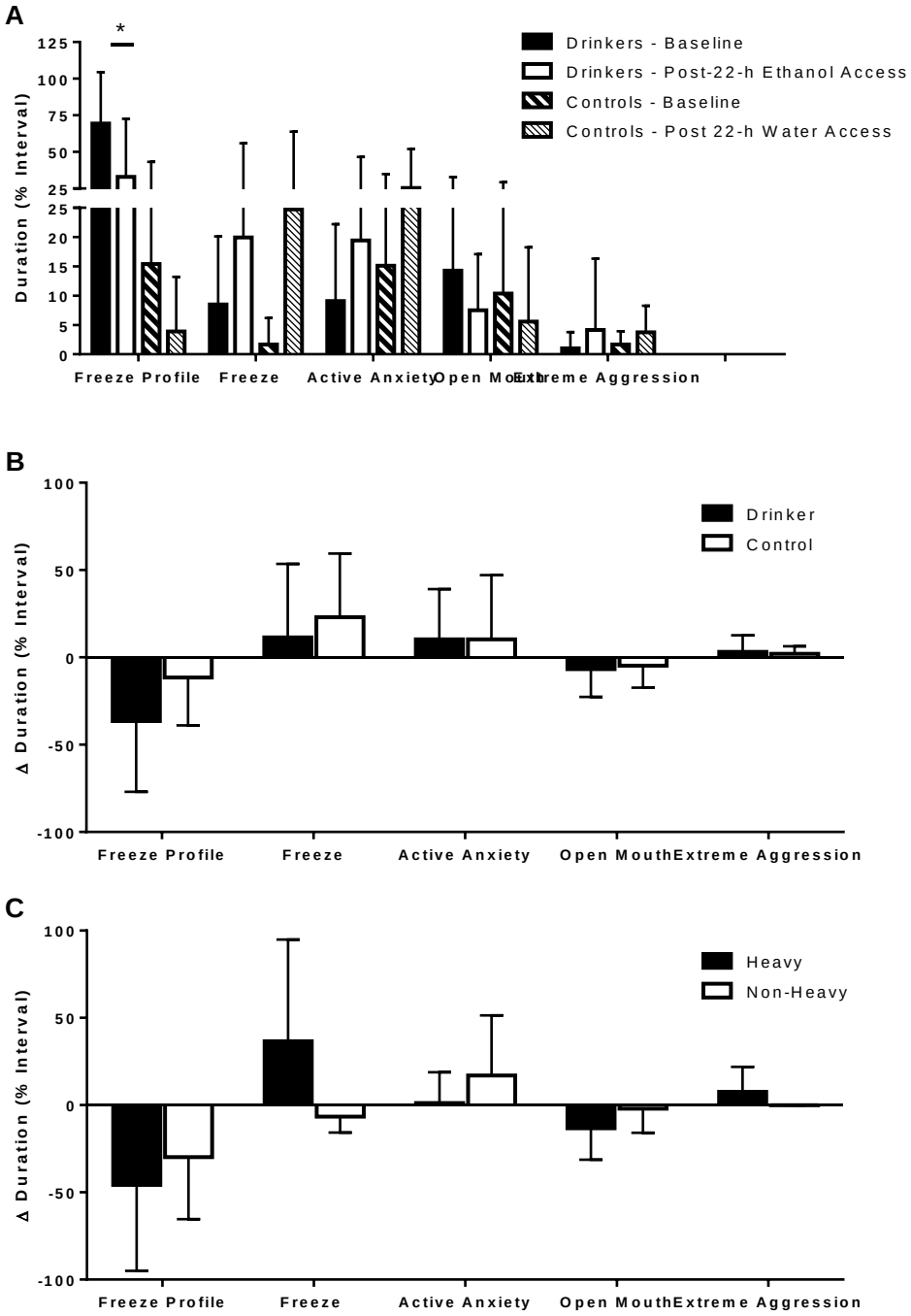
3.1.3 *Post temperament and heavy ethanol intake*

In a subset (n = 22) animals, post 22-h access behavioral tests were performed and compared to baseline behavior. Baseline and post-22-h access durations of behavior were compared within drinkers and controls with sign tests due to the non-normal nature of the behavioral variables. Monkeys with access to ethanol (n = 12) displayed significantly decreased freezing in response to the Profile after 12 months of ethanol access (Z = 2.58, p = 0.0098, Number of Non Ties = 15, Percent v < V = 13.33, Cohen's d = 1.2, r = 0.5, **Figure 9A**). No other trends or significant changes in aggressive- or anxious-like or inhibited behaviors were observed. As expected, control monkeys without access to ethanol (n = 10) did not exhibit any significant longitudinal changes in behavioral responses.

Change scores from baseline to post-22-h access were compared between drinkers and controls and between heavy (n = 5) and non-heavy (n = 7) drinkers with Mann Whitney U tests. Controls and drinkers did not significantly differ in behavioral changes observed (**Figure 9B**). Heavy and non-heavy drinkers did not significantly differ in behavioral changes observed (**Figure 9C**).

Finally, behavioral change scores were correlated with prior ethanol intake in the subjects with prior access to ethanol (n = 32) using Spearman correlations controlling for sex. The change in open mouth threat (an aggressive behavior) was negatively correlated with ethanol intake correlated with prior average ethanol intake, prior average intoxication, and intoxication at the time of test (r_s = -0.66, p = 0.027; r_s = -0.65, p = 0.032, r_s = -0.88, p = 0.0043; respectively; **Table 10**). No other behavioral changes correlated with prior ethanol intake and intoxication or intoxication at the time of test.

Figure 9: Changes in anxiety and aggression after self-administration in drinkers and controls.



Mean ± SD of **A** behavior duration at baseline HIT and post-22-h access HIT, **B** change in behavior durations compared between drinkers and controls, and **C** heavy and non-heavy drinkers. Duration presented as a % of the interval, change scores calculated as post score – baseline score. *$p \leq 0.05$ (Bonferroni corrected), statistically significant group difference. Behaviors observed during the Stare condition unless otherwise noted.

Table 10. Spearman's partial rank-order correlations (r_s, p) describing the relationship between ethanol intake and intoxication during 22-h ethanol access and change in aggressive-like, anxious-like, and behaviorally inhibited behaviors from baseline to post-22-h access temperament testing.

HIT Variables	Ethanol Intake (g/kg/d)	BEC (mg/dl)	Test BEC (mg/dl)*
ΔFreeze Profile	-0.08, 0.80	0.21, 0.51	-0.24, 0.56
ΔFreeze	0.15, 0.63	0.16, 0.62	0.16, 0.71
ΔActive Anxiety	-0.29, 0.39	-0.17, 0.62	-0.50, 0.21
ΔOpen Mouth	-0.66, 0.027[b]	-0.65, 0.032[b]	-0.88, 0.004[a]
ΔExtreme Aggression	0.18, 0.60	0.07, 0.84	-0.44, 0.28

r_s values correspond to a Spearman partial rank-order correlation controlling for sex;

[a]significant at Bonferroni corrected $p < 0.05$, [b]trend at uncorrected $p < 0.05$

*Only 8 of the 32 drinkers were assessed for intoxication at time of second HIT

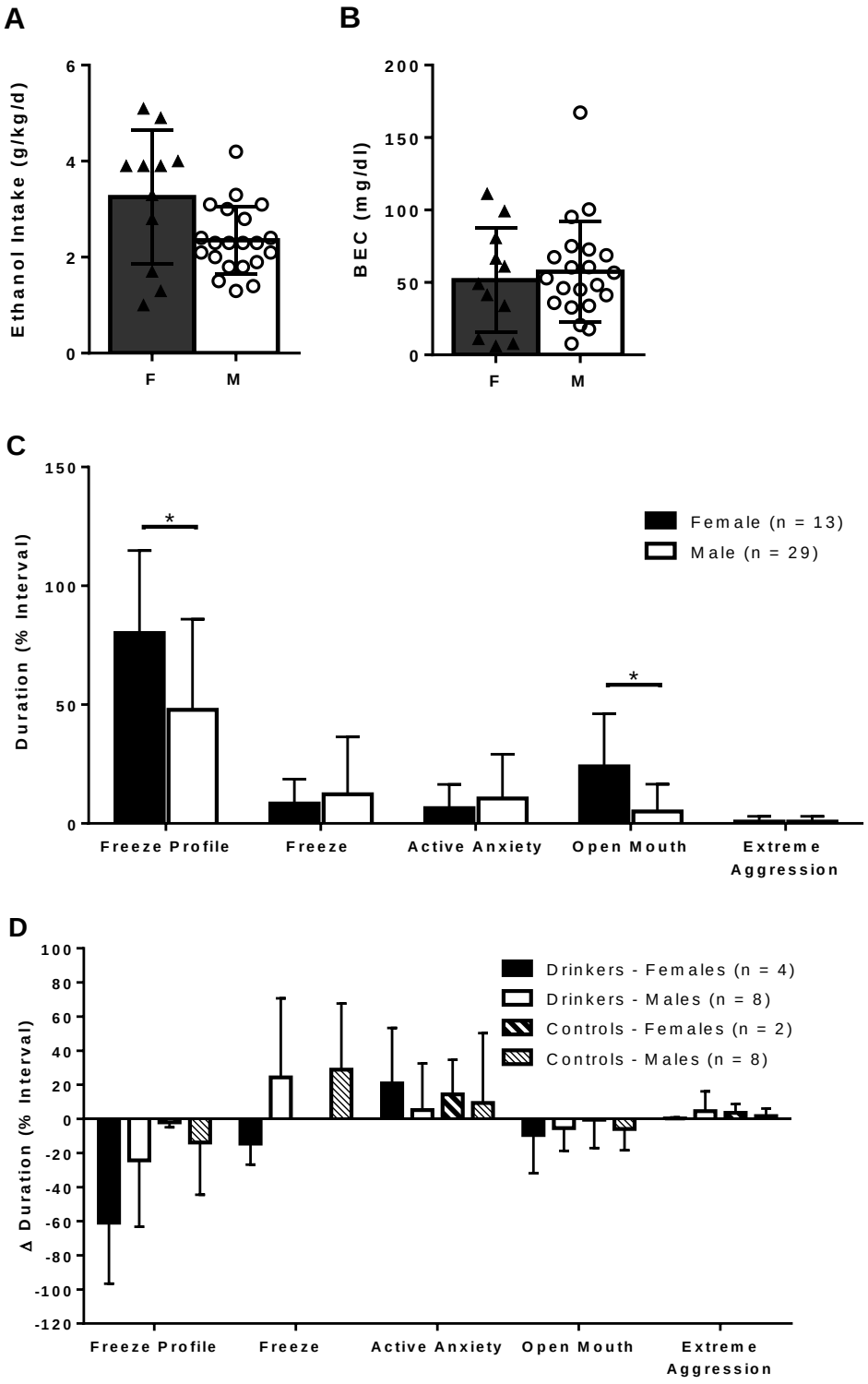
Δchange in behavior from baseline temperament test to post-22-h access temperament test (post duration – pre duration).

Behaviors observed during the Stare condition unless otherwise noted.

3.1.4 *Sex differences in ethanol consumption and temperament*

Ethanol self-administration and BECs attained by female (n = 11) and male (n = 21) monkeys did not significantly differ, though female ethanol intake was non-significantly higher (3.3 versus 2.4 g/kg/day, $t_{12.75} = 2.0$, $p = 0.062$, Cohen's $d = 0.8$, $r = 0.4$, **Figure 10A-B**). Across both drinkers and controls, female monkeys (n = 13) reacted significantly more to the HIT stimuli than male monkeys (n = 29), with significantly longer durations of freezing during the profile phase of the HIT (80.1 versus 45.0% of the interval, U = 75.0, z = 3.3, $p = 0.0009$, Cohen's $d = 0.9$, $r = 0.4$, **Figure 10C**) and open mouth threat during the stare phase of the HIT (23.3 versus 4.7% of the interval, U = 79.0, z = 3.2, $p = 0.001$, Cohen's $d = 1.0$, $r = 0.5$, **Figure 10C**). No sex differences in the change in behavior from baseline to post-22-h access were found in drinker or control monkeys (**Figure 10D**).

Figure 10. Sex differences in ethanol intake and behavior.



Mean ± SD of (A) daily ethanol intake and (B) BEC over 12 months of 22-h ethanol access and mean ± SD of individual behavior durations during baseline HIT (C) and change in behavior durations (D) plotted by sex. Mean daily ethanol intake (g/kg/day) calculated from an average of 351 days of self-administration/monkey/cohort (range 336– 384 days). Average BEC (mg/dl) calculated from 63 samples/monkey/ cohort (range 59–67 samples). Individual monkeys within each group plotted with *circles* in A and B. *$p \leq 0.05$ (Bonferroni corrected), statistically significant group difference. Behaviors observed during the Stare condition unless otherwise noted.

3.1.5 *Summary: Aim 1 results*

Overall, aggressive but not anxious or inhibited behavior at baseline was associated with heavier future drinking, partially supporting the hypothesis that higher baseline levels of aggression and anxiety would be predictive of heavier drinking. Although anxiety and inhibition were not significantly predictive of heavier future ethanol intake or intoxication, active anxiety was relatively rare in this sample, and highly anxious animals subsequently self-administered (non-significantly) more ethanol. Conversely, chronic ethanol self-administration decreased freezing (behavioral inhibition) in a non-dose-dependent manner, while aggression decreased dose-dependently. No significant alterations in behavior were observed in control subjects without ethanol access. These results again partially support the initial hypothesis, which posited that heavier drinking would increase aggression but decrease anxiety. While increased aggression was not observed, this could be due to the timing of testing, which occurred while some animals were still intoxicated (**Table 7**), but on the descending arm of intoxication. Finally, female and male subjects were largely similar in responses to the HIT and drinking patterns, although females generally responded to the HIT for longer durations and consumed more ethanol. Sex does not appear to influence the relationship between temperament and ethanol self-administration.

3.2 Aim 2: Neural correlates of temperament

3.2.1 *Temperament characterization*

As indicated in the methods and in section 3.1.1, a quartile approach was used to characterize temperament groups. 22 of the 32 subjects included in Aim 1 were scanned with rs-fcMRI, resulting in the following group characteristics. 27.3% of the subjects (6/22, female n = 3, male n = 3) were placed in the high aggression group, 27.3% of the subjects (6/22, female n = 1, male n = 5) were placed in the low aggression group, 31.8% of the subjects (7/22, female n = 1 female/6 males) were placed in the high anxiety group, 36.4% of subjects (8/22, 3 females/5 males) were placed in the low anxiety group, 18.2% of subjects (4/22, 3 females/1 male) were placed in the high inhibition group, and 45.5% of subjects (10/22, 2 females/8 males) subjects were placed in the low inhibition group. See **Table 11** for behavioral characteristics of each group. As indicated in section 3.1.1, the percentage of subjects falling into “low” categories exceeded 25% in cases with more than a quarter of the subjects eliciting no anxious, inhibited, or aggressive behaviors.

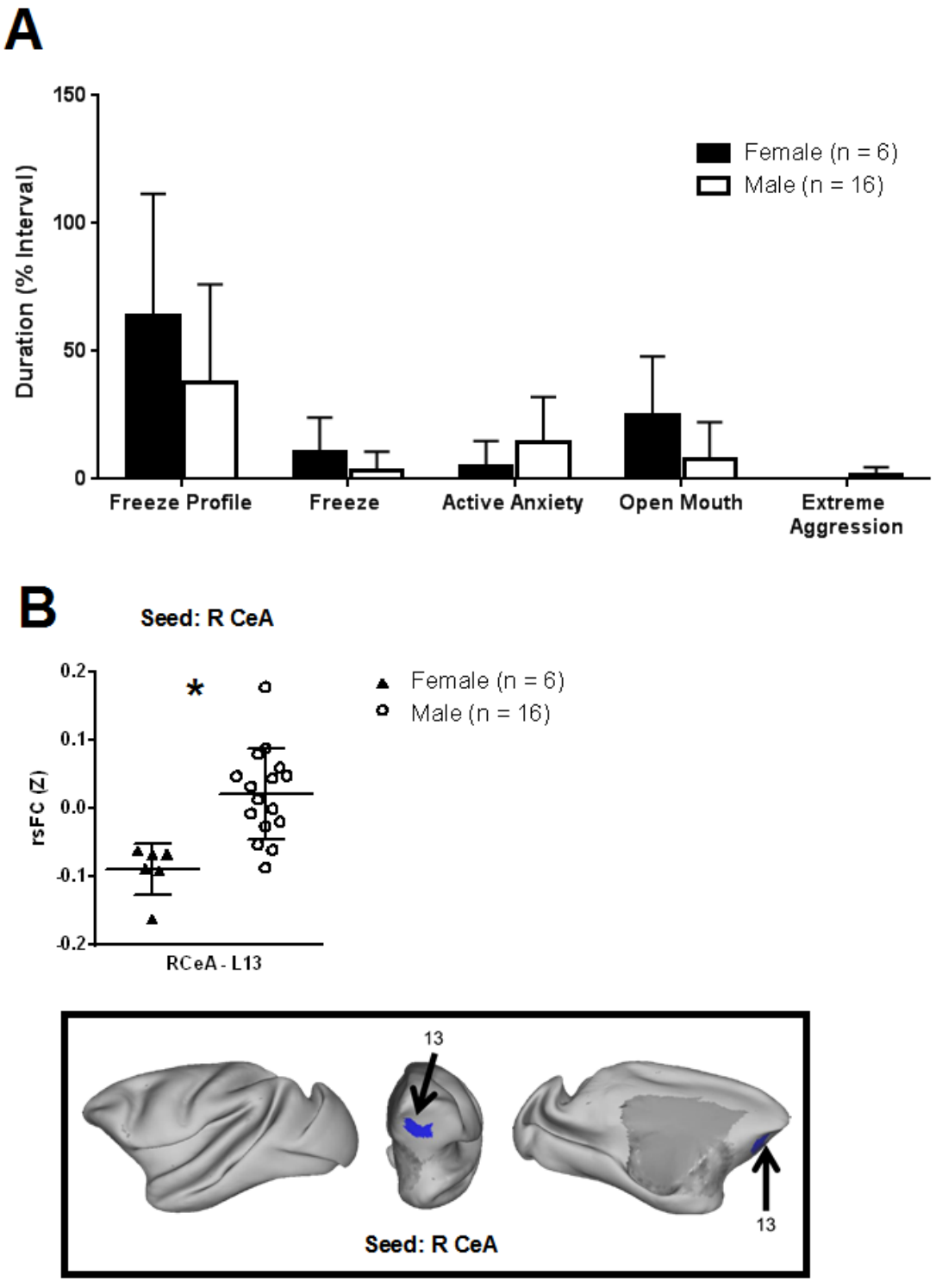
Table 11. Temperament group characteristics by sex. All measures are expressed as the mean ± standard deviation.

Status	Sex	Freezing (Total) Duration (% Interval)	Active Anxiety Duration (% Interval)	Aggression Duration (% Interval)
High Aggression (n = 6)	Female (n = 3)	76.75 ± 63.53	9.93 ± 13.16	44.79 ± 8.52
	Male (n = 3)	55.34 ± 48.58	22.24 ± 16.27	38.48 ± 16.08
Low Aggression (n = 6)	Female (n = 1)	119.62	0.00	0.00
	Male (n = 5)	53.73 ± 52.59	1.64 ±2.25	0.00 ± 0.00
High Anxiety (n = 7)	Female (n = 1)	121.9	25.09	46.16
	Male (n = 6)	24.79 ± 53.65	32.28 ± 17.25	14.62 ± 13.24
Low Anxiety (n = 8)	Female (n = 3)	72.16 ± 63.52	0.00 ± 0.00	5.00 ± 8.64
	Male (n = 5)	43.53 ± 53.65	0.00 ± 0.00	1.00 ± 2.05
High Inhibition (n = 4)	Female (n = 3)	115.26 ± 9.60	9.45 ± 13.64	27.28 ± 24.20
	Male (n = 1)	120.29	0.00	0.00
Low Inhibition (n =10)	Female (n = 2)	2.05 ± 2.90	0.72 ± 1.02	26.28 ± 37.14
	Male (n = 8)	4.10 ± 9.26	17.72 ± 21.48	8.00 ± 12.47

3.2.2 *Sex differences*

No behavioral differences were observed between male and females in this subset of 22 subjects with MRI scans. However, females generally exhibited longer durations of aggressive, anxious, and inhibited behaviors (**Figure 11A**) as observed in the larger sample (**Figure 10C**). Females also generally exhibited more strongly anticorrelated rsFC between the amygdala and PFC than males. Despite this general pattern, the only significant sex difference in rsFC was between the right CeA and left area 13 (OFC), which was significantly more anticorrelated in females (n = 6) than in males (n = 16) (0.02 v. -0.09, $t_{20} = 3.8$, $p = 0.0010$, Cohen's $d = 1.9$, $r = 0.7$, **Figure 11B**). No significant sex differences in rsFC were observed between BA or LA and PFC. All significant p's < 0.05 FDR corrected for multiple comparisons.

Figure 11. Sex differences between male and female rhesus macaques at baseline.



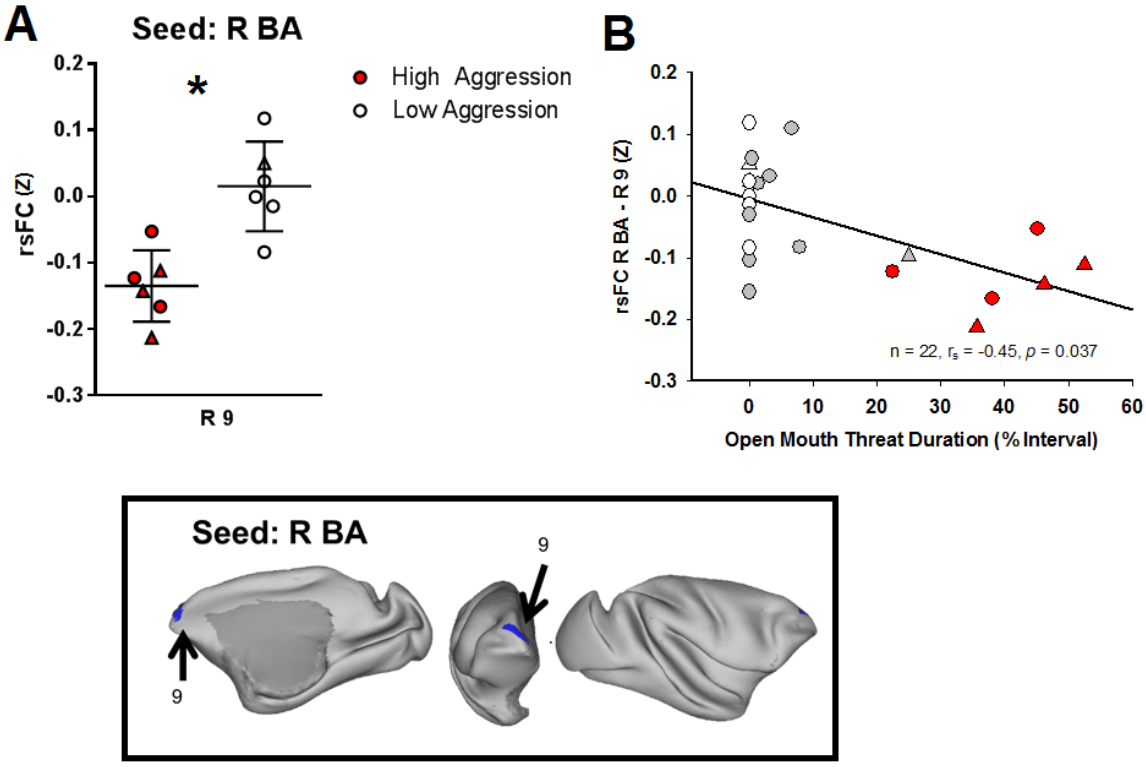
A. Non-significant sex differences in behavior observed during the HIT. **B.** Significant sex difference in amygdalocortical connectivity at rest. Significantly more anticorrelated connectivity between the left area 13 (OFC) and right CeA in female than male subjects. Markov atlas defined left area 13 depicted in blue (OFC) – brain region associated with more strongly anticorrelated rsFC with the right CeA in female subjects.

*$p \leq 0.05$ (FDR corrected), statistically significant group difference.

3.2.3 *Aggression and amygdala-PFC rsFC*

High aggression subjects (n = 6) exhibited significantly more anticorrelated rsFC between the right BA and right area 9 (dlPFC) than low aggression subjects (n = 6) (-0.13 v. 0.02, $t_{10} = 4.3$, $p = 0.0017$, Cohen's $d = 2.4$, $r = 0.8$, **Figure 12A**), but the duration of aggression did not significantly correlate with right BA to right 9 rsFC after FDR corrections for multiple comparisons ($r_s = -0.45$, 0.037, **Figure 12B**). No other significant differences in rsFC were observed between high and low aggression subjects, with no significant group differences between the CeA/LA and PFC observed. The duration of aggressive behavior did not significant correlate with rsFC between any amygdalar seeds and the PFC. All significant p's < 0.05 FDR corrected for multiple comparisons.

Figure 12. Association between aggression and amygdalocortical connectivity at rest in rhesus macaques.

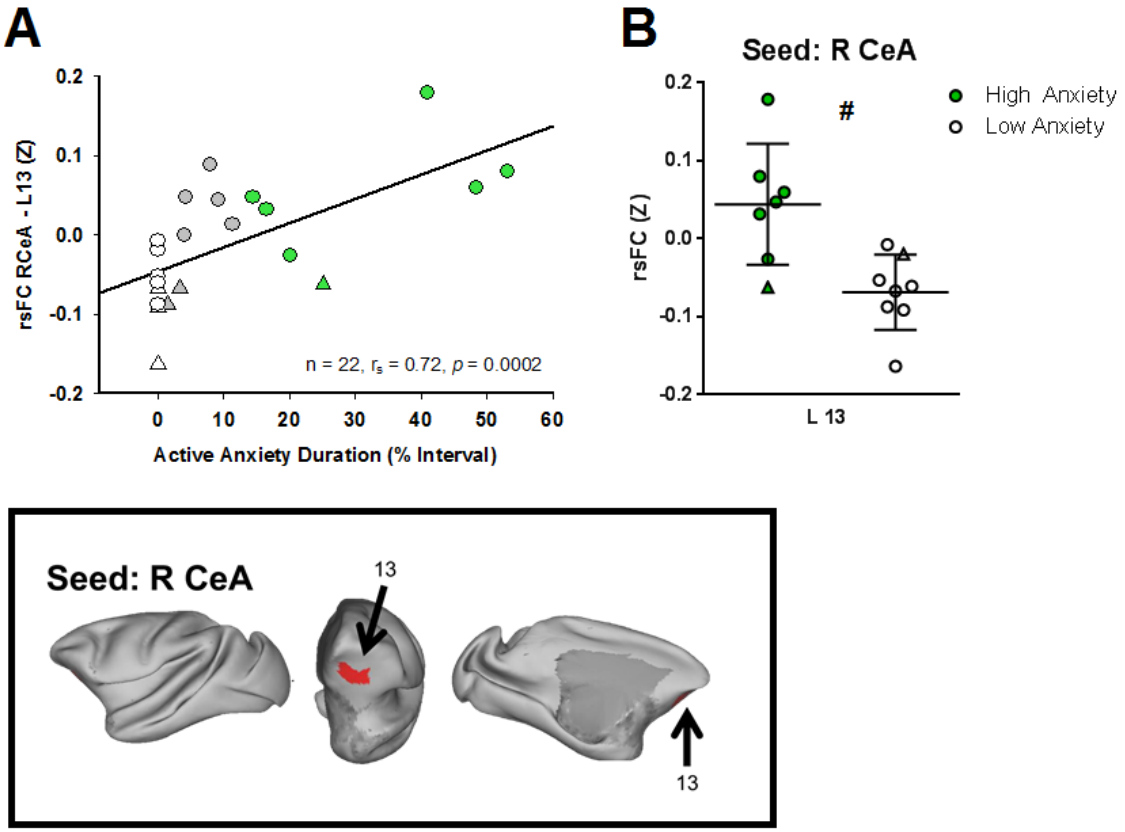


A. Significant group difference in right BA to right area 9 rsFC between high and low aggression subjects **B.** Scatter plot depicting the non-significant (after FDR correction) correlation between baseline duration of open mouth threat (aggression) duration as a percent of the stare interval and baseline rsFC between right area 9 and right BA. Markov atlas defined right area 9 (dlPFC, blue) –brain region associated with stronger anticorrelated rsFC with the right BA in subjects with longer durations of aggression. Scatter plot point color indicates subjects characterized as high aggression (red), low aggression (white), and neither high nor low aggression (grey). Shape indicates sex, with females depicted as triangles and males as circles. *$p \leq 0.05$ (FDR corrected), statistically significant group difference.

3.2.4 *Active anxiety and amygdala-PFC rsFC*

rsFC between the right CeA and left area 13 (OFC) was positively correlated with the duration of anxiety in response to the stare (n = 22, r_s = 0.72, p = 0.0002, **Table 12**, **Figure 13A**). Similarly, connectivity at rest was more positive at a trend level between the right CeA and left area 13 in high anxiety subjects (n = 7) as compared to low anxiety subjects (n = 8) (0.04 v. -0.07, t_{13} = 3.4, *p* = 0.004, Cohen's *d* = 1.6, *r* = 0.6, FDR corrected, **Table 12**, **Figure 13B**). No significant differences in rsFC between high and low anxiety subjects were observed from the BA or the LA to the PFC, nor did the duration of anxious behavior correlate with rsFC from the BA and LA to the PFC. All significant *p*'s < 0.05 FDR corrected for multiple comparisons.

Figure 13. Association between active anxiety and amygdalocortical connectivity at rest in rhesus macaques.



A. Scatter plot depicting the correlation between active anxiety and connectivity at rest between the right CeA and left area 13. **B.** Non-significant (trend) difference in right CeA – right area 13 connectivity between high and low anxiety subjects. Markov atlas defined left area 13 (OFC, red) – brain region associated with stronger positive rsFC with the right CeA in subjects with longer durations of anxiety.

Scatter plot point color indicates subjects characterized as high anxiety (green), low anxiety (white), and neither high nor low anxiety (grey). Shape indicates sex (females = triangles, males = circles). # $p \leq 0.10$ (FDR corrected), trend level difference.

3.2.5 *Behavioral inhibition and amygdala-PFC rsFC*

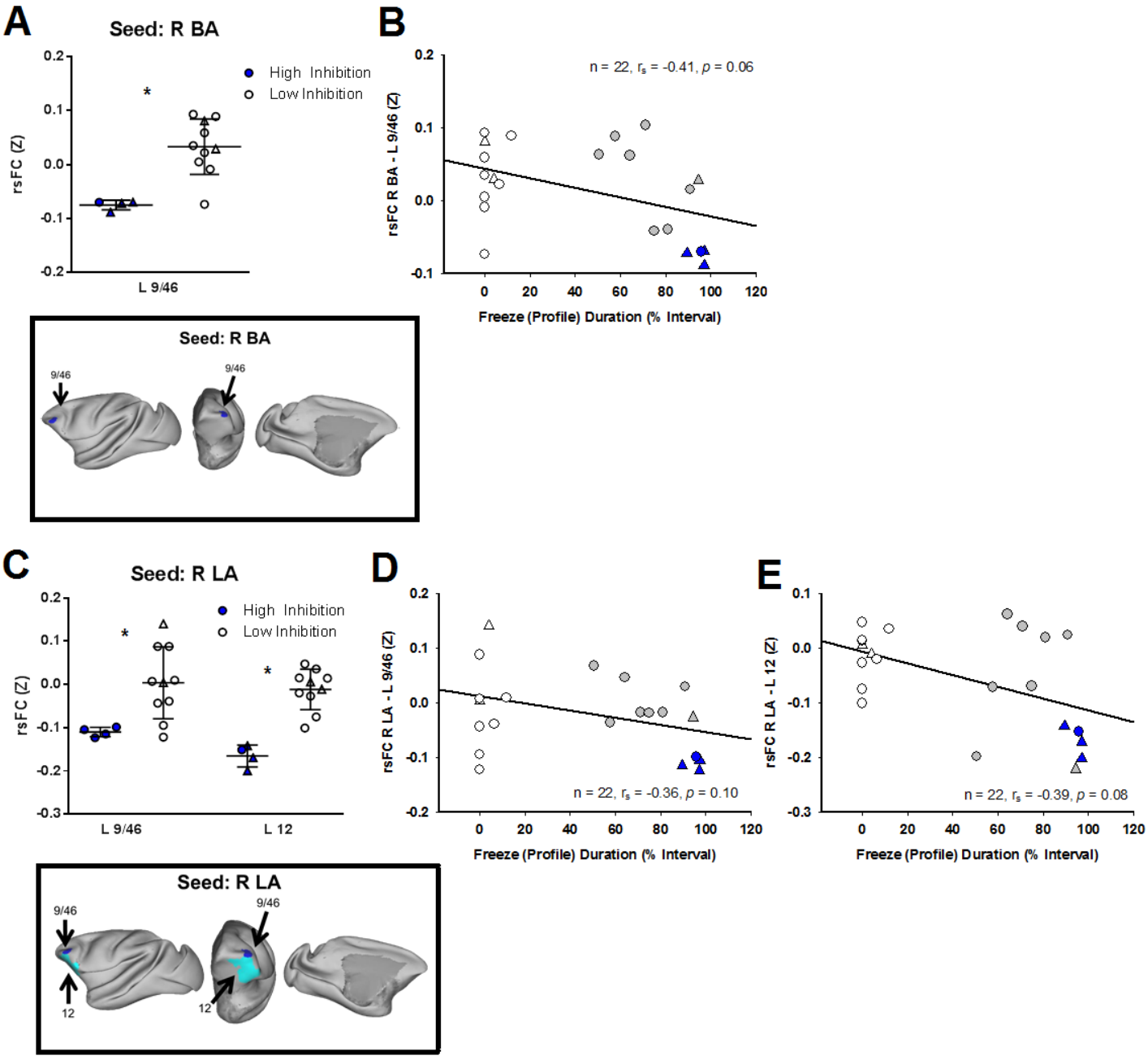
Connectivity at rest between the right BA and left area 9/46 was significantly more anticorrelated in high inhibition subjects (n = 4) than in low inhibition subjects (n = 10) (-0.07 v. 0.03, $t_{10.3} = 6.4$, $p < 0.0001$, Cohen's $d = 2.9$, $r = 0.8$, **Table 12**, **Figure 14A**). However, a significant negative linear relationship between the duration of behavioral inhibition at baseline and rsFC between the right BA and left area 9/46 was not observed ($p_{uncorrected} = 0.06$, **Table 12**, **Figure 14B**).The relationship between LA-PFC rsFC and behavioral inhibition was similar to those observed between the BA and PFC, with connectivity at rest between the right LA and left area 9/46 significantly more anticorrelated in high inhibition subjects (n = 4) than in low inhibition subjects (n = 10) (-0.11 v. 0.00, $t_{9.7} = 4.2$, $p = 0.0018$, Cohen's $d = 1.9$, $r = 0.7$, **Table 12**, **Figure 14C**). rsFC between the right LA and left area 12 was also significantly more anticorrelated in high inhibition subjects than in low inhibition subjects (-0.17 v. -0.01, $t_{12} = 6.2$, $p < 0.0001$, Cohen's $d = 4.0$, $r = 0.9$, **Table 12**, **Figure 14C**). Also similarly, the negative linear relationship observed between these connections and the duration of behavioral inhibition was not significant ($p_{uncorrected} = 0.10, 0.08$, respectively, **Figure 14D-E**). Conversely, rsFC between the CeA and PFC was not associated with behavioral inhibition. All significant p's < 0.05 FDR corrected for multiple comparisons.

Table 12. Associations between amygdala-PFC rsFC and anxiety and/or behavioral inhibition in rhesus macaques

Resting-state functional connectivity			Active Anxiety (% Interval)				Behavioral Inhibition			
Amygdala seed		PFC ROI	r_s	*p*	t	*p*	r_s	*p*	t	*p*
Right	BA	L 9/46	-0.23	0.31	0.5	0.60	-0.41	0.06	-6.4	< 0.0001*
	CeA	L 13	0.72	0.0002*	3.4	0.004	-0.23	0.31	1.8	0.10
	LA	L 9/46	-0.26	0.24	1.3	0.22	-0.36	0.10	4.2	0.0018*
		L 12	-0.06	0.78	0.05	0.96	-0.39	0.08	6.2	< 0.0001*

r_s values correspond to a Spearman partial correlation controlling for sex; t values correspond to independent t tests comparing high and low anxiety or inhibition groups; *significant at FDR corrected $p < 0.05$

Figure 14. Associations between behavioral inhibition and amygdalocortical connectivity at rest.



A. Significant group difference in right BA to left 9/46 connectivity between high and low inhibition subjects. **B**. Scatter plot depicting the non-significant negative correlation between baseline duration of freezing (inhibition) duration as a percent of the profile

interval and baseline rsFC between right area 9/46 and right BA. **C.** Significant group differences in rsFC between the right LA and left 9/46 and 12 in high versus low inhibition subjects. Markov atlas defined left area 9/46 (dlPFC, dark blue) – brain region associated with stronger anticorrelated connectivity with the right BA in subjects with higher levels of inhibition. **D-E.** Scatter plots depicting the non-significant negative correlations between baseline duration of freezing (inhibition) duration as a percent of the profile interval and baseline rsFC between left area 9/46 and 12 and right BA. Markov atlas defined left area 9/46 (dlPFC, dark blue) and 12 (OFC, light blue) – brain regions associated with stronger negative connectivity with the right LA in subjects with higher levels of inhibition.

Scatter plot point color indicates subjects characterized as high inhibition (blue), low inhibition (white), and neither high nor low inhibition (grey). Shape indicates sex, with females depicted as triangles and males as circles. *$p \leq 0.05$ (FDR corrected), statistically significant group difference.

3.2.6 *Summary: Aim 2 results*

Overall, the results of these studies partially supported the hypothesis that aggression and active anxiety would be associated with anticorrelated amygdalocortical connectivity but inhibition would be associated with higher positive amygdalocortical connectivity. Both aggression and behavioral inhibition were associated with stronger anticorrelated amygdalocortical connectivity from the BA (both aggression and behavioral inhibition) and LA (only behavioral inhibition) to the dlPFC. Conversely, active anxiety was associated with higher positive connectivity between the CeA and OFC. While it was surprising that behavioral inhibition and aggression were correlated with similar rsFC, distinct hemisphere and ROI effects were observed. Similar to the findings of Aim 1, although a single significant sex difference in baseline rsFC was observed and female subjects generally responded for longer durations in response to the HIT, the relationship between amygdalocortical connectivity and temperament did not appear to be dependent upon sex.

3.3 Aim 3: Neural correlates of heavy ethanol intake

3.3.1 *Group composition*

The same sample of 22 rhesus macaques with rsfcMRI scans from Aim 2 was used for these analyses. Of these subjects, 12 subjects had access to ethanol for 12 months (Drinkers), and 10 subjects had access to only water (Controls). Of the 12 drinking subjects, 5 were heavy drinkers (>20% of drinking days above 3g/kg) and 7 were non-heavy drinkers. See **Table 7** for individual drinking characterization.

3.3.2 *Baseline amygdala-PFC rsFC and future ethanol intake*

The regions associated with high aggression, anxiety, and behavioral inhibition in Aim 2 were not significantly associated with heavy drinking or intoxication (**Table 13**). However, connectivity at rest between the left BA and left OPAI (OFC) was significantly more highly positive in heavy drinkers (n = 5) than non-heavy drinkers (n = 7) (0.14 v. -0.04, $t_{10} = 4.8$, $p = 0.0007$, Cohen's $d = 3.0$, $r = 0.8$, **Table 13**, **Figure 15A**) but correlated weakly with average daily intake (r = 0.58, $p = 0.059$, **Table 13**, **Figure 15B**). rsFC between the left CeA and right area 8 (dlPFC) was significantly more positive in future heavy drinkers at baseline (0.11 v. -0.04, $t_{10} = 4.8$, $p = 0.0007$, Cohen's $d = 2.9$, $r = 0.8$, **Table 13**, **Figure 15C**) than in non-heavy drinkers, but again did not significantly correlate (following FDR corrections) with intake (r = 0.70, $p = 0.017$, **Table 13**, **Figure 15D**). Unlike the BA seeds, no significant differences were observed between CeA or LA and PFC ROIs between heavy and non-heavy drinkers, and no significant correlations between ethanol intake or intoxication and BA, CeA, or LA and PFC rsFC

were observed across all drinkers (n = 12). All significant *p*'s < 0.05 FDR corrected for multiple comparisons.

Table 13. rsFC correlates of future heavy ethanol intake and intoxication

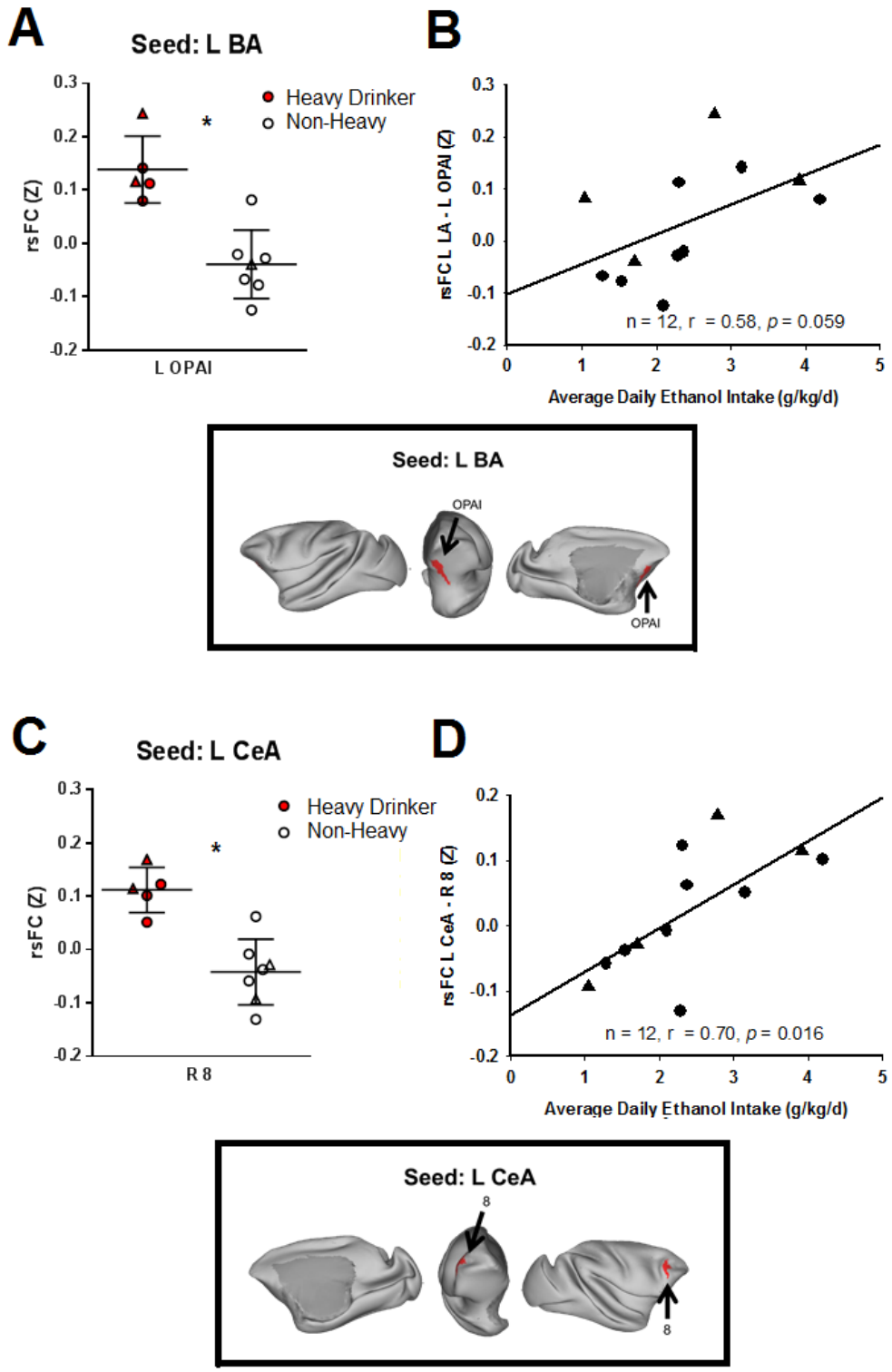
Resting-state functional connectivity			12 Month Ethanol Intake (g/kg)				12 Month BEC (mg/dl)	
Amygdala seed		PFC ROI	r	*p*	t	*p*	r	*p*
Right	*BA*	*L 9/46*	*-0.54*	*0.089*	*0.3*	*0.734*	*-0.61*	*0.046*
		R 9	*-0.06*	*0.850*	*0.3*	*0.753*	*-0.27*	*0.429*
	CeA	*L 13*	*0.07*	*0.833*	*0.5*	*0.531*	*0.18*	*0.590*
	LA	*L 9/46*	*-0.57*	*0.068*	*1.6*	*0.15*	*-0.44*	*0.217*
		L 12	*-0.26*	*0.449*	*0.4*	*0.683*	*-0.30*	*0.375*
Left	**BA**	**L OPAI**	**0.58**	**0.059**	**4.8**	**0.0007***	**0.37**	**0.261**
	CeA	**R 8**	**0.70**	**0.016**	**4.8**	**0.0007***	**0.53**	**0.096**

r values correspond to a Pearson partial correlation controlling for sex; t values correspond to independent t tests comparing heavy and non-heavy drinkers.

Italicized lines indicate connections associated with anxious and aggressive behavior at baseline in Aim 2 but not future ethanol intake, bolded lines indicate connections associated with future ethanol intake but not anxious or aggressive behavior.

*significant at FDR corrected $p < 0.05$

Figure 15. Associations between baseline amygdalocortical connectivity at rest and future heavy drinking**.**



A. Significantly higher positive connectivity between left BA and left OPAI (OFC, red) in future heavy drinkers (n = 5) versus future non-heavy drinkers (n = 7)**. B**. Scatter plot depicting the non-significant positive correlation between average ethanol intake and baseline rsFC between left BA and left OPAI (OFC). Markov atlas defined left area OPAI (OFC, red) – brain region associated with stronger positive connectivity with the left BA in heavier drinkers. **C**. Significantly higher positive connectivity between left CeA and left right area 8 (dlPFC, red) in future heavy drinkers versus future non-heavy drinkers. **D.** Scatter plot depicting the non-significant (after FDR correction) positive correlation between average ethanol intake and baseline rsFC between left CeA and right 8 (dlPFC). Markov atlas defined left area 8 dlPFC, red) – brain region associated with stronger positive connectivity with the left CeA in heavier drinkers.
Shape indicates sex, with females depicted as triangles and males as circles. *$p \leq 0.05$ (FDR corrected), statistically significant group difference.

3.3.3 *rsFC change from baseline and chronic ethanol intake*

First, in one connection predictive of heavier drinking at baseline (left CeA and right area 8, see **Figure 15** and **Table 13**), significant changes were also observed after chronic self-administration of ethanol. The percent change in connectivity at rest from baseline between the left CeA and right area 8 (dlPFC) significantly differed between heavy (n = 5) and non-heavy (n = 7) drinkers after both 6 and 12 months of ethanol access, with a decrease observed in heavy drinkers but an increase observed in non-heavy drinkers (after 6 months: -11% v. 6%, $t_{10} = 4.8$, $p = 0.0007$, Cohen's $d = 2.8$, $r = 0.8$; 12 months: -9% v. 6%, $t_{10} = 5.0$, $p = 0.0005$, Cohen's $d = 3.0$, $r = 0.8$, respectively, **Figure 16A**, **Table 14**). Similarly, the percent change in rsFC between the left CeA and right area 8 was non-significantly (after FDR correction) correlated with prior ethanol self-administration across 6 and 12 months of access (r = -0.70, $p = 0.017$; r = -0.64, $p = 0.034$; respectively; **Table 14**, **Figure 16B-C**). However, no significant differences in the change in connectivity between left CeA and right area 8 were observed when comparing all drinkers (n = 12) to all controls (n = 10) (**Table 14**). Together these results indicate a decrease in connectivity from significantly higher levels at baseline in heavier drinkers only. Conversely, the percent change in connectivity at rest between the left BA and left OPAI (OFC) was not associated with prior ethanol intake, despite the association between baseline rsFC from left BA to left OPAI with heavier future drinking (**Table 14**). Specifically, the percent change in rsFC was not correlated with past intake or intoxication, heavy drinkers did not differ in rsFC change from non-heavy drinkers, and control subjects did not differ in rsFC change from drinkers. All significant p's < 0.05 FDR corrected for multiple comparisons.

Significant differences in connectivity changes from baseline were also observed outside of the regions associated with future ethanol intake in section 3.3.2. Compared to controls (n = 10), after 6 months of 22-h fluid access, subjects with access to ethanol (n = 12) showed decreased connectivity from baseline between the right BA and right area 13 (-6.6%) whereas control subjects without access to ethanol exhibited positive increases in connectivity (8.2%) (t_{20} = 4.3, p = 0.0003, Cohen's d = 1.8, r = 0.7, **Figure 16D**). Similarly, after 12 months of self-administration, connectivity from baseline between the right LA and right area 9 was decreased in drinkers (-4.44%) but increased in controls (14.3%) (t_{20} = 4.4, p = 0.0003, Cohen's d = 1.9, r = 0.7, **Figure 16F**). However, the change in rsFC in these connections was not significantly different in heavy and non-heavy drinkers (**Table 14**), nor did it correlate with past ethanol intake or intoxication (**Table 14**, **Figure 16E,G**). Outside of the left CeA and right area 8, no significant differences in changes in connectivity from baseline were observed between heavy and non-heavy drinkers. Similarly, no significant correlations between prior intake or intoxication and the percent change in rsFC from baseline were observed. All significant p's < 0.05 FDR corrected for multiple comparisons.

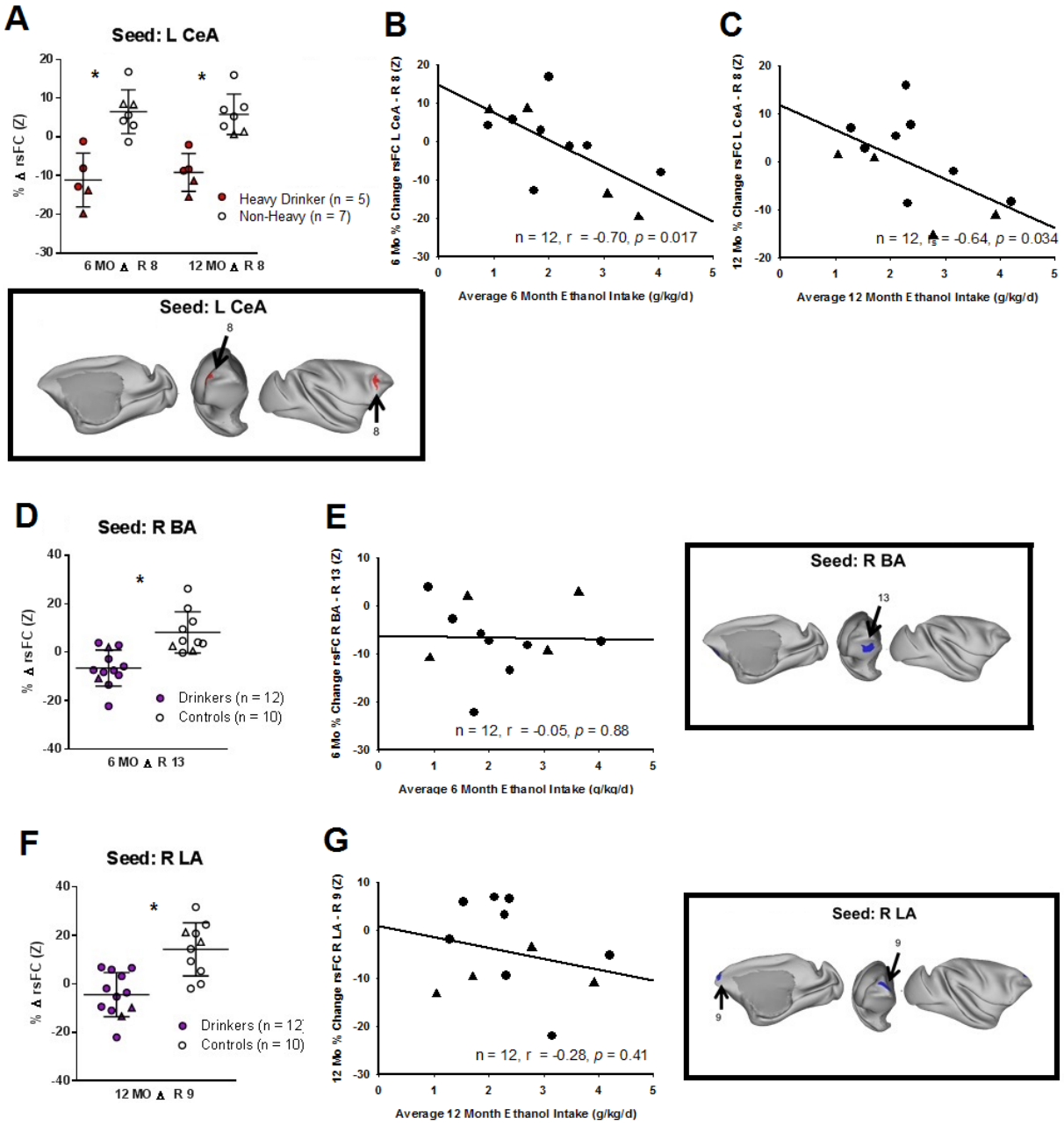
Table 14. Ethanol induced changes in amygdalocortical rsFC

		% Change rsFC		Ethanol Intake (g/kg)	BEC (mg/dl)	Drinker v. Control	Heavy v. Non-Heavy
		Amygdala seed	PFC ROI	r, *p*	r, *p*	t, *p*	t, *p*
6 mo	*L*	*BA*	*L OPAI*	*-0.29, 0.38*	*-0.06, 0.87*	*1.2, 0.26*	*2.1, 0.07*
12 mo	*L*	*BA*	*L OPAI*	*-0.16, 0.64*	*-0.02, 0.98*	*-0.4, 0.72*	*-0.9, 0.41*
6 mo	*L*	*CeA*	*R 8*	*-0.70, 0.017*	*-0.57, 0.07*	*-0.9, 0.37*	*4.8, 0.0007**
12 mo	*L*	*CeA*	*R 8*	*-0.64, 0.034*	*-0.50, 0.12*	*-0.2, 0.85*	*5.0, 0.0005**
6 mo	**R**	**BA**	**R 13**	**-0.05, 0.88**	**-0.01, 0.97**	**4.3, 0.0003***	**0.90, 0.38**
12 mo	**R**	**BA**	**R 13**	**0.44, 0.18**	**0.65, 0.03**	**-1.2, 0.24**	**-0.44, 0.67**
6 mo	**R**	**LA**	**R 9**	**-0.13, 0.71**	**-0.19, 0.60**	**2.2, 0.04**	**0.56, 0.59**
12 mo	**R**	**LA**	**R 9**	**-0.28, 0.41**	**-0.20, 0.76**	**4.4, 0.0003***	**2.1, 0.06**

r values correspond to a Pearson partial correlation controlling for sex; t values correspond to independent t tests comparing drinkers and controls and heavy and non-heavy drinkers. Italics indicate connections associated with future ethanol intake, bold indicates connections associated with past ethanol intake but not predictive of future ethanol intake in section 3.3.2. MRI timing corresponds with drinking duration (i.e. 6 mo change in rsFC correlated with 1st 6 month intake and BEC).

*significant at FDR corrected $p < 0.05$

Figure 16. Associations between the percent change in rsFC from baseline and chronic ethanol self-administration**.**



A. Significant decreases in connectivity observed after 6 and 12 months of ethanol access between the left CeA and right area 8 (dlPFC, red) in heavy drinkers versus

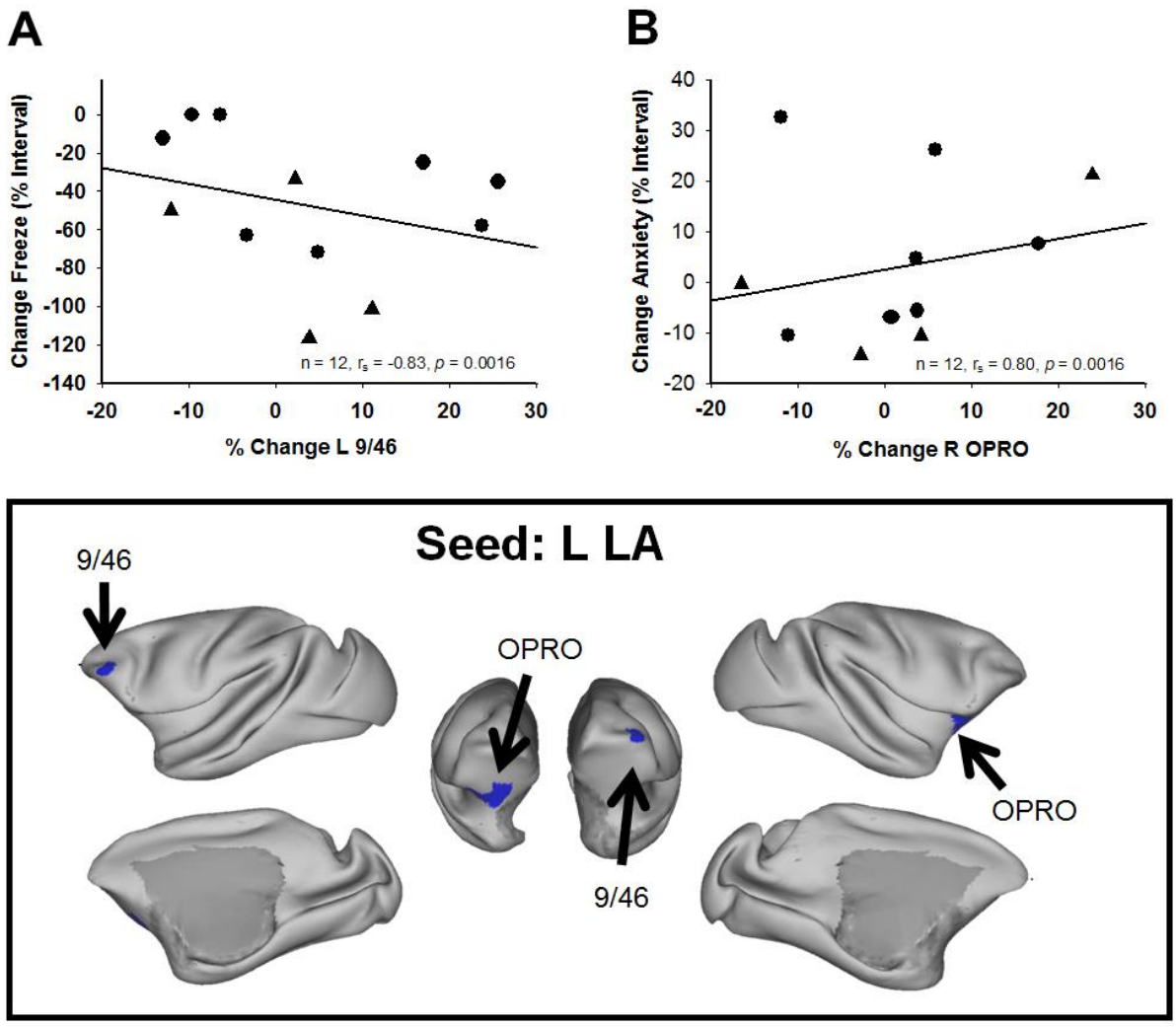
increases in connectivity in non-heavy drinkers. **B-C.** Scatter plot depicting the non-significant negative correlation between average 6 month and 12 month ethanol intake and the change in rsFC between left CeA and left 8 (dlPFC). Markov atlas defined right area 8 (dlPFC, red) – brain region associated decreased connectivity from baseline with the left CeA in heavier drinkers. **D**. Significant increases in connectivity between the right BA and right area 13 (OFC, blue) after 6 months of water access in control subjects compared to drinkers with ethanol access. **E.** Scatter plot depicting the non-significant correlation between average 6 month ethanol intake and the change in rsFC between right BA and right 13 (OFC). Markov atlas defined right area 13 (OFC, blue) – brain region associated increased connectivity from baseline with the right BA in control subjects. **F.** Significant increases in connectivity between the right LA and right area 9 (dlPFC) after 12 months of water access in control subjects compared to drinkers with ethanol access. **G.** Scatter plot depicting the non-significant correlation between average 12 month ethanol intake and the change in rsFC between LA and right 9 (dlPFC). Markov atlas defined right area 9 (dlPFC, blue) – brain region associated increased connectivity from baseline with the right LA in control subjects.

Shape indicates sex, with females depicted as triangles and males as circles. *$p \leq 0.05$ (FDR corrected), statistically significant group difference.

3.3.4 *rsFC underlying longitudinal changes in behavior*

Aim 1 demonstrated significant decreases in inhibited and aggressive behavior associated with ethanol access and heavy ethanol intake. A correlational analysis to assess possible associations between the changes in rsFC and behavior from baseline in the drinkers (n = 12) found significant correlations between changes in rsFC and changes in behavior from baseline, but not in the specific connections associated with heavier drinking at baseline or altered by ethanol access. Instead, the percent change in connectivity from baseline from left LA to left area 9/46 (dlPFC) negatively correlated with the decrease in freezing behavior from baseline (r_s = -0.83, p = 0.0016, **Figure 17A**). Similarly, the percent change in connectivity from left LA to right OPRO (OFC) negatively correlated with the decrease in active anxiety behavior from baseline (r_s = 0.80, p = 0.0016, **Figure 17B**).

Figure 17. Associations between changes in active anxiety and freezing and changes in amygdalocortical connectivity at rest from baseline in drinkers.



A. Scatter plot depicting the negative correlation between the change in freezing from baseline and percent change in connectivity at rest between the left LA and left area 9/46. **B.** Scatter plot depicting the positive correlation between the change in active anxiety from baseline and percent change in connectivity at rest between the left LA and right OPRO. Shape indicates sex (females = triangles, males = circles). INIA19 atlas defined left area 9/46 (dlPFC, blue) and right OPRO (OFC, blue).

3.3.4 *Summary: Aim 3 Results*

The hypothesis that amygdalocortical connectivity would be similarly dysregulated in aggressive/anxious subjects and future heavy drinkers at baseline was not supported by these results. However, the hypothesis that future heavy drinking would be associated with higher positive connectivity between the dlPFC and amygdala and negative connectivity between the OFC and amygdala at baseline was partially supported. While baseline connectivity associated with temperament subtypes in Aim 2 was not associated with future heavy drinking, connectivity between the CeA and dlPFC was significantly more positive at baseline in monkeys that became heavy drinkers.

Similarly, the change in rsFC from baseline associated with heavy drinking did not support the hypothesis that connectivity would be further dysregulated in the same direction of the association with heavy drinking at baseline. Instead, connectivity between the CeA and dlPFC was reduced by heavy drinking, rather than increasing as hypothesized, suggesting an adaptive change occurring with heavy drinking specifically. No other significant changes were observed between heavy and non-heavy drinkers, which did not support the hypothesis that drinking would influence amygdalocortical connectivity in the OFC. Controls exhibited significantly more increased connectivity between the BA and OFC and LA and dlPFC than subjects with ethanol access (which exhibited both increases and decreases in rsFC), suggesting that ethanol access, even at non-heavy intake levels, could influence changes in brain connectivity chronically.

Finally, the changes in behavior observed after ethanol access, as hypothesized, were mediated by similar regions as those associated with the specific behavior at

baseline, with amygdala-dlPFC rsFC correlating with the change in inhibition. However, the change in aggression was instead mediated by amygdala-OFC connectivity.

3.4 Aim 4: Cynomolgus macaque replicate

3.4.1 *Temperament group characteristics in cynomolgus macaques*

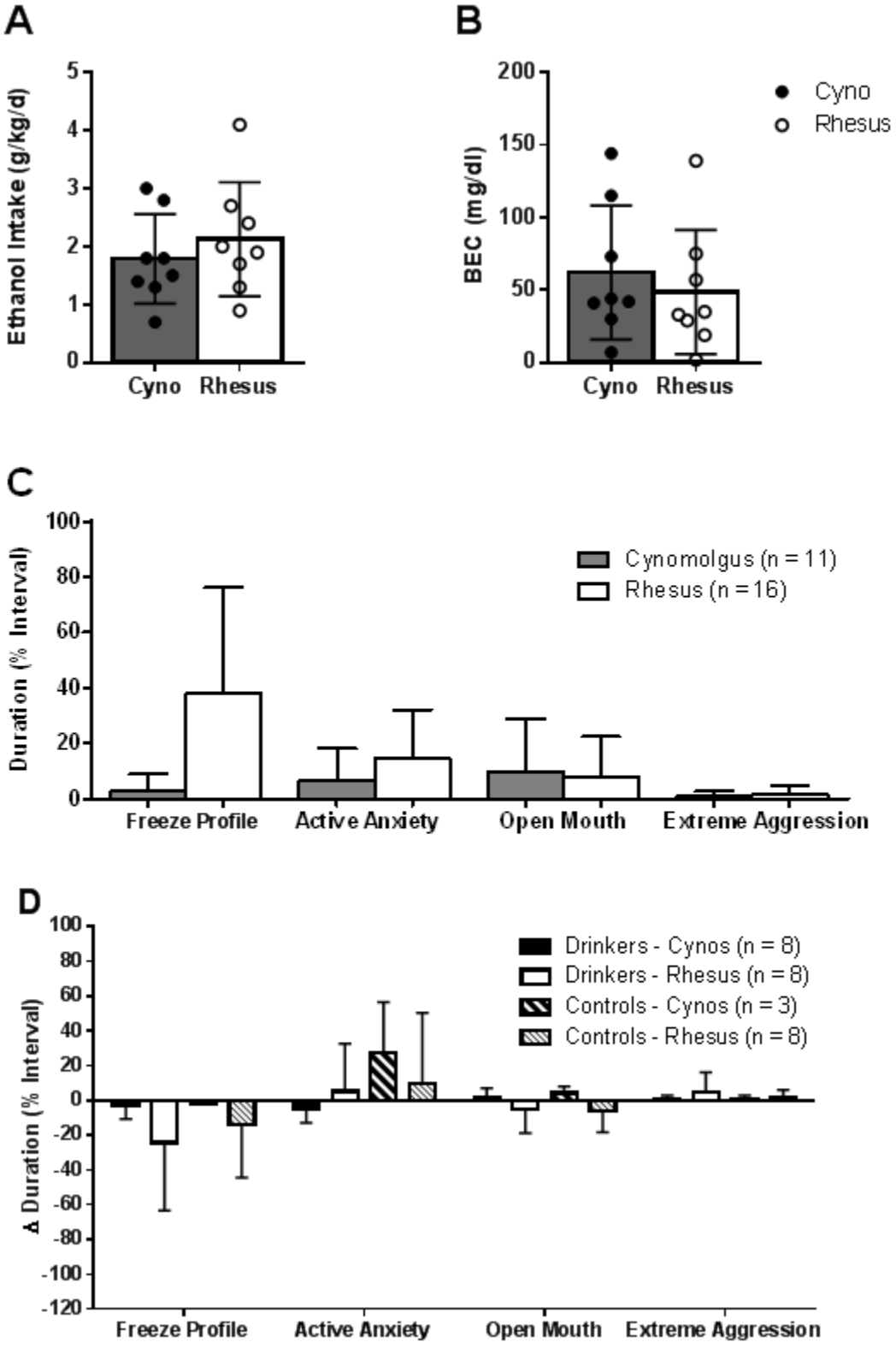
11 total subjects were included in this analysis. A quartile approach was used to characterize subjects as high or low aggression, anxiety, or inhibition, as in Aim 1. 3 subjects (27.2%) were characterized as high aggression, anxiety, and inhibition, while 6 subjects (54.5%) were characterized as low aggression and inhibition and 7 subjects (63.6%) were characterized as low anxiety. The high proportion of subjects falling in the low groups was due to the lower quartile cutoff at 0% of the interval, and the large number of subjects not exhibiting anxious or aggressive-like behaviors. Individual characterizations and ethanol intake can be found in **Table 15**.

3.4.2 *Species differences in behavior and amygdalocortical rsFC*

While no statistically significant differences in ethanol drinking, HIT behavior or brain connectivity were found between rhesus (n = 16) and cynomolgus macaques (n = 11) at baseline, trends approaching significance were observed. Rhesus macaques froze for longer durations in response to the Profile during the HIT than cynomolgus macaques (37.7% versus 2.6 % of the interval, U = 50.0, Z = -1.9, p = 0.06, Cohen's d = 1.3, r = 0.5, **Figure 18C**). No other differences in behavior approaching significance were observed, although rhesus macaques generally reacted to the HIT for longer durations (**Figure 18C**). No species differences in the change in behavior from baseline were observed (**Figure 18D**). Drinking and intoxication did not differ between cynomolgus and rhesus macaques (**Figure 18A-B**). Connectivity at rest between the right LA and left area 11 (OFC) was non-significantly (after FDR correction) more

negative at a trend level in rhesus macaques than cynomolgus macaques at baseline (-0.05 v. 0.03, $t_{24.9} = -3.3$, $p = 0.003$, Cohen's $d = 1.2$, $r = 0.5$, data not shown). No other species differences in baseline rsFC approaching significance were observed. No species differences were observed in the change in connectivity from baseline to post 6 months of 22-h access in drinkers or controls.

Figure 18. Species differences in ethanol intake and behavior.



Mean ± SD of **A** daily ethanol intake and **B** BEC over 6 (cynomolgus) or 12 (rhesus) months of 22-h ethanol access and mean ± SD of **C** individual behavior durations during baseline HIT and **D** change in behavior durations plotted by species. Individual monkeys within each group plotted with circles. No significant differences. *$p \leq 0.05$ (Bonferroni corrected), statistically significant group difference.

Table 15. Average daily intake of ethanol (4% w/v) and BEC (mg/dl) during 22-h ethanol access and anxious/aggressive status at baseline in cynomolgus macaques

Sex	Cohort	Monkey	6 Month Intake (g/kg/d)	6 Month BEC(mg/dl)	Test BEC (mg/dl)*	HIT Aggression	HIT Active Anxiety	HIT Inhibition
Male	9	53[a]	3.0	115	0	High	High	High
	9	59[a]	2.8	144	0	Low	Low	Low
	9	51	1.4	42	0	Low	Low	Low
	9	52	1.8	73	0	Low	Low	Low
	9	54	1.5	44	11	High	High	Low
	9	55	0.7	7	0	High	High	Low
	9	57	1.3	30	0	-	Low	-
	9	58	1.8	41	0	-	-	Low
	9	49	CONTROL	CONTROL	CONTROL	Low	Low	High
	9	50	CONTROL	CONTROL	CONTROL	Low	Low	High
	9	56	CONTROL	CONTROL	CONTROL	Low	Low	-

[a] Indicates that the monkey was a heavy drinker, as defined by drinking >3.0 g/kg on at least 20% of days throughout 22-h access.

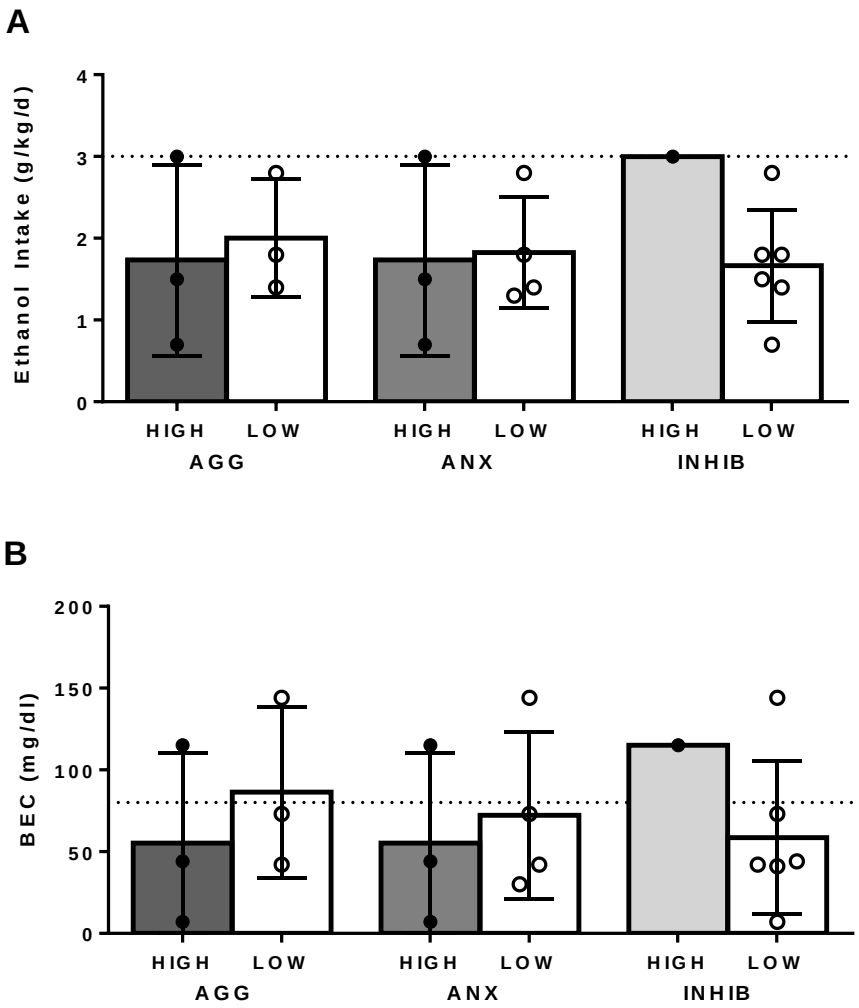
- Indicates that the monkey was neither high nor low and fell in the middle 50% of all subjects.

CONTROL indicates control monkeys without ethanol access.

3.4.3 *Baseline temperament and future heavy ethanol intake*

Unlike the associations observed between aggression and ethanol intake the rhesus monkeys (**Table 8**, **Figure 8**), anxious, aggressive, and inhibited behavior in response to the HIT were not associated with future heavy drinking or intoxication in the cynomolgus macaques with access to ethanol (n = 8). Similarly, no significant correlations between baseline durations of behavior and future ethanol intakes and BEC were found after Bonferroni corrections for multiple comparisons (**Table 16**, raw *p*-values). Finally, high and low aggression, anxiety, and inhibition subjects did not significantly differ in intake or intoxication in these subjects (**Figure 19A-B**).

Figure 19. Average daily ethanol intake and BEC by temperament group in cynomolgus macaques.



Mean ± SD of **A** daily ethanol intake and **B** BEC over 6 months of 22-h ethanol access plotted by temperament group. Mean daily ethanol intake (g/kg/ day) calculated from an average of 125 days of self-administration/monkey. Average BEC (mg/dl) calculated from 31 samples/monkey. Individual monkeys within each group depicted with *circles*.

Table 16. Spearman's partial rank-order correlations (r_s, *p*) describing the relationship between ethanol intake and intoxication during 22-h ethanol access and aggressive-like, anxious-like, and behaviorally inhibited behaviors in subjects with access to ethanol (n = 8).

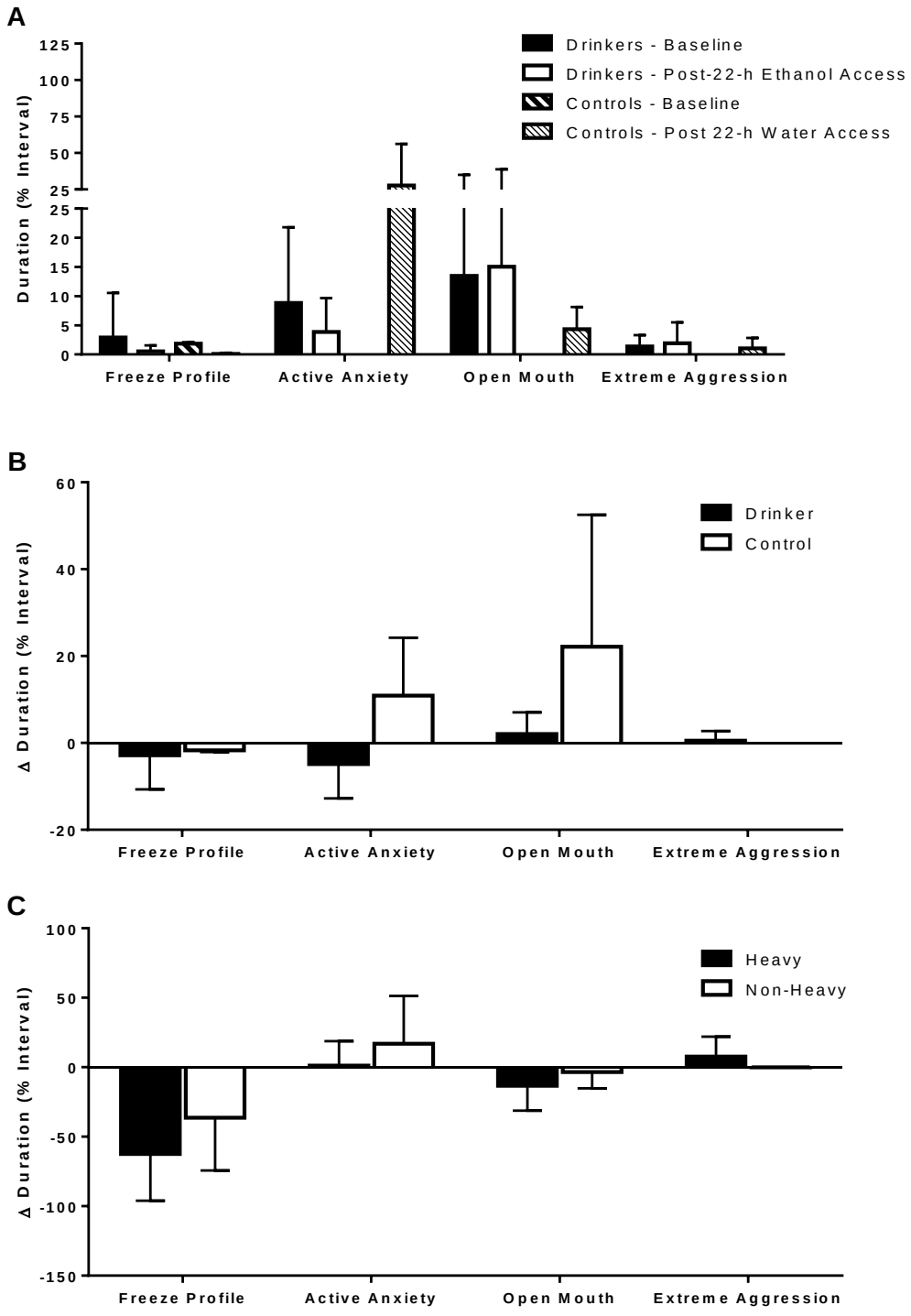
		Ethanol Intake (g/kg/d)	BEC (mg/dl)
HIT Variables	Freeze Profile	0.22, 0.60	0.08, 0.85
	Active Anxiety	0.10, 0.80	-0.10, 0.81
	Open Mouth	-0.21, 0.62	0.11, 0.80
	Extreme Aggression	0.14, 0.75	0.11, 0.80
	∆Freeze Profile	0.19, 0.66	0.07, 0.87
	∆Active Anxiety	0.44, 0.28	0.72, 0.04[b]
	∆Open Mouth	-0.63, 0.09	-0.91, 0.002[a]
	∆Extreme Aggression	-0.67, 0.07	-0.46, 0.25

r_s values correspond to a Spearman partial rank-order correlation; [a]significant at Bonferroni corrected $p < 0.05$; [b]trend at uncorrected $p < 0.05$

3.4.4 *Post temperament and heavy ethanol intake*

Despite the lack of association between drinking and baseline behavior in the cynomolgus macaques, changes in behavior from baseline were associated with past drinking behavior during 22-h access. Similar to the effects observed in the rhesus monkeys (**Table 10**, **Figure 9**), the change in open mouth threat duration was negatively correlated with past intoxication (r_s = -0.91, *p* = 0.0015, **Table 17**) but not average daily intake (r_s = -0.63, *p* = 0.09, **Table 17**), and the change in active anxiety trended towards a positive correlation with BEC (r_s = 0.72, *p* = 0.043, **Table 17**) but not average daily intake (r_s = 0.44, *p* = 0.28, **Table 17**). No significant differences between baseline and post-22-h ethanol access behavior were observed in the drinkers or controls (**Figure 20A**). Similarly, heavy and non-heavy drinkers and drinkers and controls did not significantly differ in the change in behavior observed from baseline (**Figure 20B-C**).

Figure 20. Changes in behavior in cynomolgus macaques.



Mean ± SD of **A** behavior duration at baseline HIT and post-22-h access HIT, **B** change in behavior durations compared between drinkers and controls and **C** heavy and non-heavy drinkers. Duration presented as a % of the interval, change scores calculated as post score – baseline score.

3.4.5 *Active anxiety, aggression, and amygdala-PFC rsFC*

Unlike the associations observed in the rhesus macaques subjects (**Table 12**, **Figure 12-14**), amygdalocortical connectivity at rest was not significantly associated with higher levels of active anxiety, aggression, or inhibition in the cynomolgus macaques subjects. No correlations between durations of aggressive or actively anxious behaviors and connectivity at rest between the amygdala and PFC approaching significance were observed that were not driven by a single data point.

3.4.6 *Baseline amygdala-PFC rsFC and future ethanol intake*

Similar to the associations observed in the rhesus subjects (with higher positive connectivity at baseline predicting heavier future drinking, **Table 13**, **Figure 15**), at baseline, rsFC between the left CeA and left area 13 (OFC) and left CeA and right area 12 (OFC) was significantly more positive in future heavy drinkers (n = 2) than future non-heavy drinkers (n = 6, 0.09 v. -0.03, $t_{5.9} = 5.7$, $p = 0.0013$, Cohen's $d = 3.3$, $r = 0.9$; 0.10 v. -0.05, $t_{5.4} = 6.9$, $p = 0.0007$, Cohen's $d = 4.2$, $r = 0.9$; **Table 17**; **Figure 21A**). Though non-significant after FDR corrections for multiple comparisons, rsFC between the left CeA and left area 13 and left CeA and right area 12 was also positively correlated with future ethanol intake (r = 0.82, $p = 0.010$; r = 0.80, $p = 0.017$; respectively; **Figure 21A-B**) and intoxication (r = 0.82 $p = 0.013$; r = 0.74, $p = 0.037$; respectively, **Table 17**). All significant p's < 0.05 FDR corrected for multiple comparisons.

Similarly, connectivity at rest between the left BA and left area 9/46 (dlPFC) differed at a trend level between future heavy and non-heavy drinkers (0.05 v. -0.05, t_6 = 4.9, p = 0.0027, Cohen's *d* = 3.4, *r* = 0.9), but did not correlate with future intake or intoxication (**Table 17**). Conversely, connectivity at rest between the left BA and right area 8 trended towards uncorrelated connectivity while future non-heavy drinkers were more highly positive correlated (0.07 v. -0.00, t_6 = 4.9 *p* = 0.0027, Cohen's *d* = 3.6, *r* = 0.9), but did not correlate with future intake or intoxication (**Table 17**). Connectivity between the LA and PFC at baseline was not associated with future ethanol intake or intoxication.

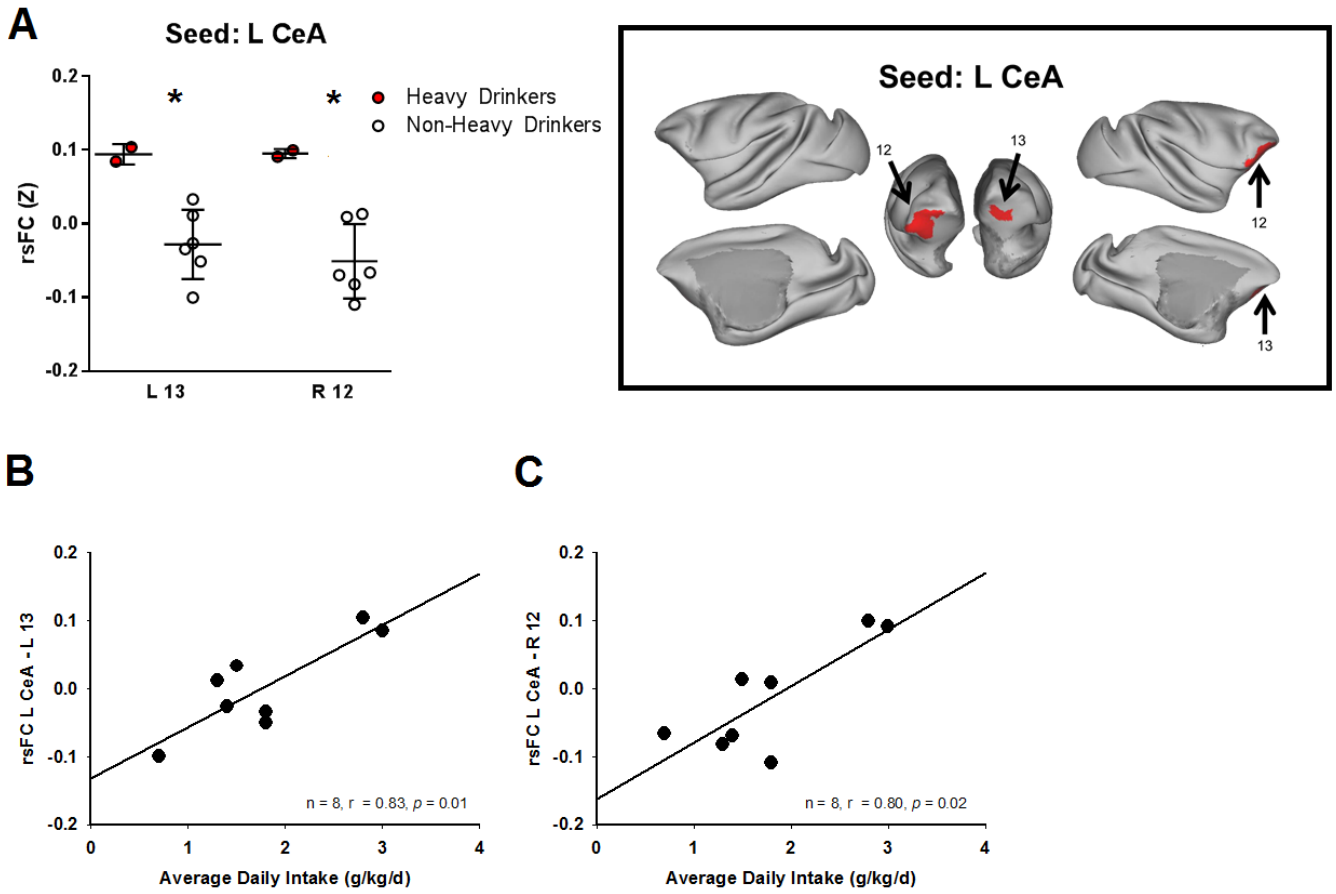
Table 17. rsFC correlates of future heavy ethanol intake and intoxication in cynomolgus macaques

Resting-state functional connectivity			6 Month Ethanol Intake (g/kg)				6 Month BEC (mg/dl)	
Amygdala seed		PFC ROI	r	*p*	t	*p*	r	*p*
Left	BA	L 9/46	0.51	0.19	4.9	0.003[#]	0.58	0.13
		R 8	0.64	0.08	4.9	0.003[#]	0.73	0.04
	CeA	L 13	0.83	0.01	5.7	0.001*	0.82	0.01
		R 12	0.80	0.02	6.9	0.0007*	0.74	0.04

r values correspond to a Pearson partial correlation; t values correspond to independent t tests comparing heavy and non-heavy drinkers.

*significant at FDR corrected $p < 0.05$, [#]trend at uncorrected $p < 0.003$.

Figure 21. Associations between baseline amygdalocortical connectivity at rest and future heavy drinking in cynomolgus macaques**.**



A. Significantly higher positive connectivity between left CeA and left area 13 and right area 12 (OFC, red) in future heavy drinkers (n = 2) versus future non-heavy drinkers (n = 6)**. B**. Scatter plot depicting the non-significant positive correlation between average ethanol intake and baseline rsFC between left CeA and left 13 (OFC). **C.** Scatter plot depicting the non-significant positive correlation between average ethanol intake and baseline rsFC between left CeA and right area 12 (OFC). Left area 13 and 12 (OFC,

red) – brain regions associated with stronger positive connectivity with the left CeA in heavier drinkers.

*p ≤ 0.05 (FDR corrected), statistically significant group difference.

3.4.7 *rsFC change from baseline and chronic ethanol intake*

Baseline rsFC connections associated with future ethanol intake were not significantly influenced by subsequent ethanol intake. However, similarly to the rhesus subjects (**Table 14**, **Figure 16**), after 6 months of ethanol access, the percent change in rsFC from baseline between the left BA and left 9/46 (dlPFC) differed between heavy drinkers (n = 2) and non-heavy drinkers (n = 6, -8.0% v. 7.6%, $t_{5.9} = 4.4$, p = 0.004, Cohen's *d* = 2.6, *r* = 0.8, data not shown) at a trend level. This connection was also associated at a trend level at baseline with future heavy drinking (see Section 3.4.6). However, the percent change in rsFC was not correlated with prior ethanol intake (r = -0.48, *p* = 0.23) or intoxication (r = -0.56, *p* = 0.15). No other differences approaching significance in the change in connectivity from baseline were observed between heavy and non-heavy drinkers. No correlations approaching significance between the change in connectivity and past ethanol intake or intoxication were observed. Controls (n = 3) and drinkers (n = 8) did not differ in the change in connectivity from baseline.

3.4.8 *rsFC underlying longitudinal changes in behavior*

Section 4.4.3 found that heavier ethanol self-administration was significantly associated with decreased aggressive behavior and non-significantly associated with increased anxious behavior after 6 months of ethanol access. In the 8 subjects with ethanol access, there were no significant associations between the percent change in amygdalocortical rsFC from baseline and the change in behavior from baseline, unlike the associations observed in the rhesus subjects (**Figure 17**).

3.4.9 *Summary: Aim 4 Results*

The cynomolgus macaque subjects served as a replicate of the rhesus subjects, and the same hypotheses were proposed. However, these hypotheses were largely not supported, with the cynomolgus subjects failing to replicate several Aims. Cynomolgus macaques responded for generally shorter durations, particularly in response to the Profile stimulus, though the differences were not statistically significant. Relatedly, Aims 1 and 2, which assessed relationships involving temperament, generally did not replicate in the cynomolgus subjects. The cynomolgus macaques did not show any predictive relationship between baseline temperament and future heavy ethanol intake, but did replicate the decreased aggression (but not decreased freezing) observed in heavier/more highly intoxicated rhesus drinkers. The associations observed between temperament and amygdalocortical connectivity in the rhesus subjects also failed to replicate, with no associations observed in the cynomolgus subjects.

However, the relationship between amygdalocortical connectivity and ethanol self-administration observed in the cynomolgus subjects was similar to the associations observed in the rhesus subjects, with both populations showing higher amygdalocortical connectivity at baseline predicting heavier drinking. Additionally, the same connection predictive of heavier drinking was non-significantly altered by subsequent ethanol self-administration, as observed in the rhesus subjects. Finally, behavioral changes after self-administration were not correlated with amygdalocortical connectivity, though this was not necessarily surprising, given the lack of association between temperament and connectivity at baseline. Overall, the cynomolgus cohort suggests that rhesus and cynomolgus macaques may have species specific relationships between temperament

and ethanol self-administration/amygdalocortical connectivity, perhaps due to species specific responses to threat, while the relationship between amygdalocortical connectivity and ethanol consumption appears similar in both species.

CHAPTER 4: General Discussion

4.1 Summary: Aims of the dissertation

Temperament is a well-established risk factor for alcohol abuse in human subjects (DeJong et al., 1993; Kessler, 2004; Skodol et al., 1999, Dick et al., 2013) associated with alterations in amygdala-PFC structure and function also observed in subjects at higher risk for alcohol abuse (Virkunnen & Linnoila., 1993; Tessner et al., 2010). However, the intrinsic neural correlates of temperament have not been established, and temperament has not been assessed longitudinally in a stable model of ethanol self-administration. Therefore, the aim of this dissertation was to establish functional neural correlates of temperament and chronic heavy ethanol intake in a unique non-human primate model of ethanol self-administration, to improve understanding of behavioral and biological predictors and consequences of alcohol abuse.

4.2 Aim 1: Behavioral predictors and consequences of heavy ethanol intake

The results of this study demonstrate that measures of temperament in monkeys are associated with future heavy ethanol consumption and intoxication, and can help disentangle the relationship between ethanol and anxious and aggressive behavior. Specifically, baseline aggressive temperament and longer durations of aggressive behavior were associated with higher ethanol self-administration and intoxication, while chronic ethanol self-administration decreased behavioral inhibition and aggression.

Interestingly, despite baseline sex differences in behavioral reactivity to the HIT (with females demonstrating higher levels of reactivity), the association between temperament and ethanol drinking phenotypes was observed across male and female subjects. Conversely, in contrast to my hypotheses, anxiety-related behavior at baseline was not associated with future ethanol intake or intoxication and no significant differences in ethanol drinking were observed between high and low anxiety or inhibition subjects.

Overall, the association between non-human primate aggression and alcohol self-administration is consistent with past research demonstrating a relationship between conduct disorder and substance abuse during adolescence, with childhood aggression and conduct disorder predicting future substance abuse (Brook et al., 1992; Boyle et al., 1999; Pardini et al., 2007; Fergusson et al., 2007; Jester et al., 2008). The correlations observed between aggressive behavior and ethanol intake and BECs in these subjects correspond with studies of adolescent children that found the magnitude of deviation in temperament from the mean of the general population to associate with the severity of drug use (Glanz and Pickens 1991; Tartar and Mezzich 1992). However, the literature on the specific relationship of alcohol with aggression in the human literature is largely non-specific regarding aggression subtypes. In this study, the predictive relationship between reactive aggression elicited by a social threat and future ethanol self-administration was specifically examined. These results found high aggression to associate with heavier future ethanol drinking independent of sex, suggesting that further research should assess the role of reactive aggression versus controlled-instrumental aggression in drinking in human subjects. Additionally, some

studies with human subjects have exclusively focused on the relationship between alcohol and aggression in male subjects (Dolan et al., 1993; Ensminger et al., 1983; Kellam et al., 1975). The results of this study suggest that aggressive behavior is an important risk factor in both male and female subjects and that more emphasis should be placed on assessing aggressive behavior in both sexes. Although alcohol self-administration did not significantly differ between actively anxious and non-actively (3.2 versus 2.3 g/kg/d, t_{21}= 2.1, p = 0.09), on average the actively anxious group exceeded the 3.0 g/kg/day threshold used to demarcate heavy versus non-heavy drinking in this model. This suggests that more than 32 rhesus monkeys from the Oregon National Primate Center Population may be needed to study how anxious temperament predisposes chronic heavy drinking outcomes in this model.

Interestingly, there was an association between active anxiety and extreme aggression (data not shown, r_s = 0.60, p = 0.0003, n = 32) consistent with studies that have found a relationship between anxiety and externalizing disorders in youth (Marmorstein 2007). Together, these data suggest that external expressions of anxiety and aggression rather than inhibition in response to stressful stimuli may be related to heavier ethanol intake. This relationship is also suggested by the association between the personality construct of behavioral undercontrol and substance use in prior research. Behavioral undercontrol includes characteristics such as negative emotionality, low constraint, risk-taking, and sensation-seeking and is also component of externalizing childhood psychiatric disorders such as conduct disorder. My data suggests that actively anxious behaviors such as teeth grinding and yawning, sometimes described as displacement behaviors (Maestripieri et al., 1992; Schino et al.,

1996), may relate to aggressive defensive behaviors elicited by the HIT and to a lesser degree future ethanol self-administration. Although past non-human primate studies have suggested a relationship between anxiety and alcohol preference (Collins and Myers 1987), my results indicate that chronic heavy ethanol intake may only relate to specific components of anxiety, particularly those encompassed by behavioral undercontrol rather than behavioral inhibition.

Interestingly, chronic ethanol self-administration did not increase aggressive behavior further from baseline levels as predicted. Instead, chronic ethanol self-administration decreased inhibitory behavior (freezing) independent of intake or intoxication, and dose-dependently decreased aggression, while control animals with no access to ethanol did not show any significant changes in behavior from baseline. The decreased aggression observed in the drinkers was likely due to the differential effects of the two limbs of the BEC curve that are characterized by distinctly different mood states and differing arousal levels (Holdstock & de Wit , 1998; Holdstock et al., 2000; Martin et al., 1993; Newlin & Thomson; 1990; Papineau et al., 1998). The 22-h/d access to ethanol was not restricted in these animals prior to the second temperament test. Due to common intake patterns (Baker et al., 2014), monkeys with measurable ethanol levels directly prior to the HIT were likely experiencing effects of the descending limb of intoxication, which is less associated with aggression than the ascending and acutely intoxicating limb (Giancola & Zeichner, 1997). While one subject included in this study was above the BEC typically required to produce aggression (80 mg/dl; see Gustafson, 1985; Pihl & Zacchia, 1986), this heavy drinking monkey was likely on the descending limb of intoxication, as his average BEC on the ascending limb was 160 mg/dl. Sex has

also been suggested to mediate the effects of alcohol on aggression, with some studies showing men but not women engaging in alcohol-related aggression (White et al., 1993; Bond & Lader, 1986; Gustafson, 1991), while others suggest that both sexes exhibit aggression following alcohol exposure (Dougherty et al., 1999). In this study, neither male nor female subjects exhibited increased aggression in the second temperament test. However, BECs prior to the HIT were not assessed in the female subjects, and therefore it is unknown whether female subjects were similarly intoxicated. Overall, the lack of acute intoxication and the decrease in aggression observed in these monkeys during testing is supported by studies that have shown that acute alcohol intake, rather than chronic, is associated with incarceration for a violent offense (Collins & Schlenger, 1988).

Chronic ethanol access also decreased behavioral inhibition, a behavior associated with anxiety. While this effect was seen only in the ethanol drinking monkeys and not in control subjects, decreased inhibition was not correlated with intoxication at the time of the test or average daily ethanol intakes, suggesting that the entire range of daily ethanol consumption in these monkeys was sufficient to reduce behavioral inhibition from baseline levels. Despite a commonly held belief that alcohol reduces anxiety in people, research has infrequently supported this hypothesis. Only large doses of ethanol of at least 1 g/kg have reliably resulted in stress-reduction in humans (Levenson et al., 1980; Niaura et al., 1988; Zeichner et al., 1983). However, in humans, many factors including gender, predisposition for heavy drinking, and tolerance have all been found to influence anxiety-reductions following alcohol exposure (as reviewed in Wilson et al., 1989). Studies with rodent subjects have been similarly inconclusive, with

both increases and decreases in anxiety-like behavior during the elevated plus maze found less than 18 hours after ethanol exposure (Kliethermes et al., 2005). As seen with aggression, the intoxication curve also influences other moods, including anxiety (Holdstock & De Wit, 1998; Holdstock et al., 2000; Martin et al., 1993; Newlin & Thomson; 1990; Papineau et al.; 1998). However, few studies have assessed anxious state on the ascending and descending limb within the same population, and results have been inconclusive (Pihl et al., 2003). However, considerable research has been performed on the biphasic effects of alcohol on cognitive processes, particularly those related to response inhibition and activation (Ostling & Fillmore, 2010; Pihl et al., 2003; Schweizer et al., 2006). It is possible that the reduction in anxiety and aggression observed in the subjects included in the current study is due to altered executive cognitive functioning, which encompasses the ability to use external feedback to moderate behavior and attention (Baddeley & Della Sala, 1998). Therefore, future studies in this model should document anxious and aggressive reactivity on both the ascending and descending limbs of the daily intoxication patterns as well as investigate ethanol-induced changes in cognitive function to fully understand the effects of chronic ethanol drinking on changes in temperament.

Most frequently, the HIT tests assess behavioral inhibition and temperament in infant subjects, which differ from the late adolescent population tested in this study. My results indicate that the HIT can also be used as an assessment tool in late adolescent-young adult subjects, although the behaviors observed may differ in type and frequency when compared to infant subjects. For example, infant subjects frequently exhibit distress vocalizations such as coos which were not observed in the subjects included in

these studies (Gottlieb and Capitanio 2013). Nevertheless, in response to a threatening situation, adult subjects show similar responses and behavioral strategies in response to threat as infants. Kalin and Shelton initially proposed two strategies: behavioral inhibition and aggression (1989), whereas more recent research has suggested additional factors such as activity and emotionality and anxiety (Kinnally et al., 2010; Gottlieb and Capitanio 2013). The results from this study suggest similar predominant strategies, using underlying cohesiveness among multiple outcome variables to create singular categories of temperament robust enough to be applied to other domains such as ethanol self-administration (see Materials and Methods section and McClintick & Grant, 2016). Interestingly, sex differences in response to the HIT were also observed.

However, when compared by sex, ethanol self-administration was comparable in male and female monkeys. Sex differences in ethanol self-administration using macaque monkeys are not always found, with reports of similar levels of self-administration between male and female cynomolgus (Pakarinen et al., 1999) and rhesus monkeys (Vivian et al., 1999) or higher levels of ethanol self-administration in male cynomolgus (Vivian et al., 2001; Grant et al., 2008b) and rhesus monkeys (Fahlke et al., 2000). However, a direct comparison between these studies is very difficult as rearing and housing conditions, age, and ethanol concentration and access differ. The relationship of temperament measures to stress and gonadal hormones will be useful to unravel the role of sex in behavioral responses and subsequently help to explain sex effects on ethanol self-administration.

In conclusion, late adolescent rhesus monkeys demonstrate individual differences in behavioral responses during temperament testing and in subsequent

ethanol self-administration. Specifically, the degree of aggression observed during testing was associated with future ethanol consumption and intoxication. On the other hand, chronic ethanol self-administration decreased aggressive behavior, independent of drinking status. The differences observed between high and low aggression subjects are analogous to the differences observed in human adolescents, with aggression serving as a potent predictor of future substance abuse (Disney et al., 1999; Boyle et al., 1999; Fothergill and Ensminger 2005, Pardini et al., 2007; Fergusson et al., 2007; Jester et al., 2008). However, the open-access conditions of this study make it difficult to test all monkeys under the same stage of intoxication to assess changes in aggression or anxiety as a result of chronic ethanol self-administration.

4.3 **Aim 2: Amygdalocortical circuitry of anxiety and aggression**

A possible role for brain network control of aggressive, anxious, and inhibited temperament was assessed by examining connectivity at rest between amygdala subnuclei and the dlPFC, dACC, and OFC in a group of male and female rhesus macaques. This study appears to be the first to use a nonhuman primate model to assess alterations in amygdalocortical connectivity at rest associated with responses to social threat. The results indicate that intrinsic fluctuations in the BOLD signal at rest between specific amygdala-PFC are associated with temperament in distinct patterns. Negative connectivity at rest between the right BA and right dlPFC was observed in high aggression subjects, versus positive connectivity in low aggression subjects. Increased positive rsFC between the right CeA and left OFC was found in high anxious subjects compared to low anxious subjects, while rsFC from the right LA to the left OFC and from

the right BA and LA to the left dlPFC was more strongly anticorrelated in high inhibition subjects versus low inhibition subjects. These results are consistent with findings from prior research with human subjects linking impaired PFC-amygdala network function to anxiety (Hahn et al., 2011, Etkin & Wager, 2007; Fulwiler et al., 2012) but also suggest that the amygdalar subnuclei and PFC connections that are included in analyses are of crucial importance.

These results cannot be directly compared to most past research due to the use of adolescent macaque subjects and assessment of anxiety and aggression via behavioral rather than self-report/diagnostic methods. Nevertheless, the impaired amygdala-PFC networks found are generally consistent with research using adult and adolescent human subjects examining pathological anxiety (Social Anxiety Disorder [SAD], Generalized Anxiety Disorder [GAD]) and aggression (Conduct Disorder [CD], trait aggression), and a single study assessing anxiety in periadolescent macaques (Birn et al., 2014). Research suggests that efficient cross-talk, or top-down and bottom-up interactions, between the amygdala and PFC is related to improved control of anxious and aggressive behavior (reviewed in Kim et al., 2011; New et al., 2009). A past study comparing emotional regulation effectiveness with functional coupling between the amygdala and medial prefrontal cortex (mPFC) found that connectivity was strengthened during cognitive reevaluation of emotional stimuli and positively correlated with self-reported effectiveness of emotional regulation (Erk et al., 2010), while another study reported beneficial anxiety outcomes to be positively associated with functional connectivity between the amygdala and ventromedial prefrontal cortex (vmPFC) (Kim et al., 2010). Interestingly, one study examining SAD found decreased rsFC between left

amygdala and right OFC in patients versus healthy controls, mirroring the negative association between right LA and left area 12 (OFC) and behavioral inhibition found in the present study. Decreased rsFC between amygdala and dlPFC was found in SAD patients (Prater et al., 2012) and monkeys with anxious temperament (Birn et al., 2014), similar to the association between anticorrelated BA/LA – dlPFC connectivity observed in these high inhibition subjects. Another study found dissociated typical amygdala-PFC rsFC and anxiety, with anxious subjects showing attenuated (typically negative) rsFC between amygdala and dmPFC and negative (typically positive) rsFC between amygdala and vmPFC in healthy subjects in absence of external stimuli (Kim et al., 2011). In these macaque subjects, behavioral inhibition and active anxiety were associated with distinct right amygdala - left PFC connections as well as dissociation between typical amygdala-PFC rsFC. While behavioral inhibition was associated with stronger anticorrelated connectivity from the BA and LA to the dlPFC and OFC, active anxiety was instead associated with stronger positive connectivity from the CeA to OFC.

Significantly less research has been performed on the functional neural correlates of aggression, with a single study each examining rs-fcMRI associations with trait aggression (Fulwiler et al., 2012) and a small number of studies investigating conduct disorder (Finger et al., 2011; Zhoue et al., 2015; Lu et al., 2015). Both Lu et al. and Finger et al. utilized whole amygdala seeds and found anticorrelated amygdala-PFC connectivity in subjects with higher levels of aggression. While these studies did find a similar directional association (anticorrelated connectivity) with aggression as observed my sample of rhesus macaque subjects, the specific ROIs associated with aggression differed, with my subjects exhibiting stronger anticorrelations between the

BA and dlPFC. Lu et al. and Finger et al., conversely, found reduced functional connectivity from the amygdala to the anterior cingulate cortex and OFC, but not dlPFC. However, it is unclear which dlPFC regions (if any) were included within each analysis, and a whole amygdala seed (rather than specific amygdalar subnuclei) were used as seed regions, which may explain the lack of associations observed between amygdala-dlPFC rsFC and aggression. Notably, this within my sample of monkeys, nominal (p > 0.01 associations between aggression and negative rsFC between amygdala and similar PFC regions were observed ([LA and BA] and OFC [area 11 and area 12]), suggesting that group differences in similar regions as those found in Lu et al. and Finger et al. may be observed in a larger sample of rhesus macaque subjects.

Given the difference in the two anxiety-related phenotypes (behavioral inhibition and active anxiety) and the lack of association observed between the two (see Chapter 1; McClintick & Grant, 2016), it is unclear whether the different functional correlates of each phenotype occur due to differing behavioral outcomes (responding actively to a threat or freezing and avoiding the threat) or due to differing functional relationships between each amygdala subnucleus and the PFC. Across all subjects in this study (n = 22), average connectivity between the right amygdala and left OFC is negative while connectivity between the right amygdala and left dlPFC is generally positive. Interestingly, these data suggest connectivity associated with maladaptive anxious behavior opposes the typical direction of connectivity of the group as a whole or non-anxious subjects (as seen in Kim et al., 2011), and suggests that the relationship of these regions with anxious behavior may be a function of the functional connectivity typically underlying each connection, rather than an effect of the specific type of

behavioral output. For example, in the case of extreme inhibition, the group as a whole generally shows positive connectivity from the BA and LA to dlPFC and OFC. However, extremely inhibited subjects exhibit negative connectivity.

While prior studies have assessed associations between anxiety (but not aggression) and connectivity from the basolateral amygdala and the centromedial amygdala to the PFC and other cortical regions (Roy et al., 2013; Qin et al., 2014; Birn et al., 2014), the lateral and basal amygdalar nuclei have not been studied separately. Additionally, many studies assess the amygdala as a single entity, which would likely mask the results observed in my sample of monkeys, and overlooks the independent functions and patterns of connectivity in each individual subnucleus. The amygdala is composed of heterogeneous nuclei and is extremely structurally and functionally complex (Roy et al., 2009). As described in **Figure 1** of the introduction, the BA/LA and CeA are generally thought to serve different functions in the amygdalar complex, and correspondingly, differ in their structural connections with the PFC. The CeA in particular does not receive direct projections from the PFC, although recent research suggests that transmission of regulatory signals can occur across more complex and indirect pathways (Ekstrom et al., 2008). The BA and LA are believed to assist associative learning processes such as fear conditioning and processing of environmental stimuli through afferents including the PFC (Phelps and LeDoux, 2005) while the CeA facilitates behavioral responses via projections including the brainstem and some cortical areas (Davis, 1997; LeDoux, 2003). Processing of emotional information through the amygdala is generally thought to proceed in a serial manner from the LA to the CeA. However, more recent advances suggest that the BA/LA and

CeA may function both independently and in parallel to mediate distinct aspects of emotional processing (Balleine, 2005; Balleine & Killcross, 2006), particularly with regard to instrumental responses to conflict (Everitt, 1992). The BA and LA project extensively and reciprocally with the PFC and other sensory regions, and also project heavily to the CeA and striatum (Cardinal et al., 2002). The CeA also receives direct sensory input from the cortex and thalamus, and can independently influence behavioral output (McDonald, 1998). Birn et al. suggested that connectivity between the CeA and dlPFC could modulate anxious behavior via a polysynaptic structural network (2014). My results suggest that each sub nuclei of the amygdala demonstrates a distinct functional relationship with the PFC, even when comparing the lateral and basal amygdalar nuclei. These results are similar to those seen in studies comparing centromedial and basolateral subnuclei (Roy et al., 2009; Baur et al., 2013), with specific functional relationships being observed from each subnuclei to cortical areas of the brain. The findings suggest unique sources for maladaptive anxious behavior across amygdala and PFC subregions.

In this sample of monkeys, the rich, direct OFC and sparse dlPFC projections to the BA/LA show reduced functional connectivity in highly inhibited monkeys, the dlPFC projection to the BA shows reduced functional connectivity in highly aggressive monkeys, and the indirect (perhaps polysynaptic) dlPFC projection to the CeA shows higher positive functional connectivity in highly anxious monkeys. A prior study in macaques found an association between decreased CeA-dlPFC rsFC and increased CeA metabolism (Birn et al., 2014), suggesting that functional connectivity may influence behavioral outcomes via metabolic activity of the amygdala. However, the

differing relationship between amygdala-dlPFC rsFC in highly anxious (higher positive rsFC) and inhibited (anticorrelated rsFC) subjects complicate this possible explanation. It is likely that the influence of dlPFC activity on the BA/LA and the CeA differ, as evidence of a direct pathway between the dlPFC and BA exists while no direct pathways between the dlPFC and CeA have been found (Freese et al., 2009; Barbas & De Olmos, 1990; Amaral & Price, 1984). However, the relationship between alterations in rsFC and metabolic activity has not been casually established, and many alternative explanations (and causal pathways) may explain the association between altered amygdalocortical connectivity and temperament. However, these findings highlight the importance of assessing the discrete role of each amygdalar subnuclei.

Given that resting-state networks are largely stable across behavioral states, levels of consciousness, and species (Gusnard et al., 2001) the monkey model can provide useful information about the neurobiology of aggressive, anxious, and inhibited behavior. Importantly, all subjects in these studies were reared similarly, and thus likely exposed to more consistent levels of stress than typical human populations. Additionally, the use of non-invasive translational brain imaging techniques such as rs-fcMRI appears useful for the study of emotional regulation in both human and monkey models. Future work to extend these findings in human subjects and task based studies will provide further evidence of the importance of distinct amygdalar subnuclei for the study of temperament and emotional dysregulation.

Overall, these results suggest that dysregulated connectivity at rest between the amygdala and PFC reflect individual differences in temperament. Aggressive, behaviorally inhibited, and actively anxious responses to threat emerge as distinct

phenotypes with discrete functional brain circuitry correlates, suggesting that behavioral phenotypes, brain hemispheres, and amygdalar and PFC ROIs should not be collapsed in future research. Additionally, this study was performed in anesthetized macaque subjects in the absence of anxiety-inducing stimuli, and provides supporting information to task-based research studying emotion regulation. These data suggest a link between anxiety- and aggression-related defensive behaviors and amygdala-PFC rsFC, establishing a role of functional networks that have been suggested to regulate emotion and stress in the expression of temperament.

4.4 **Aim 3: Amygdalocortical circuitry of heavy ethanol intake**

A possible role for brain network control of heavy ethanol self-administration was assessed by examining connectivity at rest between amygdala subnuclei and the dlPFC, dACC, and OFC in the same group of male and female rhesus macaques as in Aim 2. Despite higher levels of aggression positively correlating with heavier ethanol intake in Aim 1, baseline connectivity between the right BA and right 9, which significantly differed in high and low aggression animals in Aim 2, did not differ between future heavy and non-heavy drinkers .The connections associated with behavioral inhibition and active anxiety in Aim 2 also did not differ in heavy and non-heavy drinkers, and were not correlated with future ethanol intake or BECs. However, incorporating all ROIs into the analysis, and not limiting the analysis to those connections associated with baseline temperament, uncovered significant differences in synchronous signaling between the left BA and left OPAI (OFC) and between the right CeA and left area 8 (dlPFC). These connections are completely distinct from those

associated with temperament at baseline in terms of specific region and laterality, with right amygdalar seeds associating with temperament but left amygdalar seeds associating with heavy drinking. Similarly, although the general PFC areas (OFC and dlPFC) associated with temperament and heavy drinking do overlap, aggressive and anxious temperament generally associated with anticorrelated amygdala-PFC connectivity, whereas heavy drinking was associated with more highly positively correlated amygdala-PFC connectivity at baseline as compared to non-heavy drinkers.

The higher positive correlations between the amygdala and OFC/dlPFC observed in the heavier drinkers at baseline were somewhat surprising, both given the anticorrelated connectivity associated with temperament risk factors, and also that research assessing amygdala-PFC rsFC in individuals addicted to other classes of drugs (opioids, stimulants) consistently observed decreased rsFC strength in addicts (Gu et al., 2010; Liu et al., 2009; Upadhyay et al., 2010; Wang et al., 2010; Xie et al., 2011). However, several possible explanations for these differences exist. Firstly, differing directional relationships in the regions associated with heavy drinking and temperament at baseline may, as argued in Aim 2, be a consequence of the underlying neuroanatomical characteristics of the connections identified. This concept is especially important when considering the role of the dlPFC, which does not densely project to the BA, LA, or CeA as observed in the OFC and ACC. Instead, the dlPFC is strongly reciprocally connected with the OFC and has minor projections to the BA (Amaral & Price, 1984; Stefanacci & Amaral, 2002; Ghashghaei et al., 2007, see **Figure 1**). Despite this lack of rich structural connectivity, evidence of amygdala-dlPFC coupling has been observed during emotional regulation (Banks et al., 2007; Birn et al., 2014).

Secondly, the studies observing reduced amygdala-PFC rsFC specifically found differences in the mPFC, which was not included as an ROI in these analyses, preventing replication of their findings. Finally, the current study differs from prior research in major methodological ways, with a primary difference being the drug-naïve state of the subjects in this study. The subjects included in prior studies were known drug abusers with established drug taking histories. Therefore, the associated alterations in rsFC can only be considered associations, and are likely consequences of drug use rather than pre-existing abnormalities. The significant positive rsFC connectivity in the current study is observed in monkeys *prior to* ethanol exposure. And therefore, it is not entirely unexpected that the directional relationship of rsFC associations might differ, as factors such as acute withdrawal (Gu et al., 2010; Ma et al., 2010; Tomasi et al., 2010; Wilcox et al. 2010) and acute drug effects (Ma et al., 2010, Upadhyay et al., 2011) have been shown to influence rsFC, and likely contribute significantly to variance within and between rsFC studies.

The increased positive rsFC between the left BA and left OPAI (OFC) and left CeA and right area 8 (dlPFC) observed at baseline in the heavy drinkers may confer risk for heavier ethanol consumption via influences within the amygdalocortical network, or in associated projections. In particular, the nucleus accumbens may act as a central relay-structure to modulate information processing within the corticolimbic circuit and between the limbic and mesolimbic areas (de Olmos & Heimer, 1999; Alheid et al., 1998). Relatedly, past research has demonstrated associations between extended amygdala – nucleus accumbens function and instrumental and associative learning (Cardinal et al., 2002), anxiety disorders (Sturm et al., 2003; Bewernick et al., 2010),

and drug seeking behavior (Di Ciano & Everitt, 2004; Whitelaw et al., 1996; Ito et al., 2004; Taylor & Robbins, 1986; Robledo et al., 1996; Parkinson et al., 1999). Interestingly, Di Ciano and Everitt (2004) found that neuropharmacological disconnection of the BLA and nucleus accumbens core reduced cocaine-seeking behavior, suggesting that alterations in connectivity between the amygdala and PFC could subsequently influence drug seeking or self-administration via the nucleus accumbens. Projections between the PFC and nucleus accumbens have also been shown to alter goal-directed behaviors (Parkinson et al., 2000; McFarland & Kalivas, 2001), further suggesting that altered connectivity within any component of the limbic-cortical striatal network (including amygdala – PFC) may subsequently influence behavior. However, given the lack of research assessing brain structure and function prior to alcohol use, it is difficult to determine the specific mechanisms underlying altered alcohol or drug related behaviors. As previously mentioned, it has been suggested that changes in rsFC may influence metabolic activity within the same network (Birn et al., 2014). Given the alterations in GABA neurotransmission in the PFC in human alcoholics (see Volkow & Fowler for review), it is possible that the atypically positive rsFC between the amygdala and OFC/dlPFC may influence GABA or glutamate brain function and metabolism in the heavy drinkers prior to ethanol access, subsequently altering perception of the reinforcing effects of ethanol once the animal is exposed. However, given the general uncertainty regarding the origins and significance of fluctuations in the BOLD signal (Logothetis, 2008; Greicius, 2008) a causal role of atypical rsFC in heavy ethanol intake cannot be established.

Notably, connectivity between the left CeA and right area 8 (dlPFC) also exhibited significantly different changes in connectivity from baseline after 6 and 12 months of ethanol access. Although heavy drinking was expected to further dysregulate connectivity, connectivity instead was altered in the opposite direction. In this case, heavy drinkers exhibited, on average, a 10% decrease in rsFC as a percentage of baseline connectivity, while controls exhibited an 8% increase in connectivity, on average. This effect suggests that chronic heavy drinking may produce neuroadaptative changes in connectivity. Interestingly, within the PFC, the dlPFC is uniquely highly vulnerable to alcohol-induced neuropathological changes (as reviewed in Harper, 1998), and there is also evidence of reduced regional glucose metabolism and blood flow in the dlPFC, OFC, and mPFC in chronic alcoholics (Volkow et al., 1992; Sullivan et al., 2000). There is also evidence of reduced glial density in the dlPFC and OFC in chronic alcoholism (Miguel-Hidalgo, et al., 2010; Miguel-Hidalgo et al., 2002), suggesting that the changes in connectivity observed between the dlPFC and CeA only after chronic heavy drinking might be reflective of pathological changes in the brain producing neuronal alterations. In support of this hypothesis, impairments of the neuronal-glial unit have been associated with altered BOLD amplitudes (Walter et al., 2009) and rsFC (Horn et al., 2010).

Drinkers and controls also exhibited significant differences in the change in connectivity from the right LA to the right area 9, similar to the connection associated with aggression at baseline (right BA to right area 9; same dlPFC ROI, both seeds within the BLA). Based on the behavioral associations of this similar projection, it seemed plausible that this region could also be associated with the change in behavior

from baseline (decrease in aggression) observed in the subjects with ethanol access. However, no significant associations between the change in aggressive behavior and the percent change in amygdala-PFC rsFC from baseline were observed. Instead, the percent change in rsFC from baseline between the left LA and left area 9/46 (dlPFC) and the change in freezing behavior from baseline, and the percent change in rsFC from baseline between the left LA and right OPRO (OFC) and the change in active anxiety from baseline. Notably, these connections are similar to those associated with behavioral inhibition and active anxiety at baseline, though not identical, suggesting consistency in the regions mediating anxious behaviors.

4.5 **Aim 4: Cynomolgus macaques**

Assessment of temperament with the HIT and amygdalocortical rsFC with rs-fcMRI both at baseline and after chronic self-administration of ethanol and/or water provided the opportunity to compare species relationships across anxious and aggressive temperament, amygdala-PFC connectivity, and heavy ethanol self-administration. This analysis also provided an opportunity to independently verify the data analysis steps used with the rhesus macaques (see **Figure 6**). Non-significant species and amygdalocortical connectivity differences were observed, suggesting that rhesus and cynomolgus macaques generally exhibit similar behaviors and have similar intrinsic amygdala-PFC connectivity at rest, though the durations of behavioral responses to threat did differ non-significantly. While temperament measured at baseline prior to ethanol access was not predictive of future heavy ethanol intake, changes in aggressive behavior after 6 months of ethanol access were associated with

prior heavy ethanol intake, as observed in the rhesus macaques. Similarly, although temperament was not associated with distinct alterations in rsFC between the amygdala and PFC, amygdalocortical connectivity at rest between the CeA and OFC was predictive of future heavy ethanol intake, and also exhibited non-significant (trend) changes in rsFC after 6 months of heavy ethanol intake in the same regions associated with future heavy ethanol self-administration at baseline. Finally, in the subjects with access to ethanol, the change in freezing (behavioral inhibition) from baseline was associated with increased connectivity from baseline between the LA and OFC. Overall, these results suggest that there may be species-specific relationships involving temperament, while associations between amygdalocortical connectivity and ethanol self-administration are consistent across species.

4.5.1 *Species differences*

Average daily ethanol intakes and BECs across 6 months of access did not differ, suggesting that male rhesus and cynomolgus macaques self-administer similar levels of ethanol. As expected, cynomolgus and rhesus macaques differed in the duration of anxious and aggressive behaviors observed during the HIT, though non-significantly. Rhesus macaques generally reacted to the HIT for longer durations, and exhibited more anxiety at baseline. Rhesus subjects generally reacted more strongly to the Profile condition, freezing for 38 percent versus 3% of the interval. The lack of reactivity to the Profile condition may reflect the passive or “reserved” behavior attributed to cynomolgus subjects (Clarke & Lindurg, 1993). Given that the literature describes rhesus macaques as being more highly aggressive in response to humans

(Clarke & Mason, 1988; Sussman et al., 2013), it was surprising that the durations of aggression were nearly identical in male cynomolgus and rhesus macaques (open mouth threat duration: 10 vs. 8% of the interval; extreme aggression duration: 1 vs. 2% of the interval). It possible that the small sample sizes (n = 12 and n = 16) prevented detection of consistent behavioral differences in aggression. Changes in behavior from baseline were also similar across species, with all subjects generally exhibiting decreases in anxiety behavior in response to the Profile condition. A single trend towards a species difference in amygdalocortical rsFC at baseline was observed, with rhesus macaques exhibiting non-significantly stronger anticorrelated connectivity at baseline between the right LA and left area 11 (OFC, $p = 0.003$), suggesting that rhesus and cynomolgus macaques exhibit largely similar functional connectivity between the amygdala and prefrontal cortex, reflecting the general consistency within the amygdaloid complex described in historical neuroanatomical studies (Aggleton, 1985).

4.5.1 *Temperament as a risk factor or consequence of heavy ethanol intake in cynomolgus macaques*

Responses to the HIT were not associated with future ethanol intake. This was somewhat surprising, although temperament has never been assessed in a longitudinal ethanol self-administration model with cynomolgus macaques. However, the sample size of subjects with access to ethanol was very small (n = 8), and it is possible that this population randomly included largely non-reactive subjects. Given the method of comparing the upper and lower quartiles of all subjects, a sample pool of generally non-reactive subjects would result in a comparison of subjects that did not differ as strongly

behavior as in the rhesus subjects. A related issue was that only two heavy drinkers were included, and both were close to the cutoff for heavy ethanol consumption with average daily intakes of 2.8 and 3.0 g/kg/d. It is possible that a larger pool of cynomolgus subjects with heavier drinkers and a higher degree of reactivity could observe associations between temperament and future heavy drinking. Alternatively, given the differences in temperament between cynomolgus and rhesus macaques, it is possible that responses to the HIT are only predictive of risk in rhesus macaques. Future work to include larger sample pools with both male and female subjects will help provide evidence to support either conclusion.

Despite the lack of association between baseline behavior and future heavy drinking, the change in aggression (open mouth threat duration) was significantly negatively correlated with average daily ethanol intake and intoxication during the 6 months of ethanol access. A trend towards a positive association with the change in active anxiety was also observed . The negative association between drinking variables and the change in aggression replicated the association between decreased aggression and prior drinking in the rhesus subjects, suggesting that the effects of chronic heavy drinking consistently decrease aggression when testing during the descending limb.

4.5.3 *Amygdala-PFC rsFC and temperament*

While rhesus subjects exhibited significant associations between amygdalocortical connectivity at rest and temperament, no significant associations were observed in correlational or group comparisons in the cynomolgus macaques. However, the extremely small sample sizes of temperament groups (n = 3) resulted in very

underpowered analyses. Additionally, due to the use of a quartile approach to separate out highly anxious and aggressive animals, it is possible that the overall sample included in this study exhibited lower reactivity in response to the HIT, eliminating the opportunity to assess the neural correlates of extreme expressions of anxiety and aggression. This would also support the lack of behavioral associations with drinking described above, suggesting that subjects classified as “high” and “low” inhibition, anxiety, and aggression were not behavioral different enough to detect associations with amygdala-PFC connectivity. Unfortunately the HIT is more commonly used with rhesus macaque subjects, precluding the opportunity to compare durations of anxious and aggressive behavior with the results of past research.

4.5.4 *Amygdala-PFC rsFC and heavy ethanol intake*

The associations between amygdalocortical rsFC and heavy ethanol intake in the cynomolgus macaques were very similar to those observed in the rhesus macaques. Again, higher positive connectivity rather than anticorrelated or reduced rsFC was associated with future heavy ethanol intake. Connectivity from the left CeA to the left area 13 and right are 12 (both OFC) exhibited significantly more strongly positive functional connectivity in the two heavy drinkers. Due to the very small sample size, these results must be considered preliminary. However, it is promising that both rhesus and cynomolgus heavy drinkers exhibited higher positive amygdala-PFC rsFC at baseline. Additionally, though the effects only trended towards significance, the same regions that trended towards predicting future heavy drinking were also trended towards distinct alterations between heavy and non-heavy drinkers. More specifically, rsFC

between left LA and left area 9/46 (dlPFC) was marginally higher at baseline in future heavy drinkers (p = 0.003) and also displayed reduced connectivity after 6 months of ethanol access in heavy drinkers, whereas non-heavy drinkers were characterized by increased connectivity. Although no statistically significant alterations in connectivity were observed, these trends reflect the same relationship observed in the rhesus macaques and also within the dlPFC. Given the small sample size and lack of very heavy drinkers in the cynomolgus subjects included in this study, it is not surprising that significant changes in connectivity were not detected. Overall, the consistency in associations between amygdala-PFC rsFC and drinking outcomes seen in both cynomolgus and rhesus macaques is promising and suggests consistency in measures as well as the neurobiological relationships.

4.5.5 *Alcohol induced changes in behavior and amygdala-PFC rsFC*

Although heavy alcohol consumption significantly decreased durations of aggression in response to the HIT, these behavioral changes were not associated with changes in amygdala-PFC connectivity from baseline. This reflects the lack of association between aggression and anxiety and amygdalocortical connectivity observed at baseline.

4.6 **Limitations**

Although these studies present novel results from the first longitudinal examination of the relationship between temperament and amygdalocortical

connectivity at rest in rhesus and cynomolgus macaques, there are a few important limitations of these studies that should be addressed, including the small sample sizes, use of male and female subjects, the specificity of the hypothesis, and the assessments of anxiety and aggression.

Firstly, due to the small sample size, male and female subjects were included in the same analysis for the rhesus subjects. A single rhesus sex difference in connectivity between amygdala and PFC was observed, though non-significant differences were also observed. Sex differences in responses to the human intruder test were also observed in the rhesus subjects, with females reacting for longer durations. While sex was controlled for in the correlational analyses via partial correlations, the group effects were collapsed across sex. The role of sex in the relationship between temperament, rsFC between amygdala and PFC, and alcohol self-administration cannot be addressed in these studies, nor can the role of age, as all subjects were late adolescent. Future work addressing the role of sex in longitudinal assessments of risk factors versus consequences of alcohol use are crucial, as significant evidence of sex differences have been identified previously (for review, see Nolen-Hoeksema, 2004).

The small sample size utilized in these studies also resulted in small samples of highly aggressive subjects. Aggressive responses to the HIT are significantly rarer than anxious responses, resulting in shorter durations of aggression required to reach criteria for high aggression. This may have complicated group difference analyses of amygdala-PFC rsFC, which may be one only one significant effect was observed, and no effects were observed in the expected OFC regions. Future research seeking aggressive

subjects could help disentangle this relationship, and would more closely resemble the recruiting practices of human research.

In all resting state analyses, a select number of specific amygdala-PFC connections were explored using a seed-based analysis with a-priori hypotheses. My conclusions are therefore limited to only these regions, and I cannot assess the possible role of any other regions within the brain. The strong relationships observed between temperament and amygdala-PFC rsFC are mirrored by the frequency with which these regions are associated with anxious behavior in the literature, suggesting that these data are consistent with expected outcomes from whole brain analyses. While it is likely that heavy ethanol drinking would also be related to reward-associated circuitry in regions such as the striatum, these regions would be far less likely to mediate the relationship between temperament risk factors and heavy ethanol self-administration, and thus were not examined in these studies.

Finally, the use of a behavioral measurement of anxiety and aggression in these non-human primate subjects results in difficulty comparing these findings to the rest of the literature. To date, all research examining associations between aggression/anxiety and connectivity at rest (in any brain region) has been performed with human subjects. In these human studies, anxiety and aggression are studied as a state, trait, or symptom of GAD, SAD, or CD (Baur et al., 2013; Oathes et al., 2015; Roy et al., 2013; Kim et al., 2010; Liao et al., 2010; Baur et al., 2013; Fulwiler et al., 2015, Zhou et al., 2015). Surprisingly, there is little consensus across human subject studies regarding the direction of these associations, with several studies indicating higher positive connectivity between amygdala and cortical regions (Baur et al., 2013; Liao et al.,

2010), negative connectivity (Kim et al., 2010; Hahn et al., 2011; Pannekoek et al., 2013, Fulwiler et al., 2015) and uncorrelated connectivity (Kim et al., 2010). While methods similar to the HIT have been used to assess behavioral inhibition in children, associations with resting state networks have not been performed. The differences found in the results of this non-human primate data versus those seen in studies assessing human adult participants could be due to species or age effects, or could be due to the use of behavioral rather than clinical assessments of aggression and anxiety. Relatedly, a single behavioral measurement (the HIT) was used to assess temperament. It is possible that other behavioral assessments of aggression and anxiety in non-human primates (such as the Intruder Challenge Test) could characterize different aspects of anxiety and aggression and associate with ethanol self-administration and amygdalocortical connectivity in different patterns. As observed in a subset of the monkeys included in these studies, inhibited behavior observed during the novel objects test at baseline was not associated with future ethanol self-administration or intoxication (McClintick & Grant, 2016). Relatedly, behavioral inhibition in response to the novel objects was not significantly associated with behavioral inhibition during the HIT, suggesting that the behavioral assessment used is extremely important, and that results may not be generalizable across models (McClintick & Grant, 2016). Future research utilizing multiple measures of anxiety and aggression will help disentangle the possibility of distinct neural correlates of differing measures of anxiety and aggression.

4.7 **Future Directions**

While these studies were the first to examine intrinsic connectivity between amygdalar subnuclei and cortical brain regions in association with temperament and ethanol self-administration in non-human primates, significant opportunity for future lines of related research exist. Given that this is a translational model of alcohol abuse, an obvious future direction would be an examination of amygdalocortical rsFC in the same regions in a population of alcohol dependent human subjects. Given the absence of studies examining associations between alcohol dependence and amygdala rsFC in the literature, this work could provide insight into whether the effects observed in these macaque subjects reflect those observed in humans. The use of human subjects would also allow for task-based models, which could further probe the role of emotional regulation in this network.

Another avenue of promising research is the potential role of the hypothalamic-pituitary-adrenal (HPA) axis. Dysregulated brain connectivity within the amygdala and PFC or within other regions of the brain (such as the hypothalamus) could be a source of both altered behavior and HPA axis reactivity. A few recent papers (Veer et al., 2012; Kiem et al., 2013) have examined HPA axis reactivity and basal hormone concentrations with regard to amygdala functional connectivity and found significant relationships with the PFC. Given the strong association between HPA axis reactivity and temperament, it is possible that HPA axis reactivity could also associate with the altered rsFC observed in subjects in these studies and provide evidence for endogenous hormone modulation of functional connectivity.

The addition of fMRI and DTI analyses of amygdalocortical structure and function would also be useful additions to this work. By using only rs-fcMRI to assess neural function, conclusions cannot be drawn about activity within the amygdala or prefrontal cortex. Given the complex inhibitory and excitatory projections occurring both within the amygdala and PFC regions alone, and between the two, the lack of information about activity within each region to compare with the functional connectivity results can prevent conclusions regarding how the connectivity might relate to behavioral or drinking outcomes. Additionally, there is substantial evidence of hypoactivity in the amygdala and OFC and hyperactivity in the dlPFC during emotional processing in addicts (Wang et al., 2010; Li et al,, 2009), which, if assessed in concert with intra-amygdalar and intra-cortical connectivity provide some additional information about the communication occurring between connected regions while activity fluctuates. Similarly, structural differences in PFC laterality have been observed in subjects with increased susceptibility for developing alcohol dependence (Hill et al., 2009), and the use of non-invasive and longitudinal imaging techniques to layer with rsFC analyses would help tease apart possible outcomes of intrinsic connectivity alterations, as well as help form hypothesis regarding the development of the atypical connectivity itself.

Relatedly, the study of the projections between the dlPFC, OFC, and ACC and between the amygdalar subnuclei could also provide valuable information about the potential interactions indirectly influencing amygdala and PFC connectivity. The PFC and amygdalar regions included in these studies are densely interconnected and assessing only amygdala-PFC connectivity results in conclusions only being drawn about the major inputs and outputs of the amygdaloid nucleus. Along the same lines,

the use of whole brain analyses would determine whether the other regions outside of the amygdala-PFC network could be more strongly associated with alcohol consumption and temperament, particularly given the lack of consistency in regions associated with each variable.

Studies examining acute and chronic effects of ethanol on rs-fcMRI could provide invaluable information regarding the influence of ethanol on the brain at rest. Several studies have suggested that acute ethanol produces distinct alterations in rsFC (such as in the default mode network), even when the dose of ethanol is moderate (Zheng et al. 2015). The monkeys included in my study have ethanol access up to 1 hour prior to post-22-h scans, which can result in measureable BECs at the time of the scan. However, given that most studies have not assessed chronic and acute ethanol in the same population longitudinally, it is hard to assess the potential effects of mild intoxication in chronic heavy drinking monkeys. Understanding the influence of abstinence and chronic and acute drug exposure on rsFC networks will provide additional control for the assessment of risk versus consequence of alcohol abuse on the brain.

Finally, this model of ethanol self-administration in non-human primates provides the opportunity to test possible treatments to reverse alterations in brain connectivity and behavior associated with heavier ethanol intake. A focus on reducing amygdalocortical connectivity prior to exposure to alcohol could reduce the likelihood of becoming a heavy drinker, and thus prevent health consequences of heavy ethanol consumption. Identification of other possible regions associated with onset of heavier drinking would provide additional targets for this type of research. The use of non-

human primates, as previously discussed, provides especially helpful insight into human AUDs due to the similarities in brain structure and function and behavior. Given these similarities, the identification of biomarkers and the development of effective treatments in non-human primates could subsequently be used to prevent transition to AUDs in human subjects.

4.8 **Summary and Conclusions**

The studies included in this dissertation utilized a behavioral measurement of temperament and rs-fcMRI to assess the longitudinal relationship between intrinsic amygdalocortical functional connectivity, stress reactivity, and heavy ethanol consumption in a non-human primate model. The goal was to establish the role of anxious and aggressive temperament as predictors *and* consequences in the *same* subjects over a longitudinal period, and to assess the possible explanatory role of distinct amygdala and prefrontal cortical regions in these relationships.

This dissertation includes the first studies of the resting state functional connectivity correlates of anxious and aggressive temperaments in human or non-human primate subjects, as well as the first studies to longitudinally assess behavioral and neural risk factors and consequences of heavy ethanol use across the same non-human primate subjects. The main findings from these studies suggest that although dysregulated behavior (particularly presenting via aggression) is predictive of negative drinking outcomes, distinct patterns of connectivity at rest between discrete amygdala subnuclei and the PFC are associated with temperament and heavy ethanol use. Although an overlap between the neural circuitry associated with behavioral risk for

heavy drinking and regions independently associated with higher ethanol intakes was not observed , the altered connectivity between the central amygdala and PFC that emerges prior to ethanol access in heavy drinkers is subsequently influenced by chronic heavy drinking, with the dlPFC and OFC in particular representing areas both predictive of heavy drinking and vulnerable to alcohol-induced changes in rsFC.

The findings of these studies contribute novel information regarding the neural circuitry associated with risk for alcohol use, and can aid future research assessing the neural correlates of dysregulated emotional behavior and alcohol dependence. These data suggest that the use of non-invasive rs-fcMRI can provide valuable translational data that may help to identify and prevent biomarkers for alcohol dependence, and that corticolimbic circuitry warrants further research.

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