FOLLOW YOUR HEART: HEART RATE VARIABILITY REVEALS SEX DIFFERENCES IN AUTONOMIC REGULATION DURING ANXIETY-LIKE BEHAVIOR AND DURING ALCOHOL DRINKING IN RATS

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DEDICATION

For myself, my husband, my parents, and my brothers. And for my late grandmother, the Matriarch, whose endless curiosity about human psychology nurtured my own. L’dor V’dor.

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FOLLOW YOUR HEART: HEART RATE VARIABILITY REVEALS SEX DIFFERENCES IN AUTONOMIC REGULATION DURING ANXIETY-LIKE BEHAVIOR AND DURING ALCOHOL DRINKING IN RATS

Mental health conditions remain a substantial and costly challenge to society. Of note, women have a higher prevalence of anxiety disorders than men, and alcohol misuse in women has risen sharply in recent years. However, critical mechanisms underlying these observed sex differences remain incompletely understood. Measures of cardiac function, including heart rate (HR) and HR variability (HRV), reflect dynamic balance between the two opposing branches of the autonomic nervous system: sympathetic (SNS, “fight or flight”) and parasympathetic (PNS, “rest and digest”). Furthermore, recent evidence strongly suggests these measures are potential biomarkers for pathological states, including mental health conditions. To better understand sex differences in autonomic mechanisms related to pathological anxiety and alcohol misuse, we utilized cardiac telemetry to measure HR and HRV. This allowed observation of real-time autonomic tone in awake, freely behaving Wistar rats of both sexes. At baseline, female rats had greater HR and lower SNS influence than males, which concords with human studies. In both anxiety-like behavior and alcohol drinking studies, we observed that females tend to utilize a higher PNS influence to overcome challenge, whereas males tend to utilize higher SNS. Furthermore, female (but not male) baseline HR and HRV are related to within-task behavior, suggesting that baseline state impacts drinking and anxiety-like behavior in a sex-specific way. Again, these data concord with human research suggesting a similar PNS bias in women and SNS bias in men when responding,

especially under challenge. Taken together, these data have contributed new knowledge to sex differences in autonomic engagement, especially for anxiety states and alcohol drinking. Importantly, these findings are likely translationally relevant for the development of novel, and more personalized, therapies.

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LIST OF ABBREVIATIONS

ACh……………………………………………………………………..Acetylcholine
AIC…………………………………………………………...Anterior Insular Cortex
ANS……………………………………………………..Autonomic Nervous System
AUD…………………………………………………………Alcohol Use Disorder
CAN…………………………………………………Central Autonomic Network
CLAD…………………………………………Compulsive-like Alcohol Drinking
DMNX………………………………………………...Dorsal Motor Nucleus of X
GAD…………………………………………………...Generalized Anxiety Disorder
HFHRV……………………………………High Frequency Heart Rate Variability
HR………………………………………………………………………Heart Rate
HRV…………………………………………………………Heart Rate Variability
IBI……………………………………………………………Inter-beat Interval
NSF…………………………………………………Novelty Suppression of Feeding
LFHRV……………………………………Low Frequency Heart Rate Variability
NA ……………………………………………………………..Nucleus Ambiguus
NE………………………………………………………………Norepinephrine
NTS………………………………………………..…Nucleus of the Solitary Tract
PFC…………..………………………………………………Prefrontal Cortex
PNS..……………………………………………Parasympathetic Nervous System
RMSSD…………………………...Root Mean of the Square of Successive Differences
SDNN……………………………………………………Standard Deviation of N-N
SA node……………………………………………..………Sino-Artial Node
SNS………………………………………………………Sympathetic Nervous System

CHAPTER ONE: Introduction

1.1 Anxiety: It's Adaptive Until It Isn't

1.1.1 Defining "Anxiety"

Anxiety is regarded as an emotional response to ambiguous, uncertain, or improbable threats, and is often characterized by worry (Blanchard & Blanchard, 2008). This contrasts with fear, which is often in response to imminent, well-defined threats, (McNaughton & Zangrossi, 2008). Anxiety is a necessary emotion to have within one's response repertoire, as evidenced by its conservation across species throughout evolution (Campos et al., 2013; Blanchard & Blanchard, 2008). While one may initially believe anxiety is an emotion we should strive to eliminate, to do so would be catastrophic for the survival of the organism. For example, imagine walking along a path and seeing an undefined coiled mass ahead, and you are uncertain whether it is a snake. The individual who altered course to steer clear of that mass was more likely to avoid potential harm should the undefined coil indeed turn out to be dangerous, snake or otherwise. This individual would then have a better chance of passing their genes onto the next generation, thus increasing the likelihood of passing on this anxiety influenced avoidance strategy.

However, while anxiety is a necessary emotion, there are disorders in which the anxiety response becomes dysregulated, such that excessive worry over truly irrelevant information begins to interfere with functioning in daily life. For example, the DSM-V defines Generalized Anxiety Disorder (GAD) by constant, nonspecific, often irrational worry causing disruptions in daily life (American Psychiatry Association, 2013). Unfortunately, rates of anxiety disorders such as GAD continue to rise globally and pose a substantial cost burden on society totaling billions of US dollars per year (Chisholm et al., 2016). Curiously, anxiety disorders are often co-morbid with substance use disorders such as alcohol use disorder (AUD), where some individuals cite alcohol or drug use as a mechanism employed to regulate their anxiety (Koob et al., 2014).

1.1.2 Sex Differences in Anxiety

Important sex differences have been noted within anxiety disorders, for example women have nearly twice the prevalence of most anxiety disorders (including GAD) compared to males (Altemus et al., 2016). It is theorized that sex-specific vulnerability to anxiety disorders may be reflected in differential reproductive stressors. For example, women typically display enhanced social reasoning skills and emotional attunement with others, and this would be adaptive in the sex most poised for child-rearing (Altemus et al., 2016). However, the potential drawback of this skillset can induce more vulnerability to anxiety disorders as enhanced social perception could lead to increased rejection sensitivity and reacting more poorly to social separation (Altemus et al., 2016). From a neurobiological perspective, menstrual cycle hormones are neurologically active, effecting brain structures in addition to neuronal conductance (Williams et al., 2022). For instance, the neurological effects of cyclic hormonal shifts are implicated in premenstrual dysphoric disorder (PMDD), where hormone levels appear normal for cycle stage, and thus something about the neuronal sensitivity to these fluctuations appears critical for the disorder (Altemus et al., 2016).

Furthermore, male and female assessment of and reaction to anxiety subtly diverge. For example, a study in which otherwise healthy college students were asked to rate various threat scenarios and choose from a list of responses, females consistently rated the scenarios as more dangerous than males (Blanchard et al., 2001). Additionally, while males and females often correlated highly on their choice response to each scenario, females were more likely to choose to scream for help whereas males were more likely to choose to attack (Blanchard et al., 2001). These data also support the theory that sex differences in anxiety responses may relate to differing reproductive roles and pressures, where the "scream for help" option is the more socially inclined choice compared to "attack." Sex differences have also been noted in GAD, where males are more likely to externalize threat and report social issues, whereas women with GAD are more likely to internalize threat and report greater somatic symptoms like gastrointestinal distress and pain (Altemus et al., 2016).

Given that sex differences exist not only in presentation of anxiety but also in disorders of anxiety, it is important to scientifically determine possible mechanisms of such differences to develop efficacious, more personalized treatments. Clinical research is critical, since often the ultimate goal of scientific research is to improve human quality of life. However, stringent ethical constraints are necessary to avoid creating harm. Furthermore, human environments are subject to cultural and environmental effects beyond complete scientific control. Thus, animal models of are of immense value in order to (1) allow for critical mechanistic investigations to determine underlying biological processes, and (2) disentangle biological from socio-cultural influences on the presentation and prognosis of such disorders. Here, rodent models –particularly in the rat– are useful to model human pathology.

1.1.3 Rat Models of Anxiety-like Behavior

Defensive behaviors are those which are deployed in the face of threat, and these behaviors are highly conserved across mammalian species including humans and rodents (Blanchard & Blanchard, 2008). One of the most common species in the investigations of translationally relevant defensive behavior is the laboratory rat (Litvin et al., 2008). Although many species can be utilized for scientific research of anxiety-like behavior, an overview of that expansive literature is beyond the scope of this dissertation. Thus, we will focus specifically on rat models of defensive behavior, also referred to as anxiety-like behavior, in service of translationally relevant insights for human anxiety.

Multiple paradigms have been developed to assess aspects of rodent anxiety-like behavior and much theoretical work has been done to validate these tasks for the study of human psychopathology (Campos et al., 2013; Litvin et al., 2008; McNaughton & Zangrossi, 2008). Broadly, rodent anxiety-like behavior can be partitioned into two categories: conditioned and unconditioned responses (Campos et al., 2013; Lovick & Zangrossi, 2021). Conditioned responses involve non-ethological scenarios such as training a rat to anticipate footshock. Unconditioned responses reflect more innate, ethological reactions to stressors such as exposure to the odor of a predator, or placement in an unfamiliar territory (Lovick & Zangrossi, 2021; Campos et al., 2013; Litvin et al., 2008). The work presented in this dissertation focuses on unconditioned anxiety-like behavior paradigms. Thus, presented here is a non-exhaustive list of rat models of unconditioned anxiety-like behavior which have been helpful in determining mechanistic underpinnings of anxiety that are translationally relevant to humans.

The Open Field is one of the oldest experimental anxiety-like paradigms, and it has been modified over time (Litvin et al., 2008). The basic test involves placing the rat into a novel, uncovered, often (but not always) square shaped arena with vertical walls to prevent escape (Lovick & Zangrossi, 2021; Litvin et al., 2008). The test aims to measure "risk assessment behavior" where the rat is conflicted by an innate drive to explore novelty against an aversion to unfamiliar, potentially unsafe territory. The rat, an animal subject to predation, is especially averse to the open center away from the vertical walls (Litvin et al., 2008). Researchers then score the rat's behavior, most commonly the total distance traveled and average velocity of travel, though other behaviors such as rearing can be recorded, too. Considering sex differences, it is generally accepted that females locomote more within the Open Field (Lovick & Zangrossi, 2021). This concords with females typically having more "active" responses to stressors, such as darting or jumping, compared to males which more often freeze (Bangasser & Wicks, 2017). However, this paradigm, like virtually all others, was initially created for and validated in male rats. Furthermore, lack of consistency in experimental protocols (for example: arena size, test duration, handling procedures, amount of light, etc.) further complicates understanding of sex differences within the Open Field (Lovick & Zangrossi, 2021). Thus, there remains uncertainty and debate regarding sex differences in anxiety-like responding within this task.

The Elevated Plus Maze (EPM) and its relative the Elevated T Maze (ETM) also expose the rat to a conflict between novelty and relative safety. EPM consists of an apparatus raised above the floor (heights vary by lab) containing four equally sized corridors in the shape of a plus sign (McNaughton & Zangrossi, 2008). Two corridors are enclosed with high vertical walls. In contrast the other two corridors are open with no walls. The open and enclosed corridors are perpendicular to one another, such that adjacent to an enclosed arm is an open arm. Like the Open Field, the rat is hypothesized to prefer the relative safety of the enclosed arms to the relative vulnerability of the open arms. Researchers commonly record the time spent in the open arms versus the closed arms, and the number of transitions between the two can also be recorded (Campos et al., 2013). Rats often show increased signs of anxiety in the open arms, indexed by increased defecation and increased stress hormone (corticosterone in rats), and treatment with benzodiazepine anxiolytics increases time spent in the open arms (Pellow et al, 1986). Although the literature in rats is mixed, overall males and females appear to respond similarly in the EPM (Lovick & Zangrossi, 2021).

The ETM was born out of a desire to modify the EPM due to the lack of consistent predictive validity of EPM for serotonergic drugs, which are most prescribed for long term treatment of anxiety disorders (McNaughton & Zangrossi, 2008; Campos et al., 2013). Theoretically, the lack of efficacy of serotonergic drugs in the EPM is thought to reflect the lack of specificity of the test to differentiate anxiety from fear (McNaughton & Zangrossi, 2008). Anxiety, as defined earlier, relates more to risk assessment and aversion of a future perceived threat. Conversely, fear is thought to reflect action in response to a current or imminent threat. Within the EPM, the decision to leave the relative safety of the enclosed arm to explore the open arm is associated with risk assessment, akin to anxiety. Contrarily, retreat from an open arm into an enclosed arm reflects escape from the present aversive experience, more closely akin to fear (McNaughton & Zangrossi, 2008). Thus, the ETM was proposed to address these issues.

The ETM can be conceptualized as removing one of the enclosed arms of the EPM, leaving a "T" shape with three arms: two open arms one enclosed. The advantage of this set up is in the ability to differentiate inhibitory avoidance, which is more anxiety-like, from active escape, which is more fear-based (McNaughton & Zangrossi, 2008). The animal is placed in the enclosed arm and the latency to explore the open arms is recorded as a measure of anxiety-like behavior. Next, the animal is placed in one of the open arms and the latency to retreat to the enclosed arm is recorded as a measure of fear-like behavior (Campos et al., 2013). The predictive validity of anxiolytic drugs versus anti-panic (anti-fear) drugs is superior in the ETM compared to the EPM (McNaughton & Zangrossi, 2008).

The Light-Dark Box (LDB) consists of two equal-sized compartments, one exposed to light and the other dark, and a small entryway allowing the rat to freely move between them (Campos et al., 2013). Rats are nocturnal and possess an innate aversion to brightly lit areas. Thus, rats regularly demonstrate preference for the dark compartment. Like EPM/ETM, this task aims to measure the conflict between exploring novelty and aversiveness to display. Experimenters commonly record the latency to first enter the dark compartment, the time spent in either compartment, the number of transitions between compartments, and rearing (Lovick & Zangrossi, 2021). Recently, our lab demonstrated subtle sex differences in the LDB where the benzodiazepine diazepam significantly increased male latency to enter the dark compartment but not female (De Oliveira Sergio et al., 2021). This has been interpreted as diazepam decreasing male anxiety-like behavior, which allowed the animals to spend more time exploring the light compartment before moving to the dark, and this was not observed in females (De Oliveira Sergio et al., 2021).

The Novelty Suppression of Feeding (NSF) task is discussed at length in Chapter Two. Briefly, the NSF utilizes an arena like the Open Field, however, the NSF introduces food in the center of the arena under an aversively bright light. Prior to NSF testing, rats are food restricted to induce hunger (Campos et al., 2013). NSF creates a conflict scenario where a hungry rat must overcome its innate aversion to bright light and unprotected, open spaces to obtain food. The NSF model demonstrates good predictive validity, such that drugs that target human anxiety (e.g. serotonergic enhancing drugs and benzodiazepines) also mediate rodent performance in the task (Campos et al., 2013). Advantages of the NSF over other anxiety-like tests include (1) clearly defined motivational state based on drive to feed when hungry, and (2) multiple progressive measures leading up to food intake that can differentiate certain aspects of anxiety-like behavior (discussed in Chapter Two). Researchers most commonly measure the latency for the rat to first approach the center, the time spent in the center tolerating the light, the number of approaches to center, the latency to obtain food from the center, and the amount of food consumed during testing. Previously, our lab has shown important sex differences where females tend to show increased anxiety related to obtaining food. For instance, females demonstrated increased latency to obtain food and consumed less food within the NSF compared to males. Further, when treated with the anxiolytic diazepam, females were significantly faster to obtain food and consumed more food (De Oliveira Sergio et al., 2021). These data were interpreted to suggest female anxiety is more related to life-relevant tasks, such as obtaining food when hungry, and concords with female and male anxiety differing in subtle but significant loci.

1.2 The Problem of Maladaptive Alcohol Use

1.2.1 Maladaptive Alcohol Use and Sex Differences

Maladaptive alcohol use, broadly defined, is excessive use of alcohol that increases the risk of medical and social harms. The major psychiatric disease process associated with problem alcohol use is Alcohol Use Disorder (AUD), formerly known as Alcoholism. The DSM-V defines AUD as a "maladaptive pattern of alcohol use leading to clinically significant impairment or distress" (American Psychiatric Association, 2013). AUD poses a substantial financial burden to society, costing approximately 250 billion US Dollars per year (CDC, 2014). In the United States, excessive alcohol drinking is a leading cause of preventable death (Esser et al., 2022).

Unfortunately, rates of maladaptive alcohol use in women have risen sharply over the last several years (Carvalho et al., 2019; Grant et al., 2017). Furthermore, important sex differences exist regarding alcohol misuse and its consequences. For instance, compared to men, women escalate drinking more quickly, stabilize drinking at higher doses, and are more likely to relapse (Becker et al., 2017). Also, and perhaps a consequence of the above, women suffer disproportionately from the medical consequences of excessive drinking (Becker & Koob, 2016; Brady & Randall, 1999). In rodents, females have been shown to drink more than males (reviewed in Radke et al., 2021). Finally, alcohol misuse is often co-morbid with other psychopathologies, such as anxiety and mood disorders (Puddephatt et al., 2022) and women disproportionally suffer from these conditions (Altemus et al., 2016). Finally, few pharmacotherapies for AUD exist, and none have yet to address the sex differences specifically (Mann et al., 2018).

In sum, maladaptive alcohol use poses a substantial problem for our society and critical sex differences exist regarding the development and consequences of excessive drinking. Thus, it is of immense value to determine specific mechanisms underlying sex differences for alcohol misuse to better address these concerns.

1.2.2 Compulsive Alcohol Drinking: A Major Obstacle to Treatment

Compulsive alcohol drinking is defined as persistent intake despite known negative consequences (De Oliveira Sergio et al., 2023; Starski et al., 2023). The DSM-V criteria to diagnose AUD include several references to compulsive alcohol drinking. For instance, "continued alcohol use despite having persistent or recurrent social or interpersonal problems caused or exacerbated by the effects of alcohol" and "alcohol use is continued despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by alcohol" (American Psychiatric Association, 2013). In addition to the DSM-V, questionnaires used by clinicians to assess severity of alcohol problems --such as the AUDIT-- feature several inquiries related to compulsive drinking (Radke et al., 2021). Thus, compulsive drinking is an important facet of problem alcohol use.

1.2.3 Modeling Compulsive Alcohol Use in Rats

The use of rat models to address mechanistic questions that are translationally relevant to human disease has also been utilized in the study of compulsive alcohol drinking. A full discussion of animal models of maladaptive alcohol use is beyond the scope of this dissertation. However, specific rat models employed for the studies within this dissertation will be discussed. This includes brief discussion of developing a translationally relevant rat model that encapsulates features of compulsive-like drinking.

Our lab utilizes a long-term intermittent access to alcohol intake rat model (detailed in Chapter Two, Section 3.5.1 and Chapter Three, Section 3.2.1). This model capitulates several important facets of AUD, including escalation of intake over time (Simms et al., 2008), sensitivity to drugs used to treat human AUD (Simms et al., 2008), moderate withdrawal symptoms upon alcohol cessation (Steensland et al., 2012; Li et al., 2011), front-loading (a proxy for high motivation for alcohol) (Darvesky & Hopf 2020; Darevsky et al., 2019), and development of compulsive-like alcohol intake (reviewed in De Oliveira Sergio et al., 2023).

There are two major methods by which compulsive-like intake is measured in the rat: foot-shock resistant intake and quinine-resistant intake. Foot-shock resistant intake requires the rat to continue to drink despite aversive shock. Quinine-resistant intake involves adulterating alcohol with the bitter-tasting compound quinine, which serves as the aversion. Indeed, rats will drink quinine-adulterated alcohol in much higher quantities that quinine-adulterated water, supporting that the rewarding aspects of alcohol drive consumption under aversion (De Oliveira Sergio et al., 2023). Similar brain mechanisms are implicated in driving foot-shock and quinine-resistant alcohol intake, which both differ from intake without an aversive stimulus (reviewed in De Oliveira Sergio et al., 2023; Seif et al., 2013). Our lab prefers to use the quinine-resistant intake model, as it is technically simpler and cheaper to perform (De Oliveira Sergio et al., 2023). The curious reader is encouraged to read several recently published reviews from our lab for more information about compulsive-like alcohol intake models in rodents (De Oliveira Sergio et al., 2023; Starski et al., 2023; Radke et al., 2021).

Briefly, important sex differences in compulsive alcohol drinking have been observed. For example, our lab has recently demonstrated that licking microstructure differs during quinine-resistant drinking across sexes, where females have similar total licking compared to males despite higher consumption of alcohol both with and without quinine adulteration (De Oliveira Sergio et al., 2023b). The licking microstructure during compulsion-like responding revealed females have longer drinking bouts and appear to intake more volume per lick, thus suggesting subtle but significant sex differences in action strategy to drink despite aversion (De Oliveira Sergio et al., 2023b). These data likely reveal insights into sex-specific intake strategy differences that could partially explain the sex differences in alcohol misuse observed in humans, where females tend to have higher problems with alcohol drinking.

1.3 Heart Rate Variability: A Window into Health and Disease

1.3.1 What is Heart Rate Variability? Introduction

Heart Rate Variability (HRV) is the fluctuation in time between successive heartbeats. Over the last several decades, low HRV has been consistently shown to relate to higher all-cause mortality (Jarczok et al., 2022; Shaffer et al., 2014; Thayer & Lane, 2007; Sgoifo et al., 2015). Additionally, low HRV has been linked to a variety of disorders from seemingly disparate disciplines, such as cardiovascular disease (Sgoifo et al., 2015), myocardial infarction (Thayer & Lane, 2007), diabetic autonomic dysregulation (Ewing et al., 1985), sudden infant death syndrome (Shaffer et al., 2014), sudden unexplained death in epilepsy (DeGiorgio et al., 2010) and a variety of psychiatric disorders such as substance use disorders, depression, and anxiety disorders (discussed in Mulcahy et al., 2019). Recently, a large meta-analysis revealed that the relative likelihood of mortality (expressed as a hazard ratio) for those in the lowest quartile of HRV exceeded that for lack of sleep, sedentary lifestyle, and harmful alcohol drinking (Jarczok et al., 2022).

How can HRV, a seemingly simple measurement of variation between heartbeats, convey risk in such variegated fields? HRV is an index of neurocardiac function that reflects the overall coordination of the autonomic nervous system (Shaffer & Ginsberg, 2017; Thayer & Lane, 2009). Autonomics regulate and coordinate the most fundamental processes of an organism's biology, and thus dysfunction in this system can contribute to or be affected by issues of any organ system. It is now well supported that HRV indexes autonomic nervous system function, and mounting evidence continues to support that autonomic dysfunction is involved in a wide range of somatic and psychological disorders (Thayer & Brosschot, 2005). HRV serves as a biomarker of the very background processes (autonomic regulation) that keeps the body functioning as a unified whole. Thus, if we accept that HRV accurately indexes autonomic tone and autonomic dysfunction is implicated in a wide array of disease, then it follows that HRV is a compelling candidate for a biomarker to explore the mechanisms underlying various diseases in order to promote development of novel, efficacious therapeutics.

The heart does not act as a metronome, beating monotonously in identical pulses. On the contrary, the absolute time between each heartbeat is most often variable from one beat to the next. This phenomenon is viewed as natural flexibility built-into our physiology (Thayer et al., 2012; Thayer & Lane, 2009; Thayer & Lane, 2000; Porges, 1995; Porges, 2009; McCraty & Shaffer, 2015). Indeed, it is well accepted all the way back to Charles Darwin that appropriate adjustment to one's environment requires healthy flexibility (Porges, 1995; Porges, 2009; Thayer & Lane 2000, Thayer et al., 2012). As living beings, it is maladaptive to respond indiscriminately and identically to whatever we encounter. It is much more adaptive to interpret our environment, both internal and external, and select an appropriate response from a repertoire of possibilities. For example, one of the critical issues facing an individual with Post-Traumatic Stress Disorder (PTSD) is the misinterpretation of safe environments for dangerous ones (Sangha et al., 2020). It is as if these individuals have lost their ability to discriminate safety from peril. Indeed, in the more common affliction of Generalized Anxiety Disorder (GAD), people often realize their anxieties are misplaced or exaggerated, and yet they cannot help but excessively worry about perceived, albeit often ambiguous, threats (Thayer & Lane, 2000). Something within these populations living with anxiety disorders, be it GAD, PTSD, and others, is misaligned with their ability to select appropriate responses while engaging with their environments.

Taking this one step further, when we look at Substance Use Disorders, for example Alcohol Use Disorder (AUD), we see that many individuals utilize substances as a means of emotional regulation, for coping with their environment (Koob et al., 2014). Thus, the substance risks becoming the sole source of emotional regulation and usurps an individual's innate ability to self-regulate. Said another way, the substance can become a person's favored response for coping, which is, of course, a maladaptive way to engage with our internal and external milieu. What if there was a signal scientists and physicians alike could rely on as a litmus test outside of (and in addition to) patient self-report to provide insights into the integration of physiology and psychology? HRV is a well-supported candidate for such a biomarker. Indeed, low HRV is associated with Alcohol Use Disorder (Ralevski et al., 2019; Quintana et al., 2013; Ingjaldsson et al., 2003), GAD (Thayer et al., 1996; Lyonfields et al., 1995; Thayer & Lane, 2000) and many other pathologies of self-regulation.

1.3.2 A Brief Scientific History of Heart Rate Variability Research

Interest in heartbeat patterns and their meaning dates back millennia, with the first known writing to relate pulse patterns to pathology appearing in ancient Greece (Billman, 2011). However, until the invention of the first electrocardiogram (ECG) in the 1800s, progress in this area of research was slow, arduous, and limited (Bilman, 2011). Therefore, we will begin in the 1960s, when interest in heart rate (HR) and HR variability (HRV) strongly resurged. This was due to the coalescence of observant physicians and scientists along with technological advancements, such as the ambulatory electrocardiogram (Holter, 1961) and digital processing of data, making heart monitoring cheaper and easier to obtain than ever before. Thus, for the first time it became inexpensive and simple to obtain data not only on HR, but also on the subtle shifts in variability between heart periods, the HRV (Billman, 2011). Quickly it became apparent to researchers across a wide variety of disciplines that HRV could reveal insights into the general functional status of an organism. Specifically, it was first observed that low HRV often predicted an organism's demise.

One early example of modern translational research into HRV as a biomarker occurred in the field of obstetrics-gynecology. In the 1960s obstetrician-gynecologist Dr. Edward Hon noticed that fetal heart rate variability predicted fetal hypoxia before other symptoms became observable, affording physicians additional time to make critical choices to protect the fetus and mother (Modanlou & Hon, 2019). Following this observation, Dr. Hon and colleagues elucidated that the degree of decreases in fetal HRV tracked with the severity of fetal distress. This discovery has been so useful that fetal heart rate monitoring has become standard and standardized for use during labor and delivery to this day (Modanlou & Hon, 2019).

Contemporary with Dr. Hon, cardiologist Dr. Stewart Wolf noted the intimate connection between the heart and the brain, such that sudden cardiac death appeared to be a result of neural miscommunication from the brain to the heart (Wolf, 1967). Ahead of his time, Dr. Wolf commented that social isolation, major depression, and other threats to one's mental health presented curious risk factors for sudden cardiac death (Wolf, 1967). Thus, there had to be some connection between the brain and the heart mediating these effects. In the 1970s, Dr. Wolf became the first to demonstrate a link between diminished HRV and sudden death following myocardial infarction (Wolf, 1978). This observation has been replicated numerous times since (see Billian, 2011), eventually revealing that while myocardial infarction decreases HRV in most patients, those with the lowest HRV even within this high-risk group were more likely to die suddenly. Continuing this line of research into the present day, low HRV is now well-accepted in the field of cardiology as one of the strongest independent predictors of mortality following myocardial infarction (Billman, 2011).

After these seminal findings linking low HRV to poor health outcomes, researchers from additional fields continued to uncover additional linkages of low HRV to poor health, such as the discovery that low HRV could predict autonomic neuropathy in diabetic patients before the onset of classical symptoms (Ewing et al., 1985). Low HRV has also been consistently shown to relate to a variety of other disease risk factors, such as smoking, hypertension, work stress, obesity, and heart failure (discussed in Billman, 2011).

In sum, once technology advanced to the point of fast and inexpensive observation of HRV in humans, multiple thought-provoking lines of research emerged from disparate fields. These lines of research converged to reveal HRV as a compelling biomarker for stress and health. Today, HRV is widely accepted as an index of autonomic nervous system status, reflecting the nervous system's bi-directional communication with the heart. As such, HRV is allowing for new insights into the mechanisms of health and disease related to autonomics. These autonomic insights through HRV have become promising markers for regulatory capacity within an organism, which can ultimately allow researchers to develop novel treatments and preventative strategies to abate suffering from a variety of diseases.

1.3.3 The Autonomic Nervous System in Cardiac Function

The autonomic nervous system (ANS) is a part of the peripheral nervous system that monitors and regulates many of our involuntary physiological processes such as heart rate, blood pressure, breathing, and digestion (Carlson, 2009). The ANS consists of two major branches with opposing roles, the sympathetic (SNS, "fight or flight") and the parasympathetic (PNS, "rest and digest"). These two systems are necessary for an organism to maintain homeostasis in our complex world. For example, the SNS is critical to mobilize the body to either escape or fight some perceived danger. The SNS increases heart rate and blood pressure, dilates our pupils, expands our lungs, and diverts energy away from digestion to the skeletal muscles, all to encourage power and quickness in the face of threat. On the contrary, the PNS is necessary to perform the foundational tasks of ordinary living, such as digestion, restoration, and maintenance of the body. The PNS slows the heart rate, decreases blood pressure, constricts our pupils and airways, and stimulates the gut. Proper functioning requires both SNS and PNS branches to respond appropriately. For instance, too much PNS will mean one is unable to mobilize in the face of danger, which would likely result in death of the organism. Conversely, too much SNS prevents the body from performing necessary maintenance tasks, thus accelerating wear and tear of the organism to the point of death. Clearly, each of these systems is necessary in proper balance to ensure adequate survival of an organism.

At the level of the heart, both ANS branches converge on the sino-atrial (SA) node to influence heartbeat patterning, as the SA node is the heart's major pacemaker. As mentioned above, the SNS induces tachycardia while the PNS induces bradycardia. The SNS nerve fibers to the heart originate in the thoracic spinal cord and are activated by sympathoexcitatory nerves from the brainstem. These spinal cord preganglionic fibers synapse in paravertebral ganglia. From there, the postganglionic SNS fibers travel to the heart's SA node and increase cardiac chronotropy through the release of norepinephrine (NE) on beta-1-adrenergic receptors. This results in heart rate increases. The PNS nerve fibers to the heart begin in the brainstem, specifically the nucleus ambiguus (NA), where preganglionic nerve fibers synapse with the efferent vagus nerve, also known as cranial nerve X. From the NA, the vagus nerve travels to the heart's SA node and decreases cardiac chronotropy through the release of acetylcholine (ACh) onto muscrurinic-2-receptors. Under normal conditions, the SNS and PNS are both tonically active exerting influence on the heart, enjoining a dynamic balance of ANS control. While of course the heart is under the influence of additional regulatory mechanisms, such as humoral and hormonal influences, the ANS possesses the strongest influence on cardiac parameters (Shaffer et al., 2014; Thayer & Brosschot, 2005).

Top-down control over the ANS is regulated by many central nervous system structures, and these have been grouped together by theorists as a functional bidirectional circuit termed the Central Autonomic Network (CAN). The CAN consist of structures at all levels of the brain, from the cerebral cortex to subcortical areas to the brainstem nuclei that regulate respiration, cardiovascular control, and blood chemistry (Thayer & Lane, 2009). It is through this organizational network that the complexity of information rising from the viscera and down from the cortex coalesce to make sense of the world and how to respond to it. The CAN structures, in order of top-down regulatory control, are as follows: prefrontal cortex (PFC), cingulate cortex & anterior insula (AIC), amygdala (particularly the central nucleus, CeA), hypothalamus, periaqueductal gray, and finally the brainstem nuclei. Implicated particularly are those involved in ANS regulation: the NTS, DMNX, NA for PNS and the rostral ventrolateral medulla for SNS. The PFC at rest tonically inhibits the CeA. Inhibition of the PFC for any reason leads to disinhibition of the CeA, which then activates the SNS either directly or through PNS inhibition (Thayer & Lane, 2009). Thus, the PFC is a major guardian of top-down ANS control.

While both ANS branches are tonically active at the SA node, the heartrate is predominately under PNS control at rest. This has been demonstrated through pharmacological studies where both branches of the ANS on the heart are inhibited. For example, propranolol inhibits beta-1-adrenergic receptors of the SNS, and atropine inhibits muscarinic receptors of the PNS. Under this double blockade condition, the intrinsic heartrate of the SA node is 100bpm, which is higher than a typical healthy human HR of 60-90bpm (Thayer & Brosschot, 2005, Shaffer et al., 2014). In addition to pharmacological studies, this phenomenon has been corroborated by observations during heart transplants, where the heart removed from the body, and thus from influence of the ANS, holds a steady internal beat of 100bpm from the intrinsic activity of the SA node (McCraty & Shaffer, 2015). Thus, it is accepted that tonic resting HR in a healthy person is influenced more greatly by PNS effects.

This PNS predominance makes sense from an evolutionary perspective, as an organism must be strategic with its resources such that it cannot have the SNS constitutively overactive. Indeed, as we will discuss in Chapter Four, constitutively overactive SNS is likely an overarching response to --or cause of-- body breakdown in many pathologies, including psychopathologies. Additionally, this PNS predominance is logical when one considers the different time scales of action of the SNS versus the PNS, where PNS acts on the order of milliseconds and the SNS on the order of seconds (Nunan et al., 2010). Thus, by employing strong PNS control over the heart in resting conditions, the organism is afforded the ability to quickly release its proverbial "foot from the break" and allow for quick reaction of the SNS when threatened. In sum, the dynamic balance of SNS-PNS, where at rest PNS is predominant, is evolutionarily favorable for energy conservation without sacrificing necessary vigilance for survival.

Of course, the heart is not always in a state of PNS predominance, as that would negate the flexibility of the system. During aerobic exercise, severe psychological stress, or any number of other instances where the heartbeat exceeds 100bpm, the dynamic is often shifted to a state of SNS predominance. This allows the organism to cope with the stressor (McCraty & Shaffer, 2015) before ideally returning to the resting PNS predominant state.

Briefly, the heart also communicates back to the brain. Interestingly, the heart sends more afferent vagal impulses to the brain than any other major organ (Shaffer et al., 2014), and sensory afferents make up over half of vagus nerve fibers (Porges, 1995, Shaffer et al., 2014). Vagal afferent fibers containing sensory information from the heart travel to the nucleus of the solitary tract (NST) in the brainstem. From the NTS, these fibers transmit information to higher cortical structures such as the thalamus, hypothalamus, periaqueductal gray, amygdala and cerebral cortex (McCraty & Zayas, 2014). The effects of vagal afferents on behavior are understudied, but evidence from several groups show that sensory information from the heart can impact cognitive and emotional experiences (McCraty & Zayas, 2014; Shaffer et al., 2014; Porges, 2022).

As mentioned above, pharmacological blockade studies have been useful for identifying autonomic nervous system control over the heart. These same techniques have been employed to determine that HRV is a useful index of autonomic cardiac control, which will be discussed in the following section.

1.3.4 Calculating Heart Rate Variability

Heart Rate Variability (HRV) is the variation in time between successive heartbeats. It is created through the dynamic interplay of the opposing branches of the ANS: the SNS “fight or flight” and the PNS “rest and digest.” As mentioned earlier, the SNS quickens heart rate and the PNS slows heart rate. In addition to the ANS branches influencing HR, the PNS and SNS also exert control over the intrinsic beat-to-beat variations in HR, termed the HRV.

The initial method to determine HRV was by using simple descriptive statistics, such as standard deviation and standard deviation of the variance between successive heartbeats. These methods were termed “Time Series” domain HRV metrics. Time series domain is named because these metrics use interbeat-intervals (IBIs, ms) between heartbeats over a period of time to calculate the HRV. One such example is the SDNN, which stands for “standard deviation of the N-N" where N-N refers to the peaks between normal beats of the heart. “Normal” refers to the exclusion of beats that arise from outside the SA pacemaker node of the heart, such as ectopic beats or technological dropout/noise from recording (Shaffer & Ginsberg, 2017). SDNN is simply the standard deviation of a given set of IBIs within a period of time. Most researchers today concur that the SDNN reflects input from both SNS and PNS branches of the ANS (Shaffer & Ginsberg, 2017). Perhaps the most used Time Series HRV metric is the rMSSD, which stands for “root Mean Squared of the Successive Differences” in N-N. rMSSD is the variance in the IBIs and is calculated by first taking the difference between consecutive IBIs, then squaring these differences, then averaging these squares, and finally taking the square root of this average. rMSSD is widely accepted to reflect vagus nerve, and thus PNS, influence over the heart (Shaffer & Ginsberg, 2017).

Beginning in the 1970s, analysis of frequency domain characteristics of HRV started to be explored (Billman, 2011). Frequency domain calculations involve using Fast Fourier Transform or Autoregression techniques to break HRV down into its component frequencies, also called frequency bands. The most common frequency bands used in studies are the high-frequency component (HFHRV) and the low-frequency component (LFHRV). HRHRV (in humans: 0.15-0.40Hz, in rats: 0.75-2.5Hz) is well-accepted as reflecting PNS influences, akin to rMSSD. Indeed, HFHRV and rMSSD are often highly correlated (Visted et al., 2017; Koenig et al., 2021; Stein et al., 1994), furthering researchers' confidence in these metrics identifying similar processes. LFHRV (in humans: 0.04-0.15Hz, in rats: 0.1-0.75Hz) is like SDNN, in that it captures both SNS and PNS influences on the heart. Similarly, SDNN and LFHRV are also often highly correlated (Visted et al., 2017; Koenig et al., 2021).

ANS control over HRV has been validated using pharmacologic blockade techniques, using atropine to selectively block PNS, and propranolol to selectively block SNS at the heart. rMSSD and HFHRV are PNS-specific and are blocked by atropine. SDNN and LFHRV contain both SNS and PNS influences and are blocked by both atropine and propranolol. These observations have been validated in humans (Pomeranz et al., 1985; Toichi et al., 1997), dogs (Akselrod et al., 1985; Houle & Billman, 1999), and rats (Cerutti et al., 1991; Gehrmann et al., 2000).

Briefly, the 1990s saw the introduction of non-linear techniques for the study of HRV (Shaffer & Ginsberg, 2017; Billman, 2011). These metrics explore the unpredictability of HRV fluctuations, often through the lens of Chaos Theory, and a discussion of these metrics is beyond the scope of this dissertation. A hearty debate is still ongoing among HRV researchers to determine the precise meanings and functions of non-linear dynamical modeling of HRV.

Finally, it is important to note the HR and HRV are related, but not identical. As HR increases, there is mathematically less time between heartbeats to allow for variability. The reverse is also true, as HR decreases, more opportunity for variability exists between heartbeats. This phenomenon of HR and HRV having a strong inverse correlation is termed "cycle length dependence" (McCraty & Shaffer, 2015). However, this relationship is not one to one, meaning that within any given HR value, multiple possible HRV values exist. This will be shown in my data and can be found across the literature.

1.3.5 Heart Rate Variability and Psychopathologies

As mentioned previously, in addition to associations with cardiovascular pathology, HRV and ANS dysfunction have been noted in many psychopathologies, including depression, generalized anxiety disorder, panic disorder, post-traumatic stress disorder, and substance use disorders (Mulcahy et al., 2019; Thayer & Lane, 2000; Thayer & Brosschot, 2005; Shaffer et al., 2014; Ingjaldsson et al., 2003; Sgoifo et al., 2015). Prominent theorists in the HRV field agree that the link between HRV (and thus ANS) and heterogenous psychiatric conditions converges on a misalignment of inhibitory control within the organism (Thayer & Brosschot 2005; Thayer & Lane 2009; Thayer et al., 2012; Porges, 1995; Porges, 2009). The ideas of these theorists, and their implications, will be reviewed more thoroughly in Chapter Four. In this current section, we will briefly review research in humans and in rodents relating HRV to the psychopathologies of relevance to this dissertation: anxiety disorders and maladaptive alcohol drinking. This section will conclude with a brief discussion of what is currently known about sex differences in autonomics.

Humans with anxiety disorders consistently demonstrate lower HRV compared to healthy controls (Thayer & Lane, 2000). For example, one study (Thayer et al., 1998) compared HFHRV in individuals with generalized anxiety disorder (GAD) to non-anxious controls in a baseline relaxation state and in an experimentally induced worry state. These researchers found that regardless of relaxation or worry, GAD participants had significantly lower HFHRV compared to non-anxious controls. Additionally, for the worried state, HFHRV was reduced for both groups. These data suggest that decreased HFHRV is normal in states of worry, however the GAD participants likely overcompensate with a consistently lower HFHRV regardless of when it is appropriate (Thayer et al., 1998). Another study showed that chronic worriers possessed decreased rMSSD HRV across all experimental conditions which included an initial baseline, viewing aversive imagery, a prompt to imagine a personally relevant worry, and finally at a concluding baseline period (Lyonfields et al., 1995). Again, these results suggest that individuals with GAD consistently display lower HRV. In rats, researchers have shown reductions in HRV during conditioned expectation for a foot-shock (Inagaki et al., 2004), during social defeat stress (Sgoifo et al., 2001), and during novelty-suppression of feeding task (Frasier et al., 2023). In addition to laboratory studies from humans and rats demonstrating diminished HRV as an effect of stressors or anxiety, recent clinical work has revealed HRV has utility as a biomarker in anxiety-related illness. For example, it has been demonstrated that pre-treatment rMSSD HRV predicted success of antidepressant treatment as a function of whether the depression could be categorized as an “anxious” or “non anxious” subtype (Kircanski et al., 2020).

Low HRV has also been implicated in Alcohol Use Disorder (AUD) (Ralevski et al., 2019; Cheng et al., 2019; Quintana et al., 2013; Ingjaldsson et al., 2003). Furthermore, HRV and compulsion for alcohol has been examined. For example, one study compared high-compulsive to low-compulsive patients with AUD while asked to imagine drinking alcohol. These researchers found that high-compulsive alcoholics demonstrated lower rMSSD and self-reported more difficulty in resisting a drink compared to low-compulsive AUD patients (Ingjaldsson et al., 2003).

Recall that HRV is conceptualized as a marker of flexible engagement toward one's environment to ensure adaptation and survival (Thayer et al., 2012; Thayer & Lane, 2009; Thayer & Lane, 2000; Porges, 1995; Porges, 2009). Seen this way, it can be conceptualized that the AUD patient has maladapted to respond to cues concerning alcohol over those that are likely more beneficial. With this in mind, consider the following study that compared long-term abstinent AUD patients to more recently abstinent AUD patients. Newly abstinent patients displayed higher HFHRV in response to emotional images compared to longer-term abstinent patients, suggesting that HFHRV response during alcohol abstinence could index the recovery of emotional regulatory ability (Claisse et al., 2017). Furthermore, a different study comparing healthy controls to AUD patients showed that, while AUD patients consistently demonstrated lower HRV, AUD patients display greater rMSSD HRV increases in response to alcohol cue exposure (Ingjaldsson et al., 2003). Other researchers have shown that in treatment-seeking alcohol dependent individuals, resting HFHRV can predict craving (Quintana et al., 2013), and HFHRV reactivity to alcohol cues can even go so far as to predict relapse (Garland et al., 2012). Taken together, HRV studies present a fertile opportunity to uncover potential biomarkers for AUD and reveal previously unknown relationships between autonomic control and behavior.

Since HRV is an index of autonomic tone, it is important to briefly discuss what is known regarding sex differences in autonomic utilization. Multiple research groups have converged on the finding that females tend to bias toward PNS, and men tend to bias toward SNS (Dart et al., 2002; Sato & Miyake, 2004; Nugent et al., 2011; Koenig & Thayer, 2016; Williams et al., 2022). The reasoning behind such differences likely results from the sex hormones having differential effects on organs such as the heart. For example, estrogen has been shown to increase sensitivity to ACh, and conversely testosterone has been shown to increase NE synthesis and reduce NE clearance (Dart et al., 2002). Recall that these neurotransmitters are major players in the dynamic regulation of the ANS at target organs, such that increased ACh sensitivity would enhance PNS activity whereas increased NE availability would enhance SNS activity (see section 1.3.3).

Finally, at rest females have a higher HR and higher HRV than males, and this observation has been replicated in humans (Koenig & Thayer, 2016, Williams et al., 2019, Williams et al., 2022) and rats (Carnevali et al., 2023; Frasier et al., 2023). However, this finding appears paradoxical considering cycle length dependence, where HR and HRV are typically inversely related such that higher HR results in a lower HRV and vice versa (McCraty & Shaffer, 2015). Again, the current thinking on this apparent paradox relates back to sex hormone effects on cardiac chronotropy. Considering that the major female sex hormone, estrogen, enhances sensitivity of ACh, and ACh is the neurotransmitter which mediates PNS, it follows that females would possess greater PNS bias (and thus greater HRV) than men (Williams et al., 2022). The reasoning for the paradoxically higher HR is not yet fully understood. Considerations for natural hormone fluctuations from the female menstrual/estrus cycle and effects on HRV will be detailed in Chapter Four.

In sum, compelling evidence exists that HRV is related to various psychopathologies, including anxiety disorders and maladaptive alcohol use. Furthermore, important sex differences appear to exist in ANS regulation, where females bias toward PNS and males bias toward SNS. These sex differences are especially interesting when one considers the differential prevalence and prognoses for mental health conditions that have been outlined in the beginning of this chapter, where women have higher prevalence of anxiety disorders and worse prognoses for alcohol misuse.

1.4 Aims and Hypotheses

Alcohol misuse and anxiety disorders pose substantial issues both to the individual sufferer and to society at large. Unfortunately, the rates of alcohol misuse and anxiety disorders continue to rise, and frequently these difficulties are co-morbid. Further, important sex differences exist in the prevalence and severity of alcohol misuse and anxiety, such that women have almost twice the prevalence of anxiety disorders, report higher comorbidity of alcohol and anxiety issues, and suffer disproportionately from the medical consequences of excess alcohol consumption. Thus, there is a pressing need to identify key sex differences in the underlying mechanisms of anxiety disorders and alcohol misuse to develop more personalized, efficacious treatments.

Previous research suggests that measures of cardiac control, such as heart rate (HR) and heart rate variability (HRV), are promising candidates for the study of such mechanisms, as these serve as biomarkers for autonomic tone, and autonomic changes have been implicated in both anxiety and alcohol-related dysfunctions. However, there is a **dearth of preclinical rodent research investigating sex differences** in alcohol misuse, anxiety disorders, and their frequent comorbidity. Thus, the **central goal** of this dissertation was to develop a translationally relevant rat model, combining HR/HRV telemetry with models of alcohol misuse and anxiety-like behavior, to elucidate sex differences that are commonly observed in humans. The aims of this dissertation were to **(1)** develop and validate HR/HRV telemetry in translationally relevant rat models of alcohol misuse and anxiety-like behavior, and **(2)** characterize HR/HRV as sex specific biomarkers for alcohol consumption and aspects of anxiety-like behavior.

CHAPTER TWO: Heart rate variability measures indicating sex differences in autonomic regulation during anxiety-like behavior in rats

2.1 Introduction

Mental health conditions remain a substantial challenge to society, where anxiety and mood conditions alone extract approximately 700 billion US dollars per year globally (Chisholm et al., 2016). Additionally, epidemiological data reveal that women have nearly twice the prevalence of anxiety disorders compared to men (Altemus et al., 2014; Bangasser and Wicks, 2017; Filkowski et al., 2017; Shansky, 2018; Rincon-Cortes et al., 2019). Given that sex differences exist, it is of considerable importance to investigate the possible biological underpinnings that influence such differences, which would then facilitate the development of sex-specific and individualized treatment options. Currently, critical mechanisms that underly observed sex differences in anxiety-related conditions remain incompletely understood. Furthermore, in the study of humans, it is often challenging to truly disambiguate biological versus socio-cultural contributions to psychopathologies, including anxiety disorders (Altemus et al., 2014; Bangasser and Wicks, 2017; Filkowski et al., 2017). Thus, utilizing rodent models of anxiety-like behavior is of immense value since such models allow for the control necessary to reveal mechanistic insights into observed sex differences.

Investigations of sex differences in anxiety-like responding in rodent models have been performed by many groups (Bangasser and Wicks, 2017; Shansky, 2018; Becker and Chartoff, 2019; Rincon-Cortes et al., 2019; Scholl et al., 2019; De Oliveira Sergio et al., 2021) including our lab (De Oliveira Sergio et al., 2021). Such studies have shown that females typically locomote more (Palanza and Parmigiani, 2017; Le Moene et al.,

2020) and have differential responding patterns to stressors compared to males, where females will display "darting" or avoidance behavior, whereas males will display more freezing behavior (Bangasser and Wicks, 2017; Shansky, 2018; Becker and Chartoff, 2019; Rincon-Cortes et al., 2019). To complicate matters, nearly all anxiety-like behavior paradigms for rodents were initially developed and validated using only males (Bangasser and Wicks, 2017; Shansky, 2018; Becker and Chartoff, 2019; Rincon-Cortes et al., 2019). However, by assessing multiple measures within a given anxiety-like paradigm, one can potentially gain insights into behavioral sex differences, as our group previously showed using the Novelty Suppression of Feeding (NSF) task (detailed further below) (De Oliveira Sergio et al., 2021).

The NSF task consists of restricting the rat's food intake for 3 days prior to placement in a novel chamber which has food in the center under a bright light. The NSF task leverages the conflict between the hungry rat's drive to feed and its innate aversion to brightly lit and open spaces. One benefit of the NSF task is that there are many progressive behavioral measures prior to food consumption, such as (A) the latency to first approach the brightly lit center, (B) the number of approaches to the center, (C) the time spent in the center, (D) the latency to grab the food, and, finally, (E) the amount of food consumed during the task (illustrated in **Figure 2.1 A**). Our group recently demonstrated that females displayed greater anxiety-like behavior related to food acquisition in NSF (greater latency to grab food and less food consumed) compared to males, using a smaller NSF arena than utilized here (De Oliveira Sergio et al., 2021). We posited that these results suggest female anxiety-like behavior is increased in relation to specific, life-relevant situations, such as obtaining food when hungry in the presence of aversions.

To better understand the mechanistic basis of any sex differences in anxiety-like behaviors, our lab sought to elucidate potential physiologic underpinnings by using cardiovascular telemetry to determine autonomic state. Recently, there has been increased excitement in the use of cardiac measures, including heart rate (HR) and HR variability (HRV), as intriguing candidates for biomarkers of and/or contributors to pathological states, especially in the realm of mental health conditions (Thayer and Lane, 2009; Mulcahy et al., 2019; Ralevski et al., 2019). HR and HRV are modulated by a dynamic balance between the two opposing divisions of the autonomic nervous system: the sympathetic (SNS, "fight or flight") and the parasympathetic (PNS, "rest and digest") (Gehrmann et al., 2000; Thayer and Lane, 2009; Ralevski et al., 2019; Hamidovic et al., 2020). HR, the number of beats per minute, is straightforward to both measure and interpret. In contrast, there are multiple methods used to calculate HRV, which elucidate different aspects of autonomic influences on the heart.

Much prior work has been performed to assess and validate how particular HRV measures reflect different aspects of autonomic state, especially the relative influence of SNS and PNS. Drugs that target specific aspects of cardiac physiology have been especially valuable. For example, beta-adrenergic receptors are known to mediate SNS influences on the heart, and thus one can use the effects of beta-adrenergic receptor blockers (such as propranolol) on HRV to gain insight into the relative influence of SNS on a given HR/HRV measure. In parallel, muscarinic acetylcholine receptors are known to contribute to PNS impacts, and by using a selective blocker (such as atropine), one can better isolate PNS influences on heart measures. Indeed, such studies have been done in humans (Pomeranz et al., 1985; Toichi et al., 1997), dogs (Akselrod et al., 1985; Houle and Billman, 1999), and rats (Japundzic et al., 1990; Cerutti et al., 1991; Gehrmann et al., 2000) to demonstrate the validity of HRV indicators to discern autonomic tone.

Here, we focus on two widely used measurements of HRV, where we assess patterns of heart rate variability across a series of inter-beat intervals, the amount of time between successive heartbeats (these are called "time domain" measures). Examples of HR traces are shown in **Figure 2.2 A**, and **Figure 2.3** summarizes the HRV measures used here and how they are impacted by the drugs described above. First, we examined the rMSSD, the root Mean Squared of the Successive Differences in inter-beat intervals (IBIs), which is altered by atropine but not propranolol, and thus is considered to primarily reflect PNS influence rather than SNS. SDNN, which is the standard deviation of a series of inter-beat intervals ("NN" is the time between the peaks of two heartbeats), is altered by either propranolol or atropine. Thus, SDNN is considered to indicate a balance between SNS and PNS influences on the heart. Finally, by taking the ratio of SDNN/rMSSD, we can discern the relative impact of the SNS, since this ratio is effectively [SNS influence + PNS influence]/[PNS influence]. Thus, greater ratio is taken to indicate greater SNS influence (Gehrmann et al., 2000; Thayer and Lane, 2009; Visted et al., 2017; Ralevski et al., 2019; Hamidovic et al., 2020; Koenig et al., 2021).

A series of related measures examine HRV in the frequency domain, with the high-frequency component (HFHRV) related to PNS (similar to rMSSD), and the low-frequency (LFHRV) related to PNS-SNS balance (similar to SDNN) (Beckers et al., 2006; Henze et al., 2008; Koizumi et al., 2011; Park et al., 2017). Recent work has considered rMSSD and SDNN of high utility, in part due to analytic differences across studies of frequency-related indicators (Toichi et al., 1997; Koenig et al., 2021; Williams et al., 2022). For example, one study (Cheng et al., 2019) found reduced rMSSD but not HFHRV in alcohol drinkers, which they suggest might reflect analytic differences from other studies finding altered HFHRV. Another recent study chose rMSSD for a large meta-analysis of sex differences in HRV (Koenig et al., 2021). Nonetheless, rMSSD often correlates well with HFHRV, and SDNN with LFHRV (Visted et al., 2017; Koenig et al., 2021). Thus, we focus here on SDNN, rMSSD, and SDNN/rMSSD. By assessing across HRV measures, one can gain insight into underlying autonomic changes as a function of sex and behavioral condition.

Since HRV measures can reliably assess several aspects of autonomic state, it is thus a useful tool for the study of sex differences in autonomic regulation, especially in mental health conditions. Indeed, various studies have found sex differences in autonomic regulation, where women have higher HR than men under various conditions, including at baseline (Huikuri et al., 1996; Koenig et al., 2021; Williams et al., 2022). However, while one might think that a higher HR would automatically result in lower HRV (since less time between heartbeats leaves less opportunity for variability, mathematically), paradoxically, women tend to display a higher baseline HRV than men even though they have greater HRs (Huikuri et al., 1996; Nugent et al., 2011; Koenig et al., 2021; Williams et al., 2022). For example, in a large recent study (Williams et al., 2022), women had greater HRV than men for a given HR level, while on average women had lower SDNN but no differences in rMSSD (Nugent et al., 2011; Williams et al., 2022). Furthermore, in addition to these sex differences in basal HR and HRV, several studies have demonstrated sex differences in autonomic tone during challenge, where women tend to recruit PNS more, and, in contrast, men tend to recruit SNS more (Dart et al., 2002; Sato and Miyake, 2004; Nugent et al., 2011; Hamidovic et al., 2020; Koenig et al., 2021). Other studies also suggest sex differences in autonomic regulation at the level of different brain circuits (Mak et al., 2009; Goldstein et al., 2010; Nugent et al., 2011; Allen et al., 2015; Garcia et al., 2020). However, the functional implications of such sex differences on behavior are understudied, and much remains unknown about how autonomic measures relate to specific aspects of anxiety-like behavior, especially potential sex differences in HR/HRV measures during expression of anxiety-like behavior.

We note that the present studies include data collected from both naïve and alcohol-drinking rats. In humans, alcohol drinkers can often show autonomic differences relative to controls (Ingjaldsson et al., 2003; Ralevski et al., 2019). Thus, initially we had hypothesized there would be differences in autonomic tone not only by sex, but also by rat drinking history (e.g. "alcohol-naïve" versus near daily "alcohol-drinking" rats). However, we found no such differences in NSF behavior or their autonomic correlates in naives vs drinkers (**Figures 2.11**, **2.12**); we further explore the potential reasons for these findings in the Methods. Thus, for the current study, we collapsed data by drinking history and instead presented the data by sex, to increase the sample size and maximize the ability to observe HR/HRV differences across sex and different anxiety-like behavior measures.

As such, the present study is the first, to our knowledge, to have used cardiovascular telemetry to assess HR and HRV indicators in adult female and male

Wistar rats in relation to different behavioral measures within the NSF anxiety-like behavior task. We also compared NSF behavioral measures to HR/HRV at baseline, defined as the period of time immediately preceding the NSF task. Overall, we found significant sex differences in cardiac autonomic regulation that related to different NSF behaviors. In females, HRV measures predicted food intake and the first approach in the task, and our previous studies (De Oliveira Sergio et al., 2021) suggest that food consumption and the first movement in a novel context are of particular relevance to females. In males, HRV patterns predicted different NSF behaviors than in females, for example, where greater SNS indicators correlated with greater male intake in males who consumed food, the opposite of females. Finally, females had baseline autonomic differences that persisted into the anxiety testing period and impacted behavior, while male NSF behavior related to HRV measures within the task but not at baseline. Together, our results uncovered several important and novel sex differences in autonomic influences over anxiety-like responding, which may provide both biomarkers for and mechanistic insights into sex differences in anxiety mechanisms.

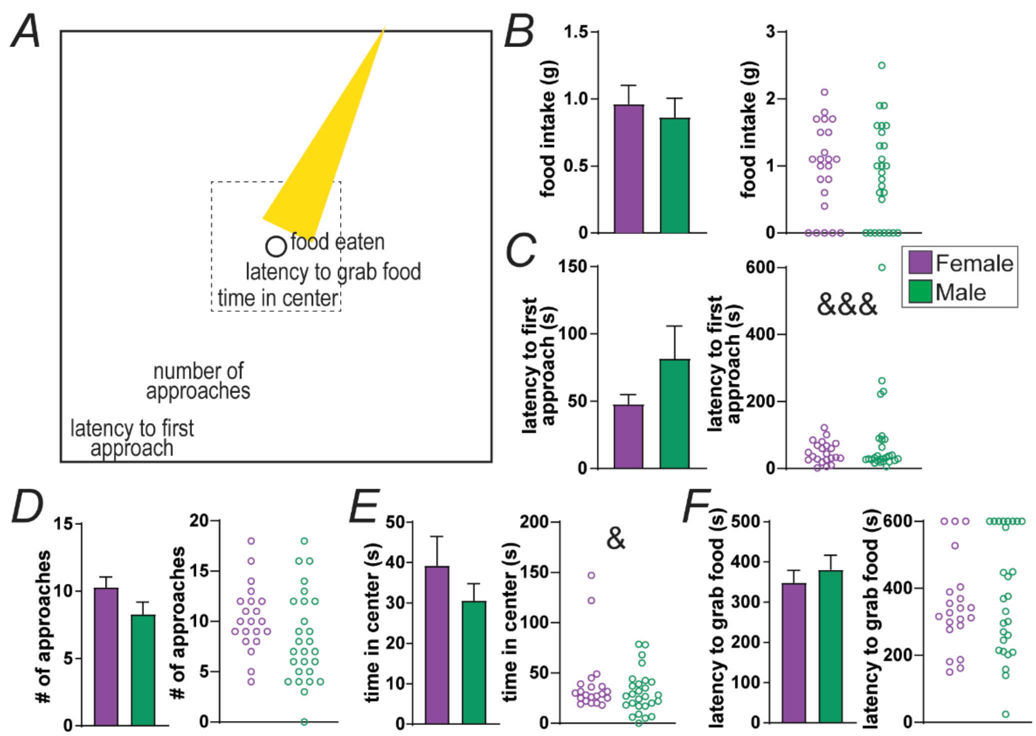

Figure 2.1. No sex differences in behavioral measures of the Novelty Suppression of Feeding (NSF) task. (A) Cartoon of the NSF arena, where a food-restricted rat has access to food in the center of the arena (circle) which is brightly lit (yellow cone). The task consists of a series of progressive measures before food consumption, including latency to first approach, the number of approaches, time in center (tolerating the light), and latency to grab the food. **(B)** Food intake. **(C)** Latency to first approach the center. **(D)** Number of approaches. **(E)** Time in center. **(F)** Latency to grab food. No average differences, although variability of latency to approach was greater in females (&&& $p<0.001$), and variability of time in center was greater in males (& $p<0.05$).

2.2 Methods

2.2.1 Animals

All experiments were performed in accordance with NIH Guidelines and approved by the Institutional Animal Care and Use Committee (IACUC) of Indiana University. Post-natal day ~50-55 male and female Wistar rats (Envigo) were singly housed in clear plastic homecages with *ad libitum* food and water in a 12hr reverse light cycle (lights off 8AM), and with both sexes in the same housing rooms. At the time of anxiety testing, rats were 6-7 month old alcohol-naïve or alcohol drinkers (see Suppl.Methods Section 2.5.1). However, we found no differences in NSF behavior or in nearly all HRV measures between drinkers and alcohol naïve rats (**Figures 2.11, 2.12).** On the other hand, we did observe greater HR and lower SDNN in females versus males (in both drinkers and naives), similar to what is seen for female and male humans (Williams et al., 2022) and other rat strains (Carnevali et al., 2023), making it likely that our telemetry is robustly assessing autonomic function. Thus, we collapsed results from drinkers and naives for all other analyses. We note that, when comparing drinkers and controls in humans, some studies find no HR differences (Rajan et al., 1998; Romero-Martinez et al., 2019) or higher HR in drinkers (Ingjaldsson et al., 2003; Seo et al., 2013; Hwang et al., 2022; Sinha et al., 2009), and many observe lower basal HFHRV in Alcohol Use Disorder (AUD) (Romero-Martinez et al., 2019; Ingjaldsson et al., 2003; Quintana et al., 2013; Karpyak et al., 2014; Cheng et al., 2019), although with some considerations. One study (Hwang et al., 2022) found no AUD vs control differences in rMSSD, HFHRV, or LFHRV, but did observe greater average entropy with AUD (a non-linear HRV measure). In addition, moderate to heavy drinkers (non-AUD) can have higher HFHRV (Mayhugh et al., 2018), and resting HRV is greater in people drinking lower levels of alcohol (1–2 drinks/day) but reduced in people consuming more than that (Karpyak et al., 2014). One possibility is that some human studies may reflect more advanced AUD stages. For example, Hwang et al., (2022) noted no HRV changes with alcohol cues, while another study (Wang et al., 2020) found rMSSD increases to alcohol cues was associated with more alcohol problems. However, Hwang et al., (2022) noted that AUDIT scores in their study were ~19, but 10-12 in (Wang et al., 2020). Thus, HRV changes may vary with the level of drinking problems, and our rats would not reflect the highest-level problem drinkers.

In addition, we note that the intermittent access alcohol model we use exhibits features related to human drinking, including escalating intake (Koob & Volkow, 2010; Simms et al., 2008), sensitivity to compounds that reduce human drinking (De Oliveira Sergio et al., 2023; De Oliveira Sergio et al., 2021b; Simms et al., 2010; Sinha et al., 2022; Steensland et al., 2012), withdrawal symptoms (although moderate) (Li et al., 2011; Steensland et al., 2012), and front loading, where strong initial drinking suggests high motivation for alcohol (Jeanblanc et al., 2019; Salling et al., 2018; Starski et al., 2023). Importantly, long-drinking Wistars also show compulsion-like intake, where drinking persists despite negative consequence (De Oliveira Sergio et al., 2023; De Oliveira Sergio et al., 2021b) that is, at least in part, mediated through an insula circuit (Seif et al., 2013; Starski et al., 2023) that somewhat similar to that observed during problem drinking in humans (Arcurio et al., 2015; Grodin et al., 2018). Thus, while rat alcohol drinking did not produce human-like changes in HRV, the drinking model we use likely captures several aspects of human addiction.

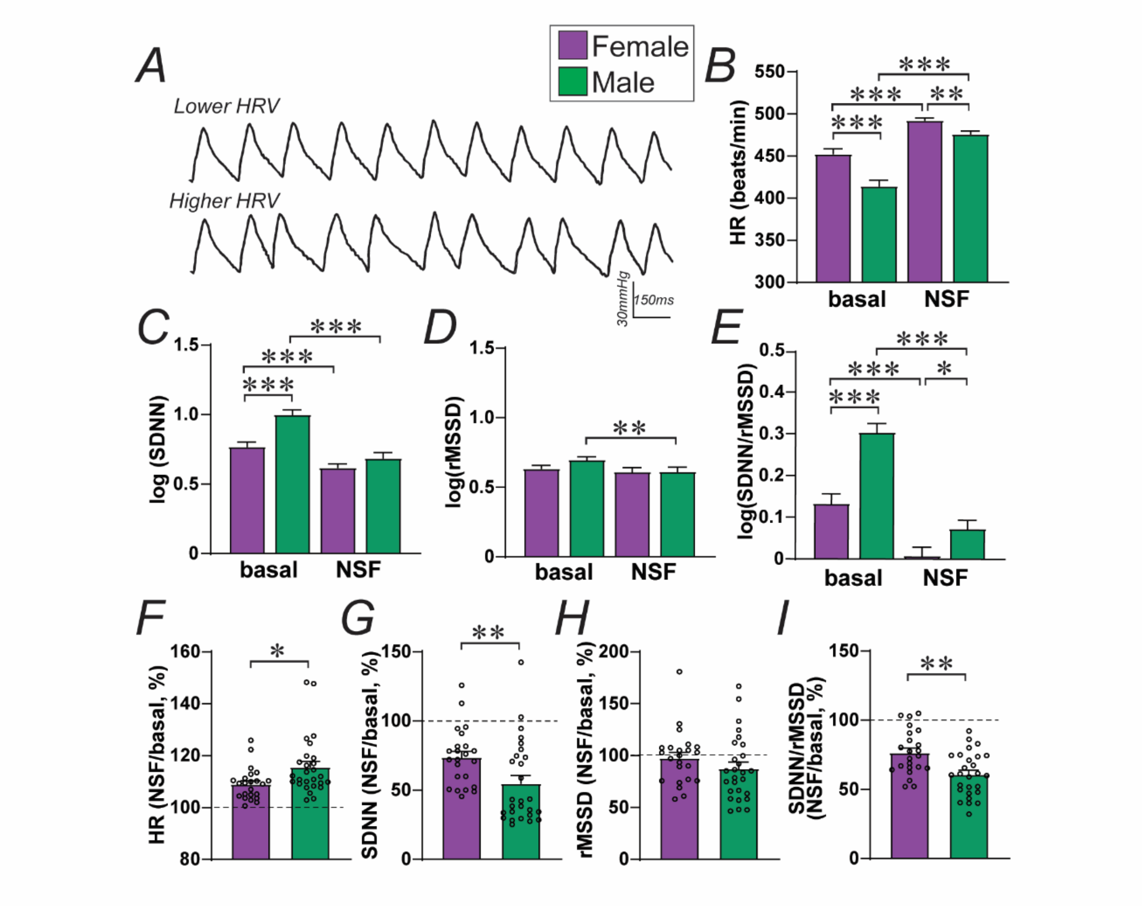

Figure 2.2. Female-male differences in basal HR/HRV measures. (A) Examples of HR traces with lower HRV (top) and higher HRV (bottom, exaggerated cartoon version). **(B-E)** Relative to males, females had **(B)** greater HR, **(C)** lower SDNN, **(D)** no rMSSD differences, and **(E)** lower SDNN/rMSSD ratio. To perform two-way ANOVA, data were log-normalized for SDNN, rMSSD, and SDNN/rMSSD. **(F-I)** Males show larger baseline-to-NSF changes in several HRV measures**,** when calculating percent change in a given measure within-rat. *,**,*** $p<0.05$, $p<0.01$, $p<0.001$.

	Potential to strongly modulate? atropine (PNS)	propranolol (SNS)
■ **IBI:** Inter-Beat Interval is the time between successive heart beats	Yes	Yes*
■ **rMSSD:** *the root mean square of IBI differences is thought to indicate more influence of PNS*	Yes	No
■ **SDNN:** *the standard deviation of IBIs is thought to indicate SNS-PNS balance*	Yes	Yes*
■ **SDNN/rMSSD:** *larger could be considered to indicate more influence of SNS*	Yes	Yes*

Yes SNS can be quite low when laying down*

Figure 2.3. Description of how to calculate different HRV measures, including how they could relate to PNS and/or SNS. Specifically, some pharmacological agents are considered to impact the SNS, through the beta-adrenergic receptors (which are blocked by propranolol), or the PNS, through the muscarinic cholinergic receptors (which are blocked by atropine) (this interpretation does require caution since laying down is related to low SNS).

2.2.2 Anxiety-like Behavior

Studies occurred in a self-standing custom-built soundproof chamber (6ft by 8ft base, 7ft high) with ventilation fans to minimize disturbance during testing. The behavioral arena was cleaned between rats with 0.025% bleach, and 15-20min air dry. Rats underwent Novelty Suppression of Feeding (NSF), with behavior videotaped for later scoring by an observer blind to the experimental condition. For NSF, rats were food restricted for 3 days. Intake was determined for ~4 days before food restriction, to determine average intake for each rat. Rats then received 80% of their normal food intake for two days, and 20% of normal intake for a third day. NSF was tested the following morning in a single 10-minute session. A 10-minute baseline session was recorded in the home cage immediately preceding NSF testing. The NSF arena was square (100cmx100cm) with 40cm high walls, custom made from black acrylic plastic. During the NSF test, a bright light shines at the center, targeting ~3g food on a 3” circular white paper (160lux at center, <60lux along edges). After all rats were run in NSF, they were given a minimum 5min in their home cage before being tested in the Light-Dark Box. Here, we present only findings from NSF. Light-Dark box analyses are ongoing, and we have considered NSF as our main task of interest, with conflict between bright light and food, while light-dark box only involves aversion to bright light (a control). NSF measures were the same as we previously assessed (De Oliveira Sergio et al., 2021) (**Figure 2.1 A**), and included latency to first approach the center, number of approaches to center, time in center, latency to first grab food, and food eaten. Latency to eat is a widely used measure for NSF tasks, without sex differences, even with some differences in NSF methods (Olivier et al., 2008; Gobinath et al., 2018; Miragaia et al., 2018; Enayati et al., 2020). The complexity of the stressor is an advantage since it is likely to reliably and fully engage the integrated systems of interest here.

2.2.3 Telemetry, HR, and HRV Analysis

Surgery is detailed in Suppl.Methods Section 2.5.2, and consisted of implanting a Stellar telemetry device (type PTA-M-C, part# E-430001-IMP-130) from TSE Systems Inc. (Chesterfield, MO). After 5-7 days of recovery from surgery, rats began food restriction. Real-time blood pressure traces were recorded using NOTOCORD-hem software (Instem, Staffordshire, UK), with all baseline data recorded in the homecage before NSF. The NOTOCORD-hem software recorded blood pressure changes at 250Hz, with pressure fluctuations related to each heartbeat. Here, we focus on HR and HRV, as determining the actual absolute blood pressure values might be more challenging relative to assessing HR.

Occasionally there were artifacts and missed heartbeats. Thus, each raw 10-minute recording was visually inspected to confirm each heartbeat was correctly detected, and to remove such data to ensure accurate reporting of cardiovascular measures. This is similar to what is typically performed in many other studies (Grippo et al., 2012; Inagaki et al., 2013; Wardwell et al., 2020; Carnevali et al., 2023) and was typically less than 2% of a session. The NOTOCORD-hem software provided the timing of each heartbeat peak. We used this time series to calculate overall HR (beats/min) and to determine inter-beat intervals (IBI, in msec) across the session. Using IBIs, the following time-domain HRV values were determined: (1) SDNN, the standard deviation of the IBIs for a given period of time, reflecting both SNS and PNS influences, and (2) rMSSD, the root mean squared of the successive differences in IBI, which reflects PNS influence (**Figure 2.3**). rMSSD was determined by taking the square of difference between successive IBI values, averaging the squares of these differences, then taking the square root of this average. We also examined the SDNN/rMSSD ratio, which provides insight into the relative SNS contribution. Importantly, recent work in humans (Williams et al., 2022) suggests the importance of not adjusting HRV measures for HR, which results in missing important sex differences.

For studies in **Figures 2.2, 2.4-2.8**, we assessed HRV across the whole NSF period, and related HRV measures to overall behavior measures. It would be useful to assess autonomic changes related to specific moments during the NSF task. For these analyses (shown in **Figure 2.9**), we ran a 16-sec sliding window program (in MATLAB) along the NSF session in which we analyzed average heart rate and the different HRV measures. For example, we first calculate the HR/HRV values centered around 8 seconds into the task (averaging across data from 0 to 16 sec in a given session). This window was then advanced in 2sec steps, and a new average was computed (e.g. where the next interval examined was from 2 to 18 sec in the session). In this way, we could assess the HRV from 8sec before to 8sec after time points across the NSF task. We then took this by-time data and centered the results from each session on the time to grab food, and examined HR/HRV measures from 150 seconds before grabbing food to 150 seconds after. In addition, we Z-scored each HR/HRV measure in a given session relative to the average and standard deviation of data from 150 to 70 sec before the time to grab food. Across all rats, there were limited changes in HR/HRV measures before the time to grab.

Analysis of longer periods of heart beats allows more precise and accurate HRV assessment. However, a 5min period is often utilized for a shorter baseline in humans (Shaffer & Ginsberg, 2017). In addition, recent work has suggested that shorter analysis windows give meaningful HRV patterns, and 60sec analysis windows are often as good as a 5min window for assessing SDNN, RMSSD, and HR (Ritsert et al., 2022; Shaffer & Ginsberg, 2017). Rodent studies have also utilized 1min HRV analysis windows (McNeal et al., 2019), including for anxiety-like tests (Grippo et al, 2012)**.** However, rat heartbeat in rats is ~4 times faster than human, and so 16 sec of rat telemetry will have about the same number of heart beats as 60 sec in humans. Thus, we chose 16 sec as a sliding window.

For one female, video tape was lost, so we only had food consumed in NSF. In a second female, video stopped early, so we only had latency to approach and food consumed. Also, we did not adjust food intake during NSF for body weight, which is consistent with other work (Gobinath et al., 2018; Miragaia et al., 2018). Finally, as an additional comparison, we examined food intake in a 30min session home cage session after NSF testing.

Since our rats were food restricted for three days before NSF testing, it is possible that food restriction itself altered HR/HRV measures. We had 23 female and 21 male rats with recording during normal food conditions (10min long), which we then compared, within-rat, to the HR/HRV measures during the 10min baseline before NSF testing. Relative to the non-food-restricted condition, rats under food restriction had ~5% lower heart rate in males ($t_{(20)}$=3.720, *p*=0.0014) and ~2% lower in females ($t_{(22)}$=1.616, *p*=0.1204 paired t-test), with a trend for sex difference in the heart rate change (MW *p*=0.0534). In addition, SDNN under restriction was ~42% greater than non-restriction in males (W=159, *p*=0.0043 Wilcoxon ranked sum), but only ~8% greater in females (W=92, *p*=0.1695), and with a sex difference in SDNN change (MW *p*=0.0202). In contrast, rMSSD increased with food restriction in both sexes vs non-restricted condition, with ~27% greater rMSSD in males (W=183, *p*=0.0007) and ~16% increase in females (W=204, *p*=0.0011), and no sex difference in rMSSD change (MW *p*=0.1902). Finally, restriction was associated with non-significant changes in SDNN/rMSSD (males: ~10% increase in restricted conditions, W=99, *p*=0.088; females: ~5% decrease, W=-54, *p*=0.4274), although there was a significant sex difference in ratio change when restricted vs non-restricted (MW *p*=0.0230). Thus, food restriction itself did have effects on HR/HRV measures. However, we note that (1) females had basal HR/HRV differences from males that mirror those seen in human and other rat studies, (2) the suggested female use of PNS, and male use of SNS, for eating food during NSF parallels sex differences in autonomic utilization in humans, (3) our previous work suggests the initiation of a task, and highly life-relevant aspects of a task (food in the NSF), are particularly important for females, and here we observe the female HR/HRV predicts the first approach and food eaten, but not more intermediate behaviors (like time in center) that are linked to autonomics in males, and (4) parallel studies of alcohol drinking when not food deprived (see Chapter 3) found that basal HR/HRV state critically regulated drinking in females but not males, and here we observed that female but not male basal autonomic state was brought into and impacted NSF behavior. Thus, even though there are some sex differences in HR/HRV changes related to food restriction, it is likely that our findings reflect meaningful sex differences in autonomic recruitment under different stages of challenge.

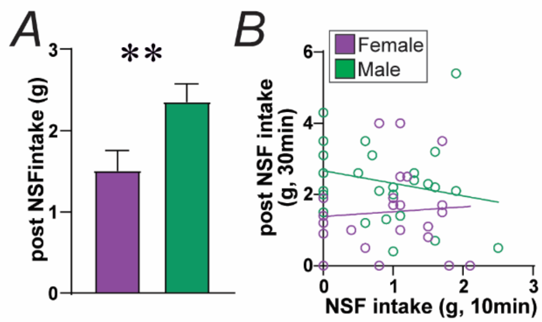

Figure 2.4. Males eat more food after NSF. Because the NSF is a brief test (10min), amount of food consumed is typically not corrected for body weight. In support of this, rats had 30min to eat food in the home cage after the NSF testing, and **(A)** male rats ate significantly more food in the post-NSF intake period than females (MW=172.5, p=0.0065), as seen in other studies. **(B)** Also, if rat intake in the 10min NSF task was titrated by body weight and nutritional need, one might predict that rats that ate more during NSF would eat less in the later home-cage intake period. However, NSF intake and later home cage intake were not correlated in females ($F_{(1,21)}$=0.118, R^2=0.006, p=0.7348) or males ($F_{(1,25)}$=1.328, R^2=0.050, p=0.2601). Also, females had a trend for greater intake post vs during NSF (Wilcoxon matched-pairs signed-rank test, diff in medians=0.600, p=0.0633), while males had significantly more intake post vs during NSF (Wilcoxon, diff in medians=1.300, p<0.0001). Thus, it is reasonable to conclude that food intake during NSF was small enough that it doesn't need to be corrected to body weight, supporting the interpretation that there was no sex difference in intake level during the NSF task.

2.2.4 Statistics

Analyses were performed using two-way repeated measures ANOVA (2way-RM ANOVA), unpaired or paired t-tests, F test (for differences in variance) or Pearson's correlations, where appropriate, using GraphPad Prism. For 2way-RM ANOVA comparing basal versus NSF measures (within-subject), and across females and males, we log-normalized the data for SDNN, rMSSD, and SDNN/rMSSD, as often done for HRV (Williams et al., 2022). For correlations, we showed the raw HRV data, and very similar overall patterns were seen when using log-normal data (data not shown). Results shown are mean ± standard error of the mean. For almost all conditions that did not show significance, the statistical information is described in the figure legends.

2.3 Results

2.3.1 Behavioral NSF Measures

The Novelty Suppression of Feeding (NSF) task was designed to assess conflict between drive for food and aversion to a bright light in a food-restricted rat, with food and light at the center of the NSF arena (**Figure 2.1 A**). The NSF task involves a 10 minute session where a rat can execute a series of behavioral measures including (1) latency to first approach the center, (2) number of approaches to center, (3) total time spent in center (tolerance of the bright light), and (4) latency to first grab food, all of which progress towards the main goal of the task, which is ultimately (5) food intake (illustrated in **Figure 2.1 A**). While the main measure of interest is typically food consumed, other measures also provide valuable insight into processing of different aspects of anxiety-like responding (see Introduction). Overall, there were no significant average sex differences between any of the NSF-related behaviors, including food intake (**Figure 2.1 B**, Mann-Whitney, MW=276, p=0.5037), latency to first approach the center (**Figure 2.1 C**, MW=297, p=0.9999), number of approaches (**Figure 2.1 D**, MW=193, p=0.0593), time in center (**Figure 2.1 E**, MW=240, p=0.3719), or latency to first grab food (**Figure 2.1 F**, MW=260, p=0.6225). However, there were significant sex differences in variance for latency to first approach ($F_{(1,26)}$=15.34, p<0.0001) and time in center ($F_{(1,26)}$=24.46, p=0.0333) (while other behavior measures were not significant, p>0.1). As we will see, latency to approach, which was more variable in females (**Figure**

2.1 C), related to HRV only in females. In contrast, time in center, which was more variable in males (**Figure 2.1 E**), was associated with HRV only in males.

2.3.2 HR and HRV Measures: Basal and Overall Changes with NSF Testing

Figure 2.2 A shows examples of heart rate traces, and **Figure 2.3** summarizes how the different HR/HRV measures are determined and interpreted. Female and male rats had significant basal differences in HR and HRV, with greater HR and lower SDNN in females, but no rMSSD differences, similar to patterns seen in humans (Williams et al., 2022) and other rat studies (Carnevali et al., 2023). For HR (**Figure 2.2 B**), there was a significant effect of sex ($F_{(1,48)}$=12.15, p=0.0011), basal versus NSF ($F_{(1,48)}$=59.35, p<0.0001), and interaction ($F_{(1,48)}$=13.17, p=0.0007) (2way-RM ANOVA). Thus, female basal HR was higher than males, and males had a greater rise in HR than females between baseline and NSF. For SDNN (**Figure 2.2 C**), there was a significant effect of sex ($F_{(1,48)}$=13.81, p=0.0005), basal versus NSF ($F_{(1,48)}$=84.48, p<0.0001), and interaction ($F_{(1,48)}$=10.68, p=0.0020). Thus, female basal SDNN was lower than males, and males had a greater drop in SDNN than females from baseline to NSF. For rMSSD (**Figure 2.2 D**), there was a significant effect of baseline versus NSF ($F_{(1,48)}$=7.903, p=0.0071) but not sex ($F_{(1,48)}$=1.054, p=0.3097) or interaction ($F_{(1,48)}$=2.396, p=0.1282), suggesting only minor changes in rMSSD related to a small decrease within the NSF task in males. Further, SDNN/rMSSD (**Figure 2.2 E**) showed a significant effect of sex ($F_{(1,48)}$=19.30, p<0.0001), basal versus NSF ($F_{(1,48)}$=139.3, p<0.0001), and interaction ($F_{(1,48)}$=12.23, p=0.0010). Scatterplots of these data are shown in the Supplemental (**Figure 2.13**). Similar patterns were observed when calculating percent change in each measure (**Figure 2.2 F-I**), with significant sex differences for HR (**Figure 2.2 F**) (MW=183, p=0.0125), SDNN (**Figure 2.2 G**) (MW=164, p=0.0038), and SDNN/rMSSD (**Figure 2.2 H**) (MW=154, p=0.0019) but not rMSSD (**Figure 2.2 I**) (MW=232, p=0.1298). Finally, post-test intake was significantly greater in males, and did not correlate with food intake during the NSF test in either sex (**Figure 2.4**). In sum, males had lower HR, greater SDNN and greater SDNN/rMSSD than females at baseline and showed larger changes in these measures within the NSF task.

2.3.3 HRV Measures and Food Intake

We first examined how HR and HRV measures might relate to food intake across all rats either from baseline or from within the NSF task. We found that female--but not male-- rats overall had HRV measures that predicted food intake. HR alone did not predict food intake, at baseline (**Figure 2.5 A**), during NSF (**Figure 2.5 B**), or as a percent change from NSF to baseline (**Figure 2.5 C**). In contrast, SDNN was significantly related to food intake in females but not in males. More specifically, in females, the relationship between food intake and baseline SDNN was almost significant (**Figure 2.5 D**; $F_{(1,21)}$=4.232, R^2=0.168, p=0.0523), but not for NSF SDNN (**Figure 2.5 E**; $F_{(1,21)}$=0.021, R^2=0.001, p=0.8850). However, females with a greater drop in SDNN from baseline to NSF (expressed as percent) had significantly greater intake (**Figure 2.5 F**; $F_{(1,21)}$=8.672, R^2=0.292, p=0.0077). In males, food intake did not relate to any SDNN measure (**Figure 2.5 D-F**).

SDNN relates to both SNS and PNS tone, whereas rMSSD only indexes PNS influence (**Figure 2.3**). Thus, any effects through PNS would also impact rMSSD. However, food intake was not correlated with any rMSSD measure in either sex (**Figure 2.5 G-I**). On the other hand, similar to SDNN (**Figure 2.5 F**), reduced SDNN/rMSSD also predicted greater food intake in females. Food intake did not relate to SDNN/rMSSD at baseline (**Figure 2.5 J**) or during NSF (**Figure 2.5 K**). However, a larger decrease in SDNN/rMSSD from baseline to NSF (expressed as percent) correlated with significantly greater food intake in females (**Figure 2.5 L**; $F_{(1,21)}$=6.694, R^2=0.242, p=0.0172) but not males (**Figure 2.5 L**; $F_{(1,25)}$=0.233, R^2=0.009, p=0.6333), and with a significant difference in slope between the sexes ($F_{(1,46)}$=4.775, p=0.0340). Lower SDNN/rMSSD ratio and lower SDNN both suggest reduced SNS and thus greater PNS influence. Thus, these findings are consistent with studies suggesting greater PNS utilization in women and SNS in men.

2.3.4 HRV Measures and Latency to First Approach the Center

While food intake is an important measure in NSF, the task is only 10 minutes, and thus behaviors more distal from actual food contact are also of interest. Thus, we next examined the latency to first approach the center. Interestingly, females showed a strong relation between several HRV measures and the latency to first approach the center, which were not observed in males. In addition, for several measures, the baseline HR/HRV level predicted subsequent latency to approach during the NSF, suggesting that the basal autonomic state of females prior to NSF continued during NSF testing and impacted behavior. In particular, in females, longer latency to approach was related to significantly higher HR at baseline (**Figure 2.6 A**; $F_{(1,20)}$=5.933, R^2=0.229, p=0.0243) and during NSF (**Figure 2.6 B**; $F_{(1,20)}$=14.18, R^2=0.415, p=0.0012), but not in males (**Figure 2.6 A,B**). Female SDNN at baseline did not correlate with approach latency (**Figure 2.6 C**; $F_{(1,20)}$=2.341, R^2=0.105, p=0.1417), while reduced SDNN during NSF did (**Figure 2.6 D**; $F_{(1,20)}$=11.43, R^2=0.364, p=0.0030). Furthermore, lower female rMSSD at baseline (**Figure 2.6 E**; $F_{(1,20)}$=5.933, R^2=0.229, p=0.0243) and during NSF (**Figure 2.6 F**; $F_{(1,20)}$=4.575, R^2=0.186, p=0.0450) both correlated with longer latency to first approach. However, in males, no HR or HRV measures correlated with latency to first approach (**Figure 2.6 A-F**). Finally, with significant effects for both rMSSD and SDNN in females, latency to first approach was not correlated with SDNN/rMSSD (**Figure 2.14**). Thus, these results suggest that basal autonomic state in females persisted into NSF testing, such that females with higher HR and reduced PNS and SNS (but no shift in SNS-PNS balance) were delayed in their first approach to center.

One consideration is that higher HR could reduce potential for variability. However, even though HR overall did correlate with lower HRV, there was some dynamic range (**Figure 2.15**). Lastly, female HR did not correlate with several other behavioral measures.

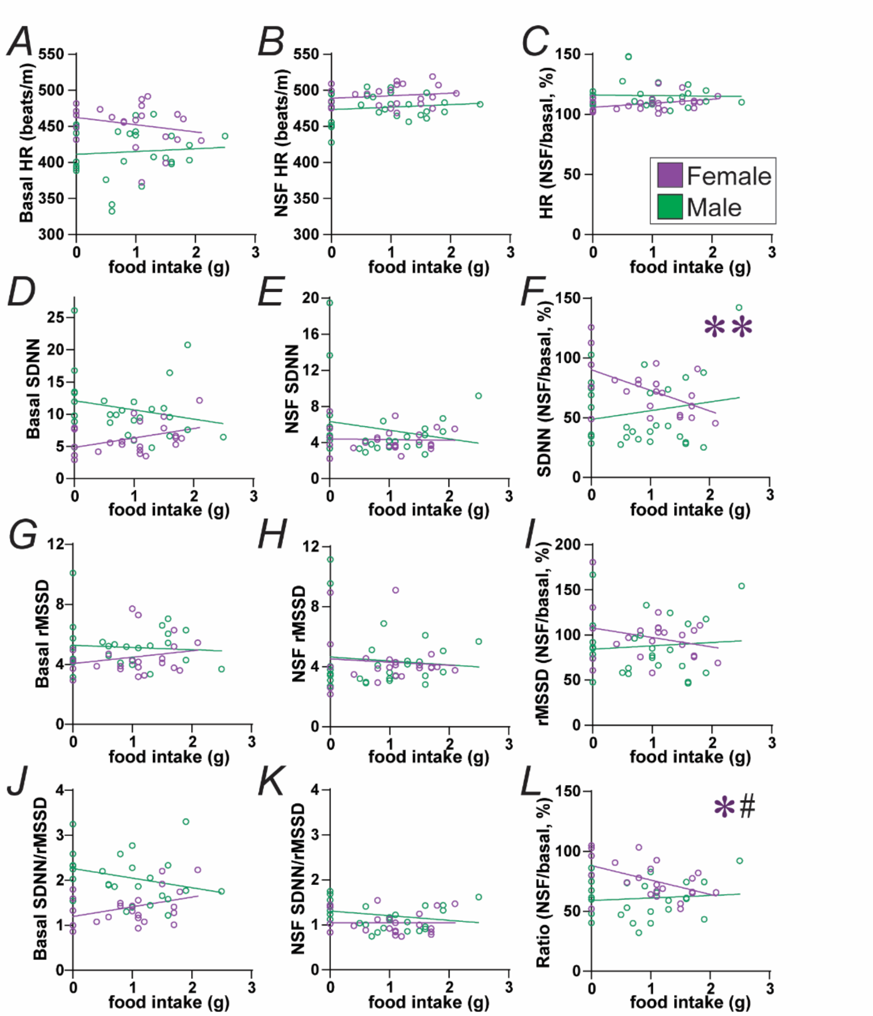

Figure 2.5. Across all rats, females with lower SNS indicators ate more food. (A-C) Food intake did not correlate with HR **(A)** at baseline (females: $F_{(1,21)}$=1.180, R^2=0.053, p=0.2897; males: $F_{(1,25)}$=0.158, R^2=0.006, p=0.6946), **(B)** during NSF (females: $F_{(1,21)}$=0.554, R^2=0.026, p=0.4651; males: $F_{(1,25)}$=0.472, R^2=0.019, p=0.3984), or **(C)** change from baseline to NSF period (females: $F_{(1,21)}$=2.944, R^2=0.123, p=0.1009; males: $F_{(1,25)}$=0.021, R^2=0.001, p=0.8851). **(D-F)** Food intake did not correlate with SDNN **(D)** at baseline (males: $F_{(1,25)}$=1.225, R^2=0.047, p=0.2789) or **(E)** during NSF (males: $F_{(1,25)}$=0.975, R^2=0.038, p=0.3330), but **(F)** females with larger drop in SDNN from baseline to NSF ate more food (males: $F_{(1,25)}$=0.824, R^2=0.032, p=0.3728). **(G-I)** Food intake did not correlate with rMSSD **(G)** at baseline (females, $F_{(1,21)}$=1.160, R^2=0.052, p=0.2938; males, $F_{(1,25)}$=0.146, R^2=0.006, p=0.7057), **(H)** during NSF (females, $F_{(1,21)}$=0.153, R^2=0.007, p=0.6992; males, $F_{(1,25)}$=0.228, R^2=0.009, p=0.6373), or **(I)** change from baseline to NSF period (females: $F_{(1,21)}$=1.547, R^2=0.069, p=0.2273; males: $F_{(1,25)}$=0.179, R^2=0.007, p=0.6759). **(J-L)** Food intake did not correlate with SDNN/rMSSD **(J)** at baseline (females: $F_{(1,21)}$=3.404, R^2=0.140, p=0.0792; males: $F_{(1,25)}$=2.197, R^2=0.081, p=0.1508) or **(K)** during NSF (females: $F_{(1,21)}$=0.000, R^2=0.000, p=0.9951; males: $F_{(1,25)}$=1.718, R^2=0.064, p=0.2019), but **(L)** females with larger drop in SDNN/rMSSD from baseline to NSF ate more food.

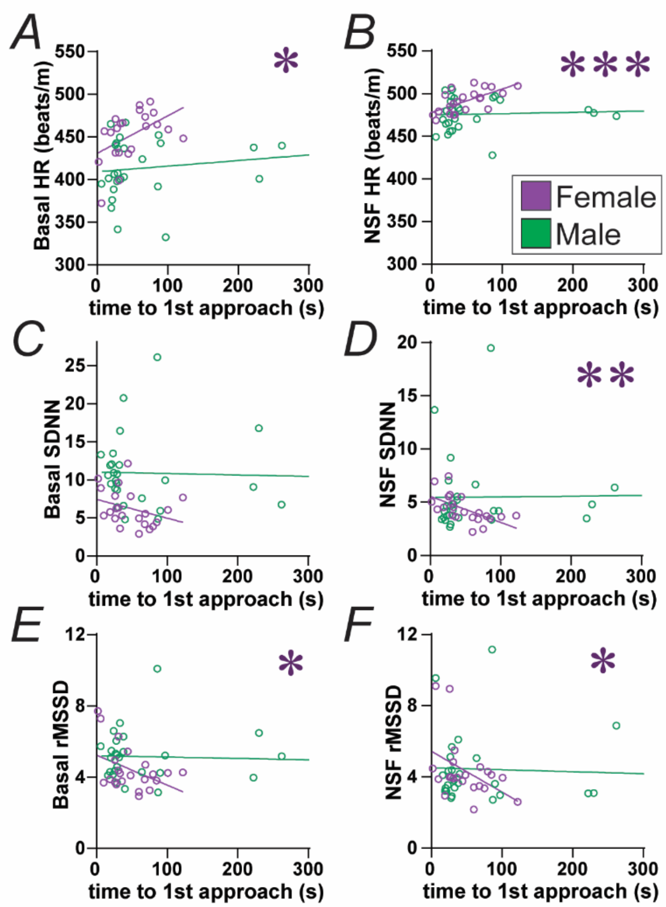

Figure 2.6. Females (but not males) with higher SNS indicators, including greater HR at baseline, had slower latency to first approach. (A,B) Females with higher HR **(A)** at baseline or **(B)** during NSF had significantly slower initial latency to approach the center (males: basal: $F_{(1,25)}$=1.413, R^2=0.053, p=0.2458; NSF: $F_{(1,25)}$=0.264, R^2=0.010, p=0.6118). **(C)** While basal SDNN did not correlate with slower latency (males: $F_{(1,25)}$=0.059, R^2=0.002, p=0.8099), **(D)** females with lower SDNN during NSF had slower initial approach (males: $F_{(1,25)}$=0.011, R^2=0.001, p=0.9175). **(E,F)** Females with lower rMSSD **(E)** at baseline or **(F)** during NSF had significantly slower initial latency to approach center (males: **E,** $F_{(1,25)}$=0.114, R^2=0.114, p=0.7386; **F,** $F_{(1,25)}$=0.115, R^2=0.005, p=0.7373). No HRV measures related to male latency.

2.3.5 HRV Measures and Number of Approaches and Time in Center

Unlike females, no HRV measure predicted latency to first approach the center in males (**Figure 2.6**). In strong contrast, several HRV indicators did predict male but not female NSF behaviors that were intermediate between the first approach and food intake, in particular the number of approaches to center and time spent in center. However, these measures did not relate to HR at basal or during NSF (**Figure 2.16**). Also, basal SDNN was not related to the number of approaches (**Figure 2.7 A**) or time in center, although with a trend in males (**Figure 2.7 C**; males: $F_{(1,25)}$=3.640, R^2=0.128, p=0.0680). In contrast, lower SDNN during NSF was correlated with significantly more approaches in males (**Figure 2.7 B**; $F_{(1,25)}$=4.634, R^2=0.156, p=0.0412) but not in females (**Figure 2.7 B**; $F_{(1,19)}$=1.192, R^2=0.059, p=0.2886). Lower NSF SDNN also correlated with significantly greater time in center in males (**Figure 2.7 D**; $F_{(1,25)}$=4.860, R^2=0.163, p=0.0369) but not females (**Figure 2.7 D**; $F_{(1,19)}$=0.035, R^2=0.002, p=0.8531), and the slope for NSF SDNN was significantly different between the sexes ($F_{(1,44)}$=4.921, p=0.0317). However, rMSSD did not relate to number of approaches at baseline (**Figure 2.7 E**) or during NSF (**Figure 2.7 F**), or to time in center at baseline or during NSF (**Figure 2.7 G,H**).

With reduced SDNN but not rMSSD related to greater male engagement with the center of the NSF arena, one might predict that lower SDNN/rMSSD would also predict higher male behavior. At baseline, ratio did not predict number of approaches (**Figure 2.7 I**) or time in center (**Figure 2.7 J**), although there was a trend in males (**Figure 2.7 K**; males: $F_{(1,25)}$=3.813, R^2=0.132, p=0.0622). However during NSF, males with lower SDNN/rMSSD had significantly more approaches (**Figure 2.7 J**; $F_{(1,25)}$=19.34, R^2=0.436,

p=0.0002) and time in center (**Figure 2.7 L**; $F_{(1,25)}$=8.727, R^2=0.259, p=0.0067). This was not seen in females (**Figure 2.7 J**; number of approaches: $F_{(1,19)}$=0.365, R^2=0.019, p=0.5529; **Figure 2.7 L**; center time: $F_{(1,19)}$=0.403, R^2=0.021, p=0.5330). Furthermore, there was a significant sex difference in slope of SDNN/rMSSD for time in center ($F_{(1,44)}$=7.576, p=0.0086). Similarly, males with a larger change in SDNN/rMSSD from baseline to NSF also had more approaches (**Figure 2.17**). Taken together, these findings suggest that males with lower SNS during the NSF (lower SDNN and ratio, but no differences in rMSSD) made significantly more approaches and spent more time in the center (tolerating the light). Females did not display these patterns, suggesting that reduced SNS could promote different aspects of behavior in females (first approach, food intake) and males (number of approaches, time in center).

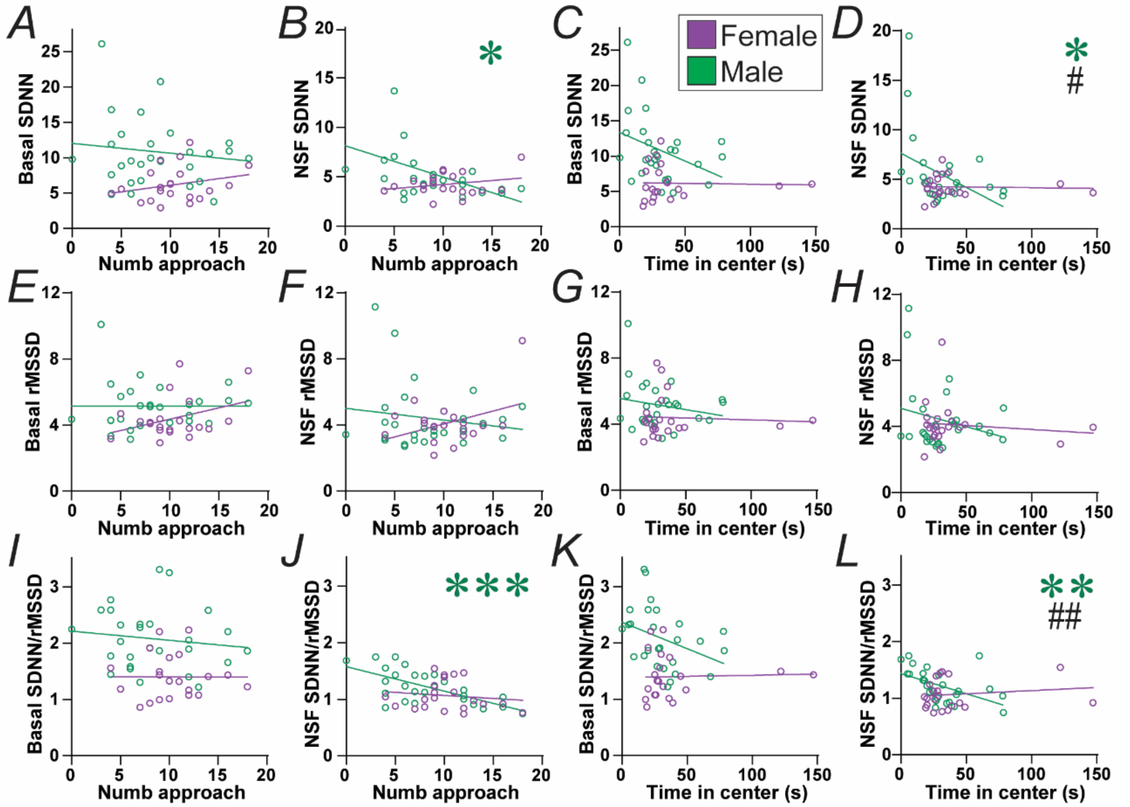

Figure 2.7. Males (but not females) with lower SNS indicators had more approaches to and time in center. (A-D) While basal SDNN did not correlate with **(A)** number of approaches (males: $F_{(1,25)}$=0.433, R^2=0.017, p=0.5167; females: $F_{(1,19)}$=1.319, R^2=0.065, p=0.2650) or **(C)** time in center (females: $F_{(1,19)}$=0.017, R^2=0.000, p=0.8986), males with lower SDNN during NSF had **(B)** more approaches and **(D)** more time in center. **(E-H)** rMSSD did not correlate any measure, at baseline **(E,G)** or during NSF **(F,H)**, or for **(E,F)** number of approaches or **(G,H)** time in center. (**E,** males: $F_{(1,25)}$=0.000, R^2=0.000, p=0.9964; females: $F_{(1,19)}$=2.800, R^2=0.128, p=0.1107; **F,** males: $F_{(1,25)}$=0.641, R^2=0.025, p=0.4309; females: $F_{(1,19)}$=3.212, R^2=0.145, p=0.0891; **G,** males: $F_{(1,25)}$=1.031, R^2=0.040, p=0.3196; females: $F_{(1,19)}$=0.071, R^2=0.004, p=0.7925; **H,** NSF: males: $F_{(1,25)}$=1.421, R^2=0.054, p=0.2444; females: $F_{(1,19)}$=0.258, R^2=0.013, p=0.6171). **(I-L)** While basal SDNN/rMSSD did not correlate with **(I)** number of approaches (males: $F_{(1,25)}$=0.454, R^2=0.018, p=0.5065; females: $F_{(1,19)}$=0.001, R^2=0.000, p=0.9784) or **(K)** time in center (females: $F_{(1,19)}$=0.022, R^2=0.001, p=0.8824), males with lower SDNN/rMSSD during NSF had **(J)** more approaches and **(L)** more time in center.

2.3.6 Exploratory Analysis: Potential Differences Between Consuming and Non-Consuming Rats

When examining **Figure 2.1 B**, we noted that a set of rats ate nothing in the NSF task and that these were somewhat segregated, as a population, from rats that ate some amount of food. Indeed, the distribution of male food intake was not normal when including non-consumers (Shapiro-Wilk=0.9177, p=0.0348 not normal), but was normal when only including consumers (Shapiro-Wilk=0.9773, p=0.8820, therefore normal). Since rats only had a 10 minute NSF period, restricting the time allowed for intake, we examined how HRV measures might relate to NSF behaviors only in rats that consumed some food during the NSF. Indeed, as shown in **Figure 2.8 A** (similar to **Figure 2.5 E**), the relation between male food intake and SDNN during the NSF could have been U-shaped, with higher SDNN in non-consumers, lower SDNN in lower-intake consumers, and somewhat higher in higher-intake consumers. For example, male non-consumers had higher SDNN/rMSSD ratio (**Figure 2.8 B**; MW=21 p=0.0024) and a trend for higher SDNN during NSF (**Figure 2.8 C**, MW=40, p=0.0583), compared to male consumers, as well as fewer approaches than consumers (**Figure 2.8 D**; MW=31, p=0.0148). This subpopulation of non-consuming rats could reflect a different profile of autonomic activation, and thus we sought to better understand how food eaten related to HR/HRV among the majority of rats that did consume some food during NSF.

After removing non-consumers, higher basal SDNN related to greater subsequent intake in females but not males (**Figure 2.8 E**; females: $F_{(1,16)}$=4.536, R^2=0.221, p=0.0491; males: $F_{(1,17)}$=0.117, R^2=0.006, p=0.7361). In strong contrast, males with higher SDNN during NSF had higher food intake (**Figure 2.8 F**; $F_{(1,17)}$=15.05, R^2=0.470,

p=0.0012), while females did not (**Figure 2.8 F**; $F_{(1,16)}$=0.738, R^2=0.044, p=0.4029). Further, for males, a smaller drop (expressed as percent) in SDNN from basal to NSF predicted greater intake (**Figure 2.8 G**; $F_{(1,17)}$=6.103, R^2=0.264, p=0.0244), with the opposite pattern in females ($F_{(1,16)}$=4.066, R^2=0.203, p=0.0609). This trend in females was similar to that in **Figure 2.5 F**, and there was a significant difference in slopes between females and males ($F_{(1,33)}$=8.732, p=0.0057). These results suggest that, in rats that consumed some food, greater food intake in males was associated with higher SNS during NSF to a point, after which too high an SNS activation resulted in no food consumption in males. In females, higher intake related to greater SNS at baseline, such that a larger SDNN drop in females from baseline to NSF predicted higher consumption.

If this interpretation were correct, we would expect that SDNN/rMSSD during NSF would predict food intake in males, while SDNN/rMSSD at baseline would predict amount eaten in females. In agreement, higher basal SDNN/rMSSD predicted greater intake in females but not males (**Figure 2.8 H**; females: $F_{(1,16)}$=5.561, R^2=0.258, p=0.0314; males: $F_{(1,17)}$=0.006, R^2=0.000, p=0.9394). Conversely, higher SDNN/rMSSD during NSF predicted greater intake in males (**Figure 2.8 I**; $F_{(1,17)}$=5.790, R^2=0.254, p=0.0278) but not females (**Figure 2.8 I**; $F_{(1,16)}$=0.639, R^2=0.038, p=0.4359). When examining the drop in SDNN/rMSSD (**Figure 2.8 H**), males had a trend for lower drop in ratio correlating with higher intake ($F_{(1,17)}$=4.131, R^2=0.196, p=0.0580) and females a trend for higher ratio drop related to greater intake ($F_{(1,16)}$=4.333, R^2=0.213, p=0.0538). However, the female and male slopes were significantly different ($F_{(1,33)}$=8.120, p=0.0075). Thus, these findings agree with the possibility that higher SNS during NSF increased intake in male consumers, while higher baseline SNS in females, and a larger SNS drop when moving to NSF, were associated with greater female intake.

2.3.7 Relationship Across NSF Behavioral Measures

Since we observed sex differences in specific NSF behaviors that related to HRV measures, it was also useful to understand how different NSF behavioral measures predicted each other. While HRV indicators were associated with female latency to first approach, and male number of approaches, neither of these behavioral measures correlated with food intake (**Figure 2.18 A,B**). In contrast, latency to grab food significantly correlated with food intake, where rats that grabbed earlier ate more food (**Figure 2.18 C**). However, latency to grab food did not relate to any HR/HRV measure (not shown). While the interpretation remains uncertain, it may relate to the briefness of the food-grabbing activity, and rats often took food back to the periphery to eat. Further studies would be required to better understand these possibilities.

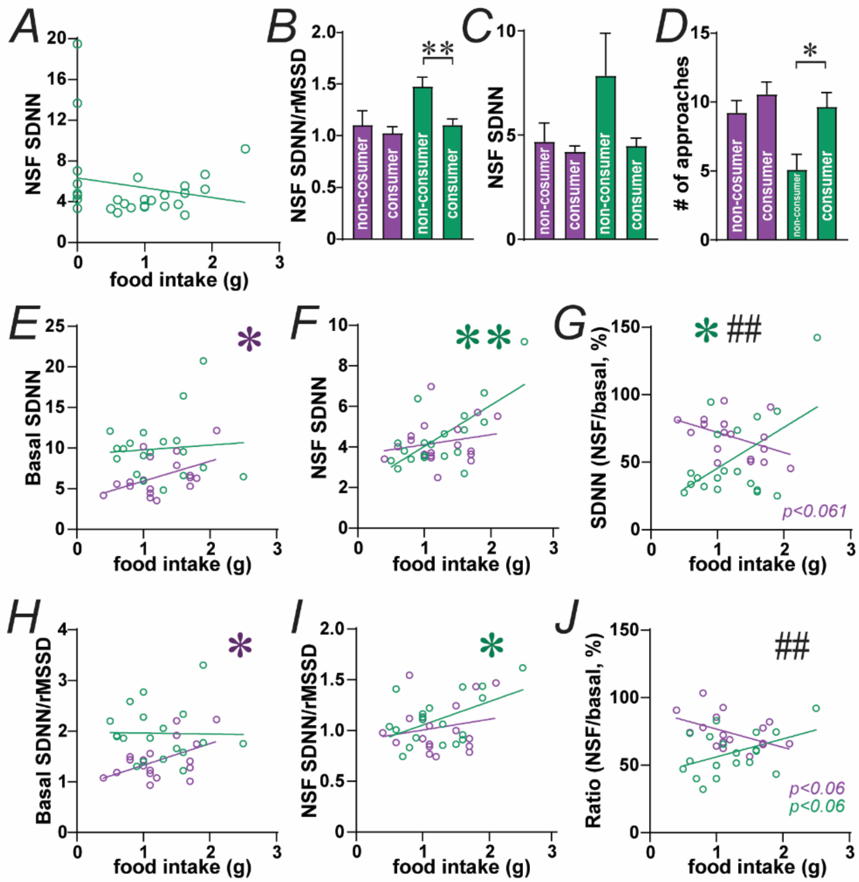

Figure 2.8. In male consumers, greater SNS indicators predicted higher food intake. **(A)** Same data as Fig.2.3F but only males, with non-consumers at 0g food intake, and consumers above that. **(B-D)** Male non-consumers had **(B)** greater SDNN/rMSSD, **(C)** trend for greater SDNN, and **(D)** fewer approaches, than males that consumed food during NSF. **(E-J)** data are for rats that consumed >0g food during NSF. **(E)** Greater basal SDNN in females, but not males, was correlated with higher intake. **(F)** Greater SDNN during NSF predicted higher food intake in males but not females. **(G)** For the drop in SDNN (from basal to NSF), smaller drop in males related to higher intake, with the opposite trend in females (bigger SDNN drop for more intake). **(H-J)** SDNN/rMSSD showed a similar pattern as SDNN, with **(H)** higher basal ratio relating to greater female intake, **(I)** greater ratio during NSF associated with more male intake, and **(J)** opposite trends in females and males (similar to **(G)**). *,** p<0.05, p<0.01. ## p<0.01 female-male difference in slopes

2.3.8 Analysis of HR/HRV Measures Across Time in the Session, Centered on the Time to Grab Food

One question is whether there were changes in any cardiac measures across the NSF session. To determine this, we averaged HR/HRV measures using a 16-sec sliding window across the session. We then centered this data around time to grab food, which was time 0, and extending from 150sec before food grab to 150sec after (see **Figure 2.9** and Methods). To assess HRV changes in the time before grab food, we generated a baseline (averaged across 150sec to 70sec before the time to grab food), and all data values were Z-scored relative to this average and standard deviation. This was done separately for each HR/HRV measure for each session. In this way, we could visualize potential changes across the pre-food grab and post-food grab periods. **Figure 2.9** shows such plots for HR (**A,B**), SDNN/rMSSD (**E,F**) and rMSSD (**I,J**).

To analyze these data, we noted visually that some periods of time might show significant differences from baseline, especially before and after grab in females for SDNN/rMSSD, and after food grab for HR in both sexes. With the great number of possible post-hoc comparisons (for an ANOVA with so many time points), we chose to examine possible HR/HRV changes in two analyses windows, one 30-40 seconds before the grab ("-30sec" grey rectangles; where females seemed to show increased SDNN/rMSSD), and the other ~20 seconds after the grab ("+20sec" white rectangles; from 18 to 22sec after grab food, around the peak of the HR drop). Indeed, both sexes showed significantly lower HR in the +20-sec analysis window versus baseline (**Figure 2.9 C,D**; 1-way repeated measures ANOVA: females, $F_{(2,18)}$=4,163, p=0.0249; males, $F_{(2,18)}$=8.400, p=0.0018; post-hoc females p=0.0202, males p=0.0120). For the other

HRV measures we used a Friedman test with Dunn's post-hoc as a non-parametric variant of 1-way repeated measures ANOVA. The SDNN/rMSSD measure in females was elevated at both the -30sec and +20sec analysis windows (**Figure 2.9 G**, Friedman stat=10.33, p=0.0057, basal vs -30sec p=0.0373, basal vs +20sec p=0.0081), with no relation in males (**Figure 2.9 H**, Friedman stat=0.4211, p=0.8102). In addition, there were no differences across analysis time points for rMSSD (**Figure 2.9 K,L**, female: Friedman stat=1.444, p=0.4857, male: Friedman stat=4.526, p=0.1040) or SDNN (**Figure 2.19**). The similar statistics for SDNN and rMSSD in females in this rank-sum measure raises the speculation that SDNN and rMSSD are more tightly coupled in females than males. Also, after the food grab and the transient HR dip, HR levels were significantly greater in females ($t_{(17)}$=2.234, p=0.0392, paired t-test), which was not observed in males ($t_{(18)}$=0.011, p=0.9911).

One final question is whether the greater SDNN/rMSSD in females before food grab relates the time to grab or food eaten measures. However, the increase in SDNN/rMSSD did not correlate with time to grab food ($F_{(1,16)}$=0.094, p=0.7631) or with food eaten ($F_{(1,16)}$=0.121, p=0.7326). Taken together, these findings suggest the possibility that HR/HRV measures were, on average, relatively consistent before the food grab, with some periods of change in some measures. As such, there was greater SDNN/rMSSD in females before and after food grab, which could suggest a sex-specific HRV regulator of food grabbing in the NSF, as well as a small but significant decrease in HR after grabbing food in both sexes.

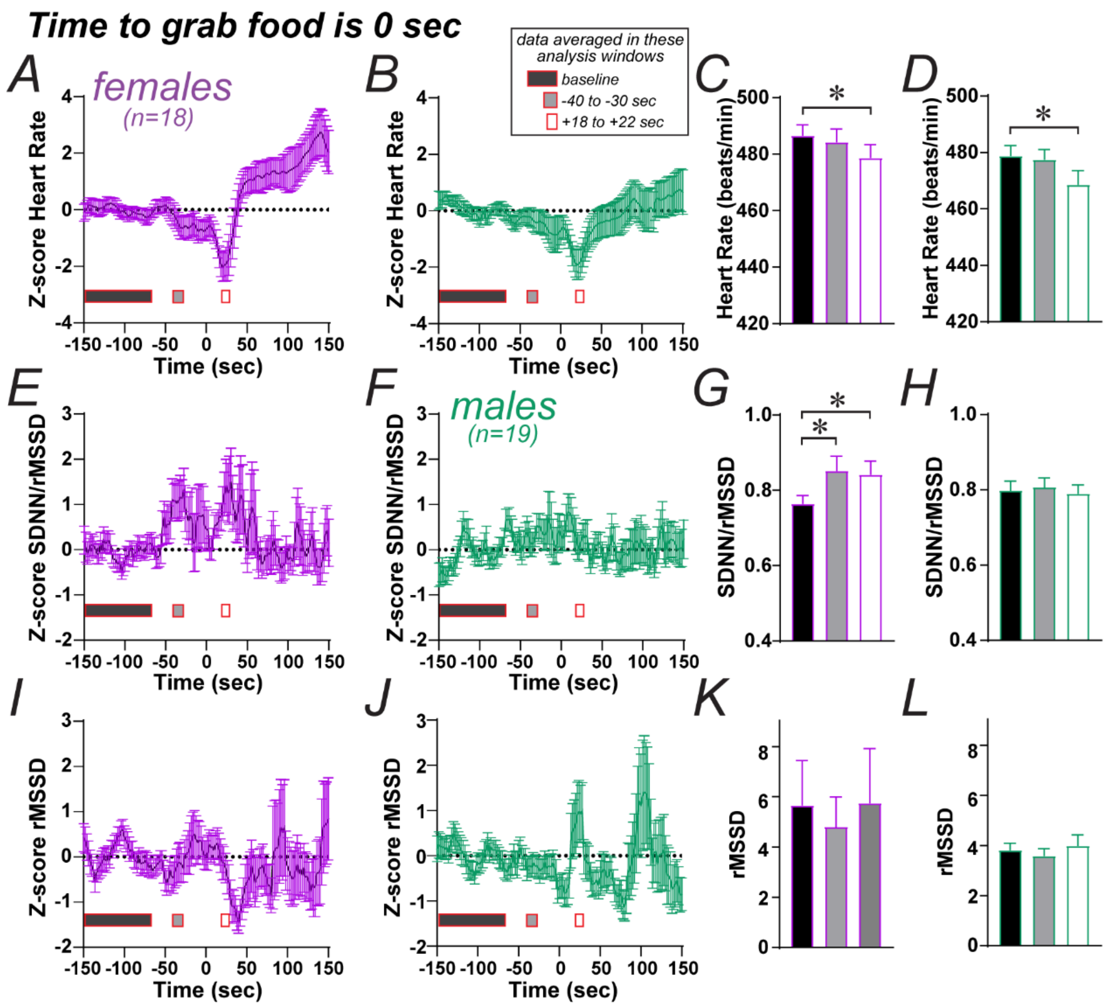

Figure 2.9. Limited changes in HR/HRV measures across time, centered on the time to grab food, an important step in the NSF. See Methods for how the by-time data were generated. **(A-D)** Both females and males show a dip in heart rate after the food grab. Note that, for a one-way ANOVA, data were first normalized by Z-score to a baseline period (150 to 70 sec before grab food), then compared across baseline, an interval 40 to 30 sec before food grab (gray rectangles in figure), and an interval 18 to 22 sec after food grab (white rectangles, about the peak of the heart rate drop after food grab). **(E-H)** Females showed an increase in SDNN/rMSSD both before and after the food grab, while males did not. **(I-L)** No changes in rMSSD before food grab. * p<0.05.

2.4 Discussion

Women have nearly twice the prevalence of anxiety disorders, but critical mechanisms underlying sex differences in expression of anxiety remain incompletely understood. We utilized cardiac telemetry to collect data on HR and HRV, since these have demonstrated potential as important biomarkers for and contributors to pathological states, including neuropsychiatric conditions. In addition, HR/HRV measures could help to better understand sex differences in anxiety-related autonomic mechanisms. Thus, we examined cardiac telemetry in food-restricted adult rats during the novelty suppression of feeding (NSF) task, which presents rats with a conflict between food and light at the center of the NSF arena, with results summarized in **Figure 2.10**. In females, basal HR was greater than in males, and basal SNS-related indicators (SDNN and SDNN/rMSSD) were lower than in males. While apparently paradoxical to have a higher resting HR in addition to a higher SDNN and SDNN/rMSSD ratio, these patterns are similar to what is seen in a large recent human study (Williams et al., 2022) and rodent study (Carnevali et al., 2023) and suggest that rat telemetry is robustly assessing HR/HRV function. While the precise reasoning for this apparent paradox is not yet fully understood, the same large human study suggests the sexes are perhaps different in their sensitivities to acetylcholine on cardiac chronotropy, which may be an effect of estrogen (Williams et al., 2022). During the NSF, females (but not males) with higher basal HR and lower PNS index (reduced rMSSD) were slower to make the first approach to center, suggesting sex differences in basal autonomic state that females brought into and impacted initial NSF behaviors. In contrast, males (but not females) had HRV patterns suggesting that reduced SNS function during NSF (lower SDNN and SDNN/rMSSD, without changes in rMSSD) led to an increased number of approaches to center and time in center. Finally, across all rats, females with lower SNS indicators consumed significantly more food, with no overall relation in males. However, there were two potentially distinct subpopulations of rats, some that consumed no food during the 10-minute NSF, and most rats that consumed some food. Non-consuming males had significantly higher SNS measures (higher SDNN/rMSSD, trend for higher SDNN) and reduced approaches than male consumers. Importantly, among consumers, males with greater SNS indicators ate more food up to a point, after which we posit SNS perhaps became active enough to inhibit food intake in the non-consuming males. This is the opposite to what we observed in females (lower SDNN and SDNN/rMSSD), with no differences in autonomic indices related to whether food was consumed within the task. Interestingly, this is congruent with human findings suggesting that women engage PNS more, and men SNS more, during autonomic regulation under challenge. Thus, our findings show novel and likely clinically relevant sex differences in HRV-behavior relationships. In addition, our results suggest that by examining HRV measures during different aspects of anxiety-like responding within the NSF task (baseline, initial approach to center, overall approaches to center, time to grab food, and food intake), we may gain new insights into sex-specific vulnerabilities and expression of anxiety-like behavior.

Many groups have suggested that women engage the PNS more during autonomic regulation under challenge, while men engage the SNS more (Dart et al., 2002; Sato and Miyake, 2004; Nugent et al., 2011; Hamidovic et al., 2020; Koenig et al., 2021), and such findings align with sex differences appreciated in HRV brain circuitry (discussed below). Our findings are, to our knowledge, the first to provide clear functional implications of the PNS-SNS sex differences using HRV indices, and, interestingly, that these HRV measures related to different aspects of anxiety-like behavior within the NSF task. Our findings might suggest that females with higher basal SNS (higher HR) and lower basal PNS (lower rMSSD) brought this autonomic state into the beginning of the NSF task, where such autonomic engagement significantly slowed the latency to first approach the center. However, females likely then disengaged from this state, since lower SNS indices were associated with greater food intake, where reduced SNS was indicated by lower SDNN and SDNN/rMSSD without changes in rMSSD, suggesting that SNS influence decreased without PNS alterations. In contrast, basal HR/HRV measures in males did not relate to any NSF behavior. Instead, males with lower SNS during NSF (reduced SDNN and SDNN/rMSSD with no significant change in rMSSD) had a greater engagement with the task center, shown through more approaches to and greater time spent in the brightly lit arena center. However, males also likely had a shift in balance, since exploratory analyses suggested that males with greater SNS either ate no food at all (non-consumers) or, for males that did consume some food, higher SNS related to greater intake level. Thus, we posit that perhaps males that engage SNS up to a point are able to consume food in the novel context, but after such point the SNS engagement is possibly overwhelming and leads to no food consumption. Such findings could be used to improve and individualize anxiety treatment, not merely by sex but also by employing personalized understanding of one's unique autonomic engagement. One such example for women would be that the different HRV changes at baseline, early in anxiety testing, and for different anxiety measures, could all come together to provide a robust constellation of biomarkers for those with particular risk for and/or expression of anxiety states.

We also note that, more generally, female rats had higher HR and lower SDNN, with no differences in rMSSD. These patterns were similar to what has been reported in humans (Williams et al., 2022) and rats (Carnevali et al., 2023). The presence of these basal differences suggests that our telemetry was accurately and robustly assessing HR and HRV parameters and validates that other HRV differences we observed are likely to be translationally and clinically relevant. However, we also note the underlying mechanisms for the apparent paradox in female autonomics, with great HR and also HRV, remain unclear, several studies have posited that sex differences in cardiac acetylcholine regulation could contribute to this apparent paradox (Dart et al., 2002; Williams et al., 2022).

It is also interesting that female HRV measures were particularly related to the first challenge-related movement in the task (the latency to first approach the center in addition to food intake). In this regard, our previous work suggests that female anxiety-like behavior is only greater than males for aspects of a task that might be considered most life-relevant, including food intake in NSF and the first movement in the novel context (De Oliveira Sergio et al., 2021) (also discussed in the Introduction). We note that our present studies did not observe sex differences in the level of these NSF behaviors, unlike the previous study (De Oliveira Sergio et al., 2021). Several factors may contribute to this, including use of a larger NSF arena here (100x100cm here, 60x60cm in De Oliveira Sergio et al., 2021), and where rats in the present work had undergone telemetry surgery ~10 days before NSF testing, both of these factors could plausibly increase stress or anxiety in the rat. Nonetheless, even though we did not observe behavioral differences here, the relation between female HRV and first latency to approach the food as well as food intake concurs conceptually with our previous work. In particular, in our previous work we had compared female and male rat behavior in NSF and in the Light-Dark Box (LDB), which we consider a control task since it involves aversion to bright light, but no food restriction (De Oliveira Sergio et al., 2021). Importantly, we used a moderate dose of the anxiolytic diazepam (Bodnoff et al., 1989; Shephard & Broadhurst, 1982) to assess whether any sex differences actually reflect anxiety (e.g., vs locomotion). Interestingly, only a subset of anxiety measures show sex differences. In NSF, these are measures directly related to food (latency to grab food, amount of food eaten), where females show greater anxiety, and diazepam causes more anxiolysis in females. In strong contrast, measures less directly related to food (such as time in the center) are similar in females and males. In addition, LDB responding is largely comparable in females and males, except for the first movement in the task, moving from the lit chamber into the dark (a novel context), where females had more anxiety-like behavior (De Oliveira Sergio et al., 2021).

Taken together, we have interpreted these findings to indicate that females are more impacted by aspects of an anxiety-like task that could be considered more life-threatening or life-relevant, with largely similar anxiety-like expression in females and males for behaviors more distal to the central focus (e.g., grabbing food). Interestingly, results from other studies add additional support for this possibility. When a shocker is placed in the home cage bedding, females bury the shock probe more quickly than males, and diazepam slows latency-to-bury in females more than males; however, there are no sex differences in other behaviors more distal to direct interaction with the shocker (Olvera-Hernandez & Fernandez-Guasti, 2011). Females also have greater anxiety and bigger effect of anxiolytics with predator odor (Shephard and Broadhurst, 1982; Shepherd et al., 1992). Thus, we hypothesize that females are specifically impacted by life-threatening conditions (food when food restricted; initial uncertainty in a novel context; direct shock; imminent danger). In the present study, we did not observe overt behavioral differences in the first approach to center or for food intake. However, the initial approach in females was strongly delayed when basal HR was higher (and rMSSD lower), while food intake was related to lower SNS indicators (lower SDNN, lower SDNN/rMSSD ratio, no significant change in rMSSD). In contrast, more intermediate behaviors, such as number of approaches and time in center, were not sex-different in the previous study (De Oliveira Sergio et al., 2021), and were not correlated with HRV measures in females, but only in males. One implication is that, even with sex-similar behavior and diazepam sensitivity in the number of approaches and time in center, there were still sex differences in autonomic mechanisms related to these behaviors. Thus, our present HRV studies provide novel insights into underlying mechanisms that would not be detectable with behavioral and even pharmacological methods. Indeed, in some ways, the similar NSF behavior was a strength, since it allowed us to infer that anxiety-like behaviors were related to and likely influenced by different autonomic drivers in females and males rather than differences in overt behavior.

One key question is whether the greater HR in females, especially during NSF, might reduce HRV simply by mathematically reducing potential for variability, which could confound accurate interpretation of HRV measures. At present, we cannot definitively answer this question. Greater HR did relate to reduced HRV (**Figure 2.15**), but HR only correlated with one behavioral measure, latency to first approach, and only in females. Especially during NSF, when HR was higher in both males and females, we note that other behaviors (number of approaches, time in center) were related to lower SDNN in males but not in females. Thus, it is unlikely that HR differences explained all the observed sex-related patterns, although we cannot rule out, for example, that the relation between lower rMSDD and slower first approach (**Figure 2.6 E,F**) is at least in part impacted by greater HR reducing variability.

When our group initially planned these experiments, we had expected to see not only sex differences in anxiety-like responding, but also hypothesized that we would appreciate differences based on alcohol drinking history. This was in part due to results from the human literature that suggest humans with alcohol use disorder have altered basal HRV (Ingjaldsson et al., 2003; Ralevski et al., 2019) compared to healthy controls. Furthermore, HRV in alcohol use disorder patients can reliably predict relapse (Nugent et al., 2011). Thus, we had initially hypothesized that rat drinking history would also alter HRV responses, at baseline and during anxiety-like behavior. However, we found no such differences by drinking history, only by biological sex (**Figures 2.11, 2.12**). We believe this is due to the model of alcohol drinking we utilize in our rats, which does not recapitulate alcohol dependance but rather facilitates compulsive-like drinking, a critical barrier to treatment in addiction disorders (De Oliveira Sergio et al., 2023; Starski et al., 2023) (and see Methods). We choose to model compulsion rather than dependence in part since many problem drinkers are not dependent (Esser et al., 2014). Thus, the model of alcohol drinking utilized by our lab may not be severe enough to replicate findings from certain human alcohol studies (Discussed further in De Oliveira Sergio et al., 2023; Starski et al., 2023).

Another important caveat of the present study is the use of singly housed animals, since single housing is a stressful stimulus and can affect cardiovascular functioning (Sgoifo et al., 2014; Cruz et al., 2016) and anxiety-like behavior (discussed in De Oliveira Sergio et al., 2021). However, part of our reason for single housing was to support the larger goal of understanding interactions between alcohol and anxiety, where single housing is often required for alcohol drinking rat studies. Furthermore, as mentioned above, we had initially hypothesized that alcohol drinking history could affect HR/HRV responses in anxiety-like behavior, and thus planned the study using our typical alcohol drinking protocols which require single housing rats.

It is also worth noting that our rats were food restricted for 3 days prior to testing, and food restriction could plausibly alter cardiovascular measures and increase stress. Indeed, as detailed in the Methods, food restriction did lead to some changes relative to free feeding. However, other groups such as Inagaki and colleagues found that food restriction alone did not alter HRV in rats (Inagaki et al., 2005). Thus, the important question of the role of food restriction on HRV remains open, and future work is needed to further clarify these possible effects.

Another important consideration is how autonomic regulation can vary across the menstrual cycle in humans, and estrous cycle in rats. Many studies find clear sex differences in baseline HR and HRV in freely cycling women (Nugent et al., 2011; Hamidovic et al., 2020; Williams et al., 2022), as we (**Figure 2**) and others (Carnevali et al., 2023) observed in rats. However, estrogen can increase PNS activity in humans

(Huikuri et al., 1996; Goldstein et al., 2005; Goldstein et al., 2010) and rodents (Dart et al., 2002). Also, we find clear sex differences in anxiety-like behavior in freely-cycling females versus males (De Oliveira Sergio et al., 2021), where we examined a large sample of females, in different cohorts tested across several days, in a partial, although likely incomplete, attempt to sample across estrous stages. Rodent studies show reduced anxiety-like behavior in proestrus (Palanza et al., 2001; Amodeo et al., 2018; Miragaia et al., 2018), although other groups find no estrous influence in elevated plus maze or open field (Henricks et al., 2017; Scholl et al., 2019), shocked licking (De Jesus-Burgos et al., 2016), or in light enhancement of startle (Davis et al., 2010), and ovariectomy alters NSF performance (Mahmoud et al., 2016). In humans, women exhibit anxiety differences across the menstrual cycle. During aversion responding, there is greater central amygdala (and other regions) activity when estrogen is low versus high, with brain activity under low estrogen more similar to males, suggesting that higher estrogen may reflect an anti-stress mechanism (Goldstein et al., 2005; Goldstein et al., 2010; Jacobs et al., 2015; Wheelock et al., 2021). However, one human study found that HFHRV showed greater sex differences (~45%) compared to cycle stage differences (~16%) (Sato and Miyake, 2004). Thus, some conditions show clear association with estrogen levels, while others may not vary across the menstrual and estrous cycles. Future studies are needed to examine more carefully the relation between estrous cycle and autonomic regulation during anxiety-related conditions.

A final critical question is whether there are sex differences in brain circuits underlying anxiety and autonomic regulation. Many human studies have noted sex differences in expression of affect related behaviors, including neural correlates of emotional reactivity (Young et al., 2013; Lee et al., 2014; Kogler et al., 2015; Filkowski et al., 2017), e.g. with greater amygdala in women and anterior insula in men in an emotion-evoking task (Filkowski et al., 2017). Males also have larger cortisol increases than women during stressors (Wheelock et al., 2021). Other work suggests greater top-down regulation of emotion in men versus women (Mak et al., 2009; Domes et al., 2010; Filkowski et al., 2017), perhaps reflecting greater differences in effort and/or cognitive strategies (Domes et al., 2010; Filkowski et al., 2017). Further, better emotional regulation is related to positive insula-amygdala connectivity in women, with negative connectivity in males (Wu et al., 2016). Also, greater PNS function is associated with greater amygdala activity in women (but not men) under cognitive challenge (Nugent et al., 2011) and at rest (Nugent et al., 2011; Allen et al., 2015), and greater amygdala and insula activation predicts more negative affect during emotion tasks in women but not men (Mak et al., 2009). As noted above, amygdala activity reflects basal SNS activation by negative images in women during lower estrogen stages of the menstrual cycle (Goldstein et al., 2005; Goldstein et al., 2010). In addition, while few studies examine sex differences in HRV circuitry in neuropsychiatric conditions, women with depression (but not men) have higher insula and amygdala activity that relates to lower HFHRV during negative affect (Garcia et al., 2020). Finally, it is likely that brainstem areas (Oppenheimer & Cechetto, 2016) also contribute to differences, and sex differences are observed in important arousal systems (noradrenaline (Reyes et al., 2014), orexin (Grafe et al., 2017)) where females do not show habituation to receptor stimulation, leading to protracted sensitivity to stressors. Thus, there are likely important sex differences in brain circuit regulation of anxiety-like behaviors and autonomic regulation. However, there are several challenges when interpreting such studies, including difficulty in disentangling biological versus social influences (Altemus et al., 2014; Filkowski et al., 2017), and where similar behavior may involve different brain areas (e.g. human (Goldstein et al., 2005), rodent (Simone et al., 2018)). Also, some studies find few sex differences, including in insula activation to stress cues (Seo et al., 2011; Seo et al., 2017). Thus, sex differences in autonomic regulation under challenge, and underlying brain circuits, remain of great and clinically relevant interest, but are still incompletely understood. Future studies, both in humans and animals, are needed to further disentangle these questions.

Taken together, our studies provide novel and likely clinically relevant insights into sex differences in autonomic mechanisms that contribute to specific aspects of basal and anxiety-like conditions. Since women have nearly twice the risk of developing an anxiety disorder, and human studies suggest sex differences in use of PNS versus SNS, our results provide important insight into when and how such sex differences manifest before and during anxiety-like behavior.

	Females			Males			Behavioral Difference
	HR	HRV-SNS	HRV-PNS	HR	HRV-SNS	HRV-PNS	
Basal Differences	> males	< males (SDNN, ratio)	no relation				
Slower first approach	↑HR	uncertain	↓ PNS (rMSSD,SDNN)	--	--	--	Females less variable
More approaches	--	--	--	--	↓ SNS (SDNN, ratio)	--	
More time in center	--	--	--	--	↓ SNS (SDNN, ratio)	--	Males less variable
More food intake (all rats)	--	↓ SNS (SDNN, ratio)	--	--	--	--	
More food intake (eaters)	--	↓ SNS (SDNN, ratio)	--	--	↑SNS (SDNN, ratio)	--	

Figure 2.10. Summary of sex differences in the relation between HRV patterns and specific NSF behaviors. For females, greater HR at baseline and during NSF predicted slower initial latency to approach, while lower SNS correlated with greater food intake. In males, lower SNS was associated with increased number of approaches and time in center, while greater SNS predicted higher food intake in rats that consumed food. "- -" indicates no relation between HRV and behavioral measures.

2.5 Supplemental Materials

2.5.1 Alcohol Drinking Protocol

Rats first drank under a 2-bottle choice intermittent access to alcohol paradigm, with access to alcohol (20% v/v), or water in a second bottle. Alcohol access began on Monday, Wednesday, and Friday at ~1 hour into the dark cycle and lasted 16-24 hours each day. Following ~3 months of IA2BC, rats were switched to drink alcohol (20% v/v) or water for 20min/day Monday-Friday. Non-drinking rats lived in the same room but never had access to alcohol.

2.5.2 Telemetry Surgery

Alcohol was withheld from rats for approximately 48-72 hours prior to surgery to prevent complications. Using antiseptic surgical techniques, rats were put under isoflurane anesthesia and implanted with a telemetry device (type PTA-M-C, part# E-430001- IMP-130) from TSE Systems Inc. (Chesterfield, MO), with instruction and assistance from TSE personnel. The telemetry device consisted of a silicone elastomer transmitter (8.3 mm in by 16.5 mm x 4 mm), and a thin, plastic-sheathed wire which had a small sleeve at the distal tip which detected changes in blood pressure within the artery. The surgery required two incisions, one in the midline of the abdominal cavity (to place the transmitter), and a second where the abdomen meets the left thigh to access the left femoral artery. The femoral artery was carefully dissected from the adjacent femoral vein and femoral nerve, and then dilated through topical application of 2% injectable lidocaine. Suture silk was used to temporarily occlude the femoral artery, and then a small needle puncture was made into the vessel. The wire with telemeter at the end was inserted into the blood vessel, the silk suture was loosened slightly, and the wire tip advanced until it sat approximately between the iliac bifurcation and the renal arteries within the abdominal aorta. This was assisted by a removable trocar which led the wire. Once the wire was in place, the suture silk was lightly tied to the femoral artery to keep the wire from slipping and to assist in closing the small puncture to prevent bleeding. To confirm placement within the abdominal aorta, real-time blood pressure trace was assessed using NOTOCORD-hem software (Instem, Staffordshire, UK). As the aorta is the major arterial vessel emerging from the heart, it provides accurate information about HR and blood pressure. Nonabsorbable sutures were used to attach the transmitter unit to the inner musculature of the abdominal wall. Finally, the animal is sewed up with absorbable sutures, provided pain relieving drugs (carprofen 5mg/kg and buprenorphine 0.03mg/kg), and placed in their homecage for recovery.

2.5.3 Supplemental Figures

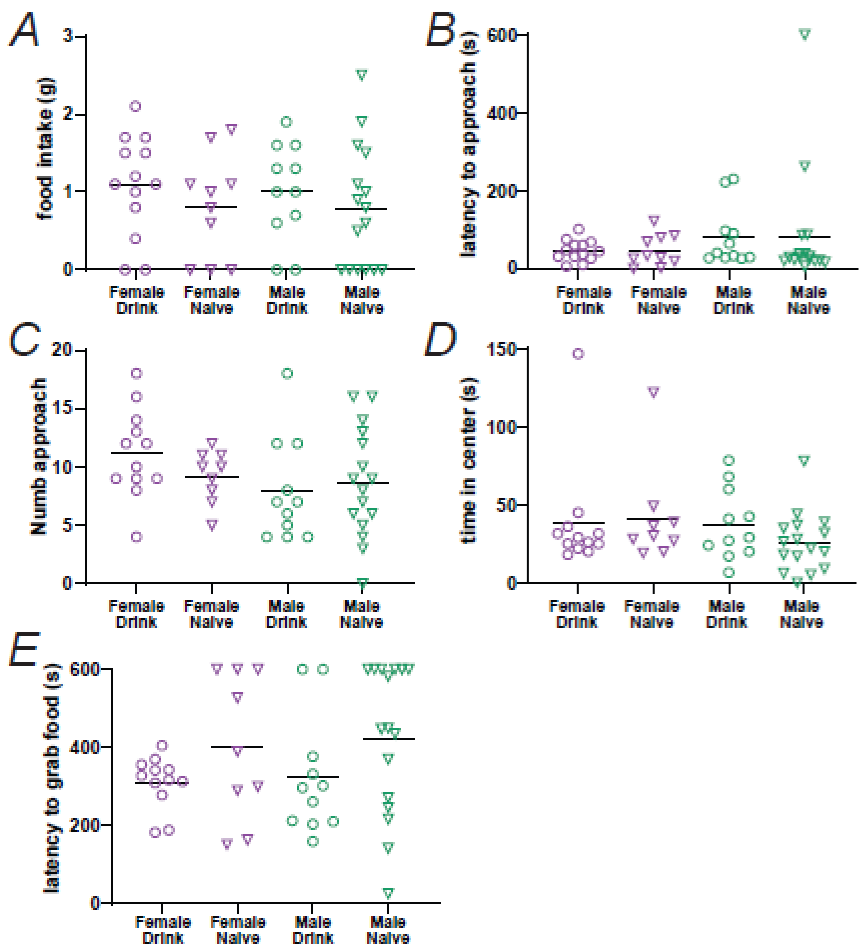

Figure 2.11. No average behavioral differences between drinkers and non-drinkers in **(A)** food intake (Fs<1.56, ps>0.2), **(B)** latency to approach food (log, Fs<2.3, ps>0.13), **(C)** number of approaches (Fs<2.35, ps>0.13), **(D)** time in center (log, Fs<1.99, ps>0.16), or **(E)** latency to grab food (log, Fs<0.9, ps>0.3).

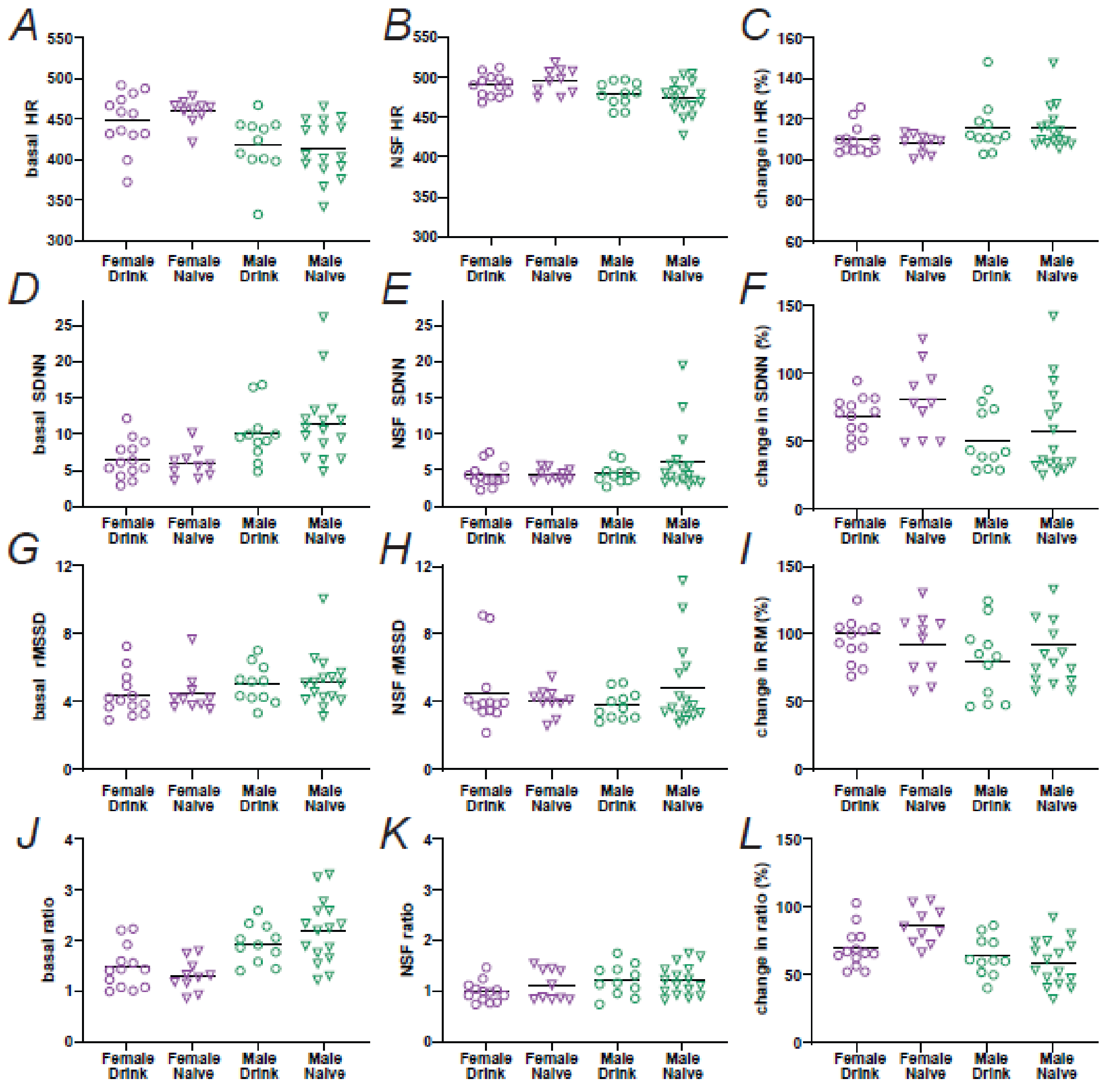

Figure 2.12. No average HR/HRV differences between drinkers and non-drinkers for nearly all measures. (A-C) HR **(A)** at baseline (sex: F(1,46)=16.17, *p*=0.0002, other Fs<0.9, ps>0.3), **(B)** during NSF (sex: F(1,46)=10.96, *p*=0.0018, other Fs<1.1, ps>0.3), and **(C)** % change from basal to NSF (log, sex: F(1,46)=6.399, *p*=0.0149, other Fs<0.3, ps>0.6). **(E-G)** SDNN **(E)** at baseline (log, sex: F(1,46)=22.08, *p*<0.0001, other Fs<0.7, ps>0.4), **(F)** during NSF (log, Fs<1.5, ps>0.2) , and **(G)** % change from basal to NSF (log, sex: F(1,46)=10.90, *p*=0.0019, other Fs<0.7, ps>0.4). **(H-J)** rMSSD **(H)** at baseline (log, sex: F(1,46)=3.645, *p*=0.0624, other Fs<0.2, ps>0.7), **(I)** during NSF (log, Fs<1.5, ps>0.2), and **(J)** % change from basal to NSF (log, Fs<2.7, ps>0.11). **(K-M)** SDNN/rMSSD **(K)** at baseline (log, sex: F(1,46)=27.12, *p*<0.0001, other Fs<1.97, ps>0.18), **(L)** during NSF (log, sex: F(1,46)=4.246, *p*=0.0450, other Fs<0.8, ps>0.4), and **(M)** % change from basal to NSF (log, sex: F(1,46)=13.51, *p*=0.0006; interaction: F(1,46)=5.289, *p*=0.0260; drinker-vs-naïve: F(1,46)=0.885, *p*=0.3518). Thus, there was an effect of drinking condition for percent change in SDNN/rMSDD, although with multiple corrections, this would not be considered significant.

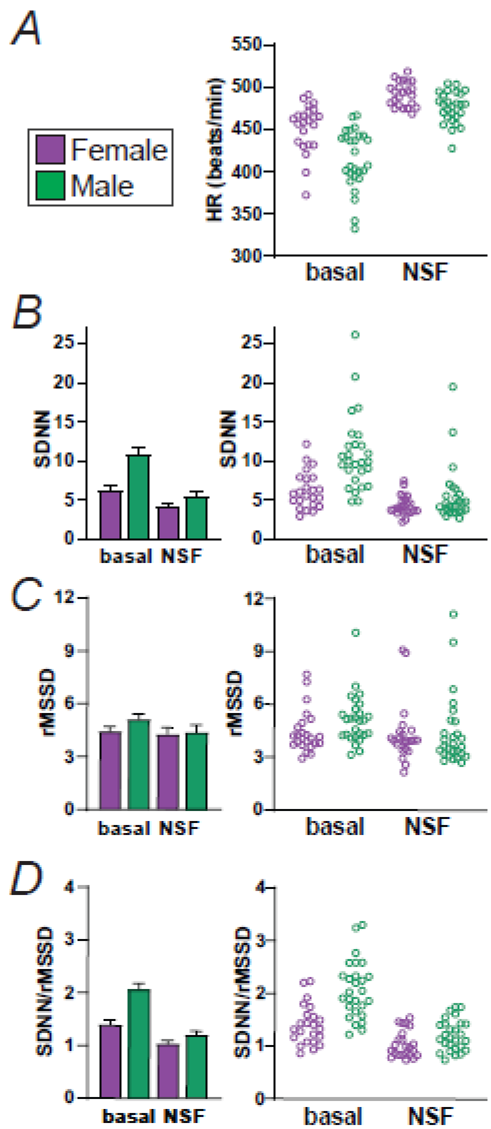

Figure 2.13. **Raw data and scatter plots for basal and NSF HR/HRV measures.** Also, we ran two-way ANOVAs on the basal-vs-NSF HRV measures, even though some groups for each measure were not normal, to compare basal versus NSF measures (within subject), and across females and males. Results for SDNN and SDNN/rMSSD were similar to log-normalized data (**Figure 2**). For SDNN, there was a significant effect of sex ($F(1,48)=22.19$, $p<0.0001$), basal versus NSF ($F(1,48)=127.1$, $p<0.0001$), and interaction ($F(1,48)=5.961$, $p=0.0184$). Thus, female basal SDNN was lower than males, and males had a greater drop in SDNN than females. However, there were no significant changes for rMSSD (sex: $F(1,48)=0.984$, $p=0.3262$; basal-NSF: $F(1,48)=3.659$, $p=0.0618$; interaction: $F(1,48)=1.621$, $p=0.2091$). Even so, SDNN/rMSSD showed a significant effect of sex ($F(1,48)=20.91$, $p<0.0001$), basal versus NSF ($F(1,48)=103.3$, $p<0.0001$), and interaction ($F(1,48)=17.56$, $p=0.0001$).

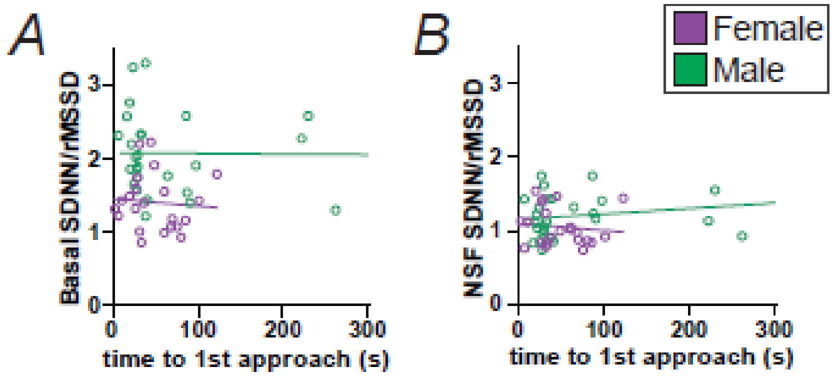

Figure 2.14. First approach latency. With significant effects for both rMSSD and SDNN, there was no association between latency to first approach and SDDN/rMSSD, **(A)** at baseline (females: $F_{(1,20)}$=0.123, R_2=0.006, p=0.7294; males; $F_{(1,25)}$=0.008, R_2=0.000, p=0.9309), or **(B)** during NSF (females: $F_{(1,20)}$=0.267, R_2=91 0.013, p=0.6110; males: $F_{(1,25)}$=2.651, R_2=0.096, p=0.1160.

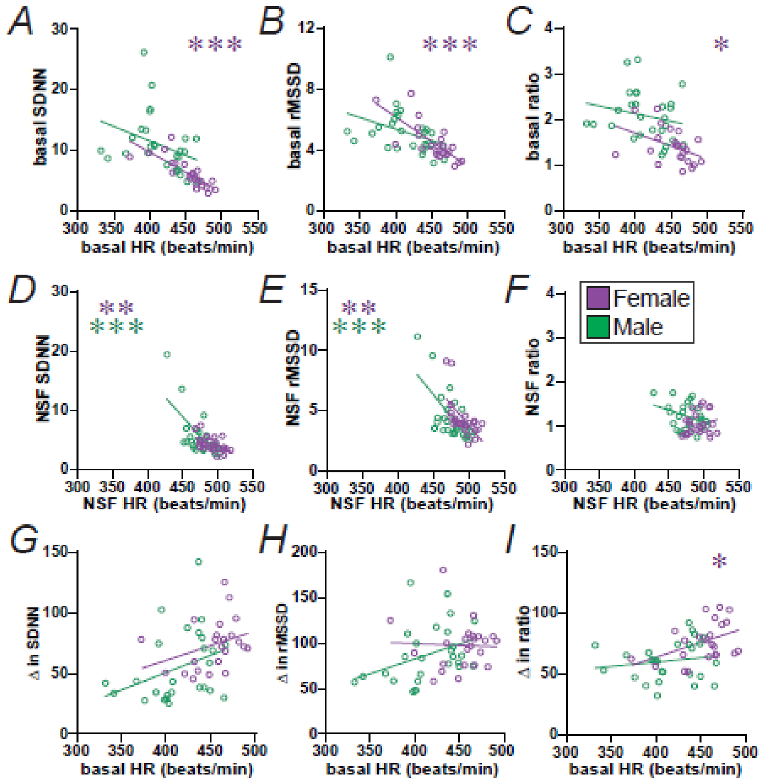

Figure 2.15. Examining whether higher HR was associated with reduced HRV. A mathematical relationship which could impact HRV interpretations. **(A-C)** For baseline HR measures, female HR (and trends in males) was associated with **(A)** lower SDNN (female $F_{(1,21)}$=37.11, R^2=0.639, p<0.0001; male $F_{(1,25)}$=3.514, R^2=0.123, p=0.0726), **(B)** lower rMSSD (female $F_{(1,21)}$=27.49, R^2=0.567, p<0.0001; male $F_{(1,25)}$=4.218, R^2=0.144, p=0.0506), and **(C)** lower SDNN/rMSSD ratio (female $F_{(1,21)}$=4.632, R^2=0.181, p=0.0432; male $F_{(1,25)}$=1.227, R^2=0.047, p=0.2785). **(D-F)** For NSF HR measures, both sexes had significantly lower HRV with higher HR, including for **(D)** SDNN (female $F_{(1,21)}$=8.368, R^2=0.285, p=0.0087; male $F_{(1,25)}$=21.96, R^2=0.468, p<0.0001) and **(E)** rMSSD (female $F_{(1,21)}$=12.55, R^2=0.374, p=0.0019; male $F_{(1,25)}$=20.88, R^2=0.455, p<0.0001), but not **(F)** SDNN/rMSSD (female $F_{(1,21)}$=2.606, R^2=0.110, p=0.1214; male $F_{(1,25)}$=3.308, R^2=0.117, p=0.0809) which may be due to concurrent decreases in both SDNN and rMSSD. Together, data in **(A-F)** suggest that HRV was lower under conditions with higher HR, females at baseline and NSF, and males during NSF. **(G-I)** Even so, basal HR did not correlate with **(G)** the change in SDNN (NSF minus basal, female $F_{(1,21)}$=0.954, R^2=0.043, p=0.3398; male $F_{(1,25)}$=3.226, R^2=0.114, p=0.0846), or **(H)** change in rMSSD, although a trend in males (female $F_{(1,21)}$=0.035, R^2=0.002, p=0.8539; male $F_{(1,25)}$=3.835, R^2=0.133, p=0.0614), and where **(I)** higher basal HR correlated with smaller change in SDNN/rMSSD in females ($F_{(1,21)}$=4.812, R^2=0.186, p=0.0397) but not males ($F_{(1,25)}$=0.666, R^2=0.026, p=0.4221). Thus, these data support the possibility that higher HR was associated with reduced HRV, which might impact HRV patterns seen with latency to first approach food. On the other hand, results in **(G,H)** suggest that there was some dynamic range for HRV measures to change, separate from basal HR. *,**,*** p<0.05, p<0.01, *** p<0.001.

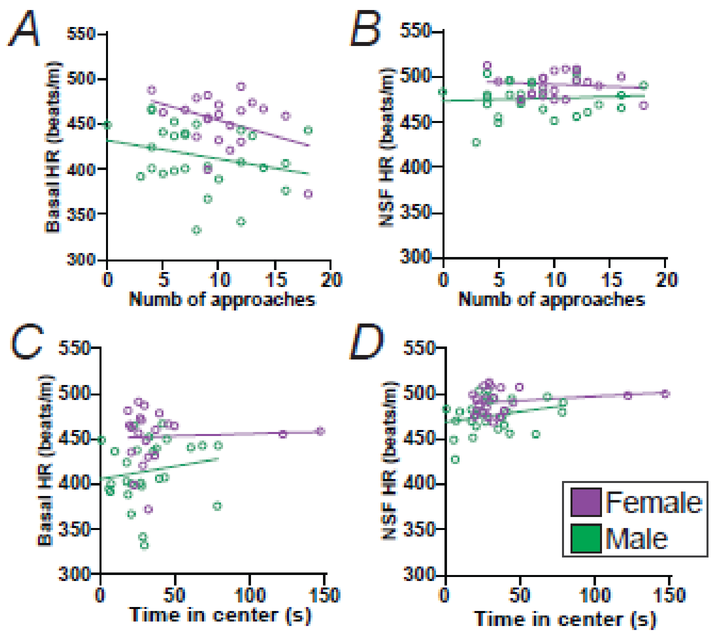

Figure 2.16. HR, number of approaches and time in center. HR did not relate to **(A,B)** number of approaches **(A)** at baseline (males: F(1,25)=1.824, R2=0.068, p=0.1889; females: F(1,19)=3.511, R2=0.156, p=0.0764) or **(B)** during NSF (males: F(1,25)=0.159, R2=0.006, p=0.6939; females: F(1,19)=0.270, R2=0.014, p=0.6092). **(C,D)** HR was also not correlated with time in center **(C)** at baseline (males: F(1,25)=0.732, R2=0.028, p=0.4005; females: F(1,19)=0.053, R2=0.003, p=0.8205) or **(D)** during NSF (males: F(1,25)=1.826, R2=0.068, p=0.1887; females: F(1,19)=0.991, R2=0.050, p=0.3320).

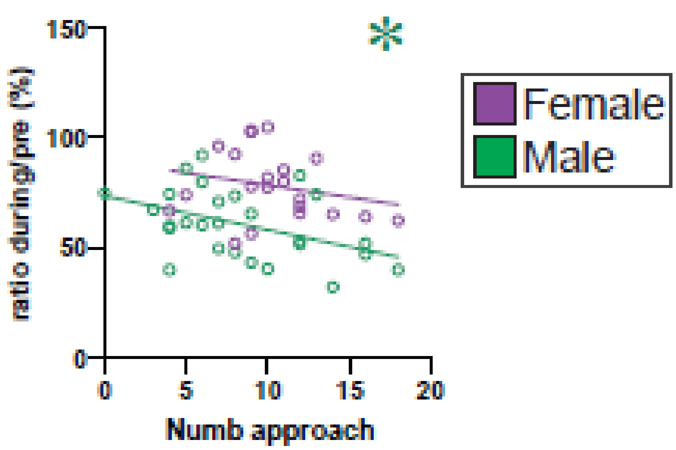

Figure 2.17. Males with a larger change in SDNN/rMSSD from baseline to NSF had more approaches (F(1,25)=5.938, R2=0.192, p=0.0223), which was not observed in females (F(1,19)=1.095, R2=0.055, p=0.3085).

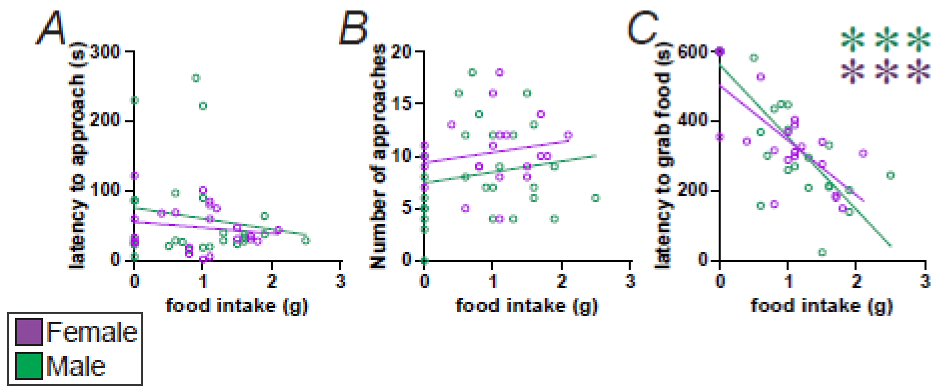

Figure 2.18. Relation between different NSF behaviors. (A) After removing a male outlier (600s latency to approach), there was no relation between food intake and latency to first approach in females (F(1,20)=0.596, R2=0.029, *p*=0.4491) or males (F(1,25)=2.201, R2=0.081, *p*=0.1504). **(B)** No relation between food intake and number of approaches in females (F(1,19)=0.697, R2=0.035, *p*=0.4142) or males (F(1,25)=0.714, R2=0.028, *p*=0.4063). **(C)** Food intake was significantly and negatively correlated with latency to first grab food in females (F(1,19)=21.94, R2=0.536, *p*=0.0002) and males (F(1,25)=55.64, R2=0.690, *p*<0.0001). However, no HR/HRV measure correlated with latency to grab food (not shown). *** *p*<0.001.

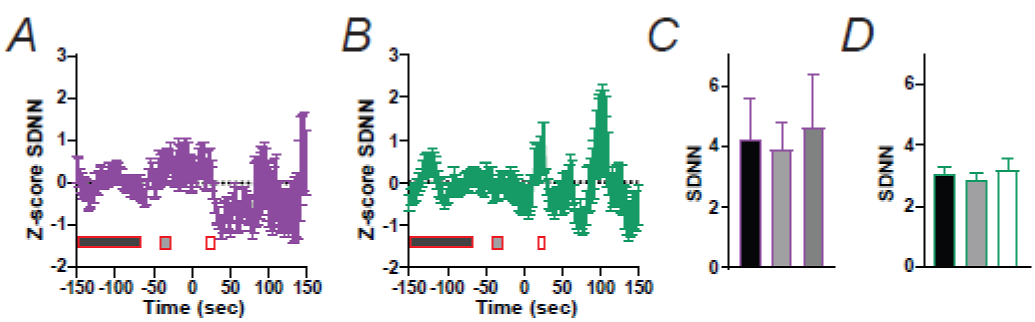

Figure 2.19. SDNN measures across the session, centered on the time to grab food. See Figure 2.9 legend for details. No differences across analysis time points for SDNN (**C,D,** female: Friedman stat=1.444, *p*=0.4857, male: Friedman stat=0.947, *p*=0.9474).

CHAPTER THREE: Sex Differences in Heart Rate Variability Measures that Predict Alcohol Drinking

3.1 Introduction

Alcohol Use Disorder causes many medical and social harms (CDC, 2014; Manthey et al., 2021; Aslam & Kwo, 2023) and has become a leading cause of death (Esser et al., 2022). Importantly, excessive drinking in women has risen dramatically in recent years (Grant et al., 2017; Carvalho et al., 2019), and women have higher risk for alcohol problems and higher comorbidity of Alcohol Use Disorder and mood disorders (Brady & Randall, 1999; Becker & Koob, 2016). Thus, it is of great importance to determine biological underpinnings of these observed sex differences to better treat alcohol intake with a more personalized approach. Compulsive alcohol drinking, where consumption persists despite negative consequences, can be a key driver of problem intake, and is often a major obstacle to treatment (De Oliveira Sergio et al., 2023; Starski et al., 2023). However, despite the substantial harms, and growing evidence that important sex differences exist within human drinking problems, there continue to be limited pharmacotherapies, and no treatments individualized by biological sex (Mann et al., 2018). Thus, there is a critical unmet need to develop novel, personalized interventions, which would be greatly aided by identifying sex-specific mechanisms that promote alcohol intake.

In the present study, we examine changes in heart rate (HR) and heart rate variability (HRV) in relation to voluntary alcohol drinking and biological sex. HR and HRV are of broad interest, since such measures may reflect important biomarkers for (Thayer & Lane, 2009; Mulcahy et al., 2019) and contributors to many neuropsychiatric conditions including alcohol use disorder (Gehrmann et al., 2000; Ingjaldsson et al., 2003; Thayer & Lane, 2009; Kircanski et al., 2019; Ralevski et al., 2019). Indeed, basal HR or HRV (Ingjaldsson et al., 2003; Ralevski et al., 2019; Hwang et al., 2022; but see Mun et al., 2008; Claisse et al., 2017) and HRV changes to alcohol stimuli (Jansma et al., 2000; Ingjaldsson et al., 2003; Mun et al., 2008; Garland et al., 2012; Claisse et al., 2017; Wang et al., 2020; Leganes-Fonteneau et al., 2021) can predict greater drinking problems or intake. Also, treatments that successfully alter alcohol drives can influence cardiac reactivity, which may help in the development of cardiac indicators that predict the efficacy of a given treatment for an individual (Milivojevic et al., 2020). In addition, rodent alcohol drinkers show changes in HR related to alcohol reinforcement and seeking (Bell et al., 2002; 2008) and differing sensitivities to the rewarding aspects of alcohol (Ristuccia & Spear, 2008). Thus, it is of critical importance to further examine the utility of HR and HRV as biomarkers that can reveal important information about an individual's neuropsychiatric state. Rodent work can be of immense value, helping to disentangle the impact of biology, such as sex, from sociocultural influences that are unavoidably present in human work.

HR and HRV are regulated by a dynamic balance between the two branches of the autonomic nervous system (ANS): the sympathetic (SNS, "fight or flight") and parasympathetic (PNS, "rest and digest") systems (Gehrmann et al., 2000; Thayer & Lane, 2009; Ralevski et al., 2019; Hamidovic et al., 2020). HR, the number of beats per minute, is widely examined, since it is simple to assess and interpret. There are also a number of methods to calculate HRV, ranging from simple arithmetic to more complex frequency analyses. Considerable work has assessed how particular cardiac indicators might reflect autonomic state, and drugs targeting specific aspects of cardiac physiology have been especially helpful in elucidating this. In particular, beta-adrenergic receptors (blocked e.g. by propranolol) mediate SNS effects on the heart, while muscarinic acetylcholine receptors (blocked e.g. by atropine) mediate PNS, and studies in humans (Pomeranz et al., 1985; Toichi et al., 1997), dogs (Akselrod et al., 1985; Houle & Billman, 1999), and rats (Cerutti et al., 1991; Gehrmann et al., 2000) used such drugs to validate HRV indicators.

Here, we focus on two such HRV measures in the time domain, which assess patterns across a series of inter-beat intervals (the amount of time between successive heart beats, abbreviated IBI), and are relatively simple to calculate (**Figure 3.1**). First, we assessed rMSSD, the root Mean Squared of the Successive Differences in inter-beat intervals, which is altered by atropine but not propranolol and is thus considered to primarily reflect PNS contributions. Second, we assessed SDNN, the standard deviation of inter-beat intervals, which is impacted by both drugs, and is thus widely considered to reflect the influence of both SNS and PNS actions on HRV; also, higher SDDN/rMSSD ratio can indicate greater SNS influence (Gehrmann et al., 2000; Thayer & Lane, 2009; Visted et al., 2017; Ralevski et al., 2019; Hamidovic et al., 2020; Koenig et al., 2021) . For example, alcohol drinking can increase SNS activation (Ristuccia & Spear, 2008; Ralevski et al., 2019; Leganes-Fonteneau et al., 2021), and thus would show an increase in SDNN, SDNN/rMSSD, and HR. There may be no change in rMSSD (reflecting a level of tonic PNS that is maintained during alcohol), or PNS influences could drop during SNS increases (indicated by reduced rMSSD) and causing further increases in SDNN/rMSSD. Importantly, while SDNN reflects the total variability in the time between heart beats, rMSSD involves squaring the time between heart beats, which increases the influence of longer inter-beat intervals, and capturing a level of variability beyond standard deviation (Volpes et al., 2022). Thus, by careful examination of the impact of experimental conditions, including autonomic-targeting drugs, the field has developed HRV measures that, with appropriate caution and care, can together provide insight into underlying autonomic changes.

We note that HRV is often assessed using spectral analysis of HRV fluctuations, where the high-frequency components (HFHRV, 0.75-2.5Hz in rat) are considered to relate to PNS (like rMSSD), while the low-frequency components (LFHRV, 0.1-0.75Hz in rat) can indicate PNS-SNS balance (like SDNN) (Beckers et al., 2006; Henze et al., 2008; Koizumi et al., 2011; Park et al., 2017). However, some recent work has considered rMSSD and SDNN as perhaps more robust than frequency measures, in part due to analytic differences across frequency studies (Koenig et al., 2021; Toichi et al., 1997; Williams et al., 2022). One study found reduced rMSSD in drinkers, but no HFHRV changes, which they suggest might reflect different analysis methods from studies finding altered HFHRV (Cheng et al., 2019). Another study chose rMSSD for a large meta-analysis of sex differences in HRV (Koenig et al., 2021). Also, rMSSD often correlates highly with HFHRV, and SDNN with LFHRV (Visted et al., 2017; Koenig et al., 2021). Thus, SDNN, rMSSD, and their ratio, have been broadly validated and considered useful to gain insight into PNS and SNS activity.

Thus, HRV can provide useful indicators for different aspects of autonomic state, and an opportunity to delve deeper into the realm of sex differences in autonomic regulation, especially for different neuropsychiatric conditions. Interestingly, many studies find sex differences in autonomic function. Women have higher HR than men under several conditions (Huikuri et al., 1996; Nugent et al., 2011; Koenig et al., 2021; Williams et al., 2022), including for heavy drinkers (Sucharita et al., 2012). However, even though having a faster HR might reduce HRV (Ingjaldsson et al., 2003), women often display greater basal HRV (Huikuri et al., 1996; Nugent et al., 2011; Koenig et al., 2021). Indeed, a recent study found that women have greater HRV for a given HR level relative to men (Williams et al., 2022), and another study replicated that pattern in Groningen rats (Carnevali et al., 2023). While the exact mechanism for this paradox is not yet known, it has been posited that the effects of estrogen on cardiac chronotropy may provide a plausible explanation (Williams et al., 2023). In addition, several studies find sex differences in autonomic systems recruited during challenge, especially where women utilize the PNS more, while men engage the SNS more (Dart et al., 2002; Sato and Miyake, 2004; Nugent et al., 2011; Hamidovic et al., 2020; Koenig et al., 2021). HRV brain circuits also differ in relation to biological sex (Mak et al., 2009; Goldstein et al., 2010; Nugent et al., 2011; Allen et al., 2015; Garcia et al., 2020). However, the functional implications of sex differences in autonomic regulation remain understudied, especially for alcohol drinking.

Thus, we have performed the first study to our knowledge to uncover potential sex differences in HRV indicators that predict alcohol drinking levels in adult Wistar rats. We determined HR/HRV measures at baseline, at drinking onset (before most alcohol would have time to enter the blood stream; Doyon et al., 2003; 2005), and across the drinking period (when alcohol could have direct effects on cardiac measures; Ristuccia & Spear, 2008; Ralevski et al., 2019; Leganes-Fonteneau et al., 2021). We then related these HRV measures to alcohol consumption levels and found an association between intake and PNS indicators in females and SNS indicators in males. Also, when alcohol was on board, several measures only related to compulsion-like versus alcohol-only drinking, and in a sex-specific manner. These agreed with our previous behavioral studies (Darevsky et al., 2019; Darevsky & Hopf, 2020) suggesting that aversion-resistant and alcohol-only intake utilize different cognitive-emotional action strategies. Taken together, the present study contributes new insights into sex differences in autonomic regulation that are novel and likely translationally relevant for the treatment of problem drinking.

	Potential to strongly modulate? atropine (PNS)	*Potential to strongly modulate?* propranolol (SNS)
IBI: Inter-Beat Interval is the time between successive heart beats	Yes	Yes*
rMSSD: *the root mean square of IBI differences is thought to indicate more influence of PNS*	Yes	No
SDNN: *the standard deviation of IBIs is thought to indicate SNS-PNS balance*	Yes	Yes*
SDNN/rMSSD: *larger could be considered to indicate more influence of SNS*	Yes	Yes*

Yes SNS can be quite low when laying down*

Figure 3.1. Description of different HRV measures, and how they relate to PNS and/or SNS. In particular, some effects of pharmacological agents are considered to alter the impact of SNS, through beta-adrenergic receptors (blocked by propranolol), or the PNS, through muscarinic cholinergic receptors (blocked by atropine), although this interpretation requires some caution (e.g. where certain postures are associated with low SNS).

3.2 Methods

3.2.1 Animals and Alcohol Drinking Methods

All experiments were performed in accordance with NIH Guidelines and approved by the Institutional Animal Care and Use Committee (IACUC) of Indiana University. PN45-50 female (n=7-16/group) and male (n=10-18/group) Wistar rats (Envigo) were singly housed with *ad libitum* food and water in a 12hr reverse light cycle (lights off 8AM). After 2 weeks, rats were initiated to alcohol drinking methods, which followed those we previously used (Seif et al., 2013; Darevsky et al., 2019; Darevsky & Hopf, 2020; De Oliveira Sergio et al., 2021). All drinking (including HRV testing) began ~0.5-2hr after the onset of the dark cycle and occurred within the homecage.

Rats first drank under a 2-bottle choice intermittent access to alcohol paradigm, with access to alcohol (20% v/v), or water in a second bottle. Alcohol access began on Monday, Wednesday, and Friday and lasted 16-24hr. Following ~3 months of intermittent access, rats were switched to drink under two-bottle choice for alcohol (20% v/v) or water for 20min/day Monday-Friday (**Figure 3.2 A**). After ~1 month of this 20min/day intake, rats began drinking 20min/day but with 2-3 alcohol-only days and 2-3 days of compulsion-like alcohol drinking (CLAD) per week (in randomized order); we model CLAD here by adulterating 20% alcohol with 10mg/L of the bitter-tasting quinine as done previously (Seif et al., 2013; Darevsky et al., 2019; Darevsky & Hopf, 2020; De Oliveira Sergio et al., 2021a; 2023). Rats had at least 3 CLAD sessions before telemetry surgery to ensure habituation to the novelty of quinine-alcohol. After surgery, rats had telemetry recording during 3 alcohol-only sessions and 3 CLAD sessions over 2 weeks, with at least 1 alcohol-only day between CLAD sessions (although, for some rats, the device failed before all drinking sessions were recorded). We recorded multiple sessions per condition per rat, as we often do (Seif et al., 2013; De Oliveira Sergio et al., 2021; 2023), to reduce variability by calculating average values across sessions from a given rat for a particular condition.

3.2.2 Telemetry Surgery and Recording

Rats were implanted with a Stellar telemetry device (type PTA-M-C, part# E-430001-IMP-130) from TSE Systems Inc. (Chesterfield, MO) (**Figure 3.2 B**). Drinking studies began after 3-5 days of recovery. Telemetry surgery is detailed in Suppl. Methods Section 3.5.1. Briefly, the telemetry device has a silicone elastomer transmitter (8.3mm x 16.5mm x 4mm), and a thin, plastic-sheathed wire with a small sleeve at the distal tip that detects changes in blood pressure within the artery. This wire was inserted through a small slit in the femoral artery, then guided into the abdominal aorta until it sat approximately between the iliac bifurcation and the renal arteries, as illustrated in **Figure 3.2 B**. The transmitter unit was attached to the inner musculature of the abdominal wall. Blood pressure traces visualized during insertion of the catheter were used to assure detector placement within the abdominal aorta.

Blood pressure traces were recorded and visualized using NOTOCORD-hem software (Instem, Staffordshire, UK) at 250Hz, with pressure fluctuations related to each heartbeat (cartoon illustration: **Figure 3.2 C**). Baseline data were recorded in the homecage ~0.2-1hr before alcohol access. We assessed HR/HRV, since determining absolute blood pressure values was more challenging to assess (due to possible basal offsets in blood pressure measures) relative to changes in cardiac measures within a recording session. Raw recordings were visually inspected to remove artifacts, missed heartbeats, and ectopic heartbeats, as is typically done in other studies (Grippo et al., 2012; Inagaki et al., 2013; Carnevali et al., 2023). NOTOCORD-hem software output the timing of each successive heartbeat peak. This time series was used to determine HR (beats/min) and also the sequence of interbeat intervals (IBI, in msec) across a given session. We then used IBIs to determine time domain HRV measures, including (1) SDNN, the standard deviation of the interbeat intervals, reflecting SNS and PNS influences, and (2) rMSSD, the root mean square of the successive differences in interbeat interval, a PNS indicator (see Introduction and **Figure 3.1**). rMSSD was determined by taking the square of differences between successive IBI values, averaging the squares of these differences, then taking the square root of this average. Finally, we determined SDNN/rMSSD ratio, which is taken to indicate the relative strength of SNS contributions, since this ratio is effectively [SNS influence + PNS influence]/[PNS influence]. Importantly, recent human work (Williams et al., 2022) has indicated the critical importance of not adjusting HRV measures for HR, since this can result in missing important sex differences.

Although alcohol-only and CLAD recording sessions were the typical 20min/day schedule, telemetry was recorded for the first 10min of alcohol access. We did this for two reasons: (1) our rats overwhelmingly frontload their alcohol drinking during the first minutes of alcohol access (Darevsky et al., 2019; Darevsky & Hopf, 2020; Starski et al., 2023). In addition, (2) we wanted to compare alcohol-related HRV patterns with cardiac measures during other 10min long behavioral tests occurring in our lab, such as anxiety-like models (De Oliveira Sergio et al., 2021; Frasier et al., 2023). Importantly, to directly compare HRV values across behaviors, HRV indicators must be calculated across the same duration of time (Shaffer & Ginsberg, 2017). Rats were undisturbed during the recordings, as wireless telemetry removed the need to handle rats, and all drinking occurred in the homecage.

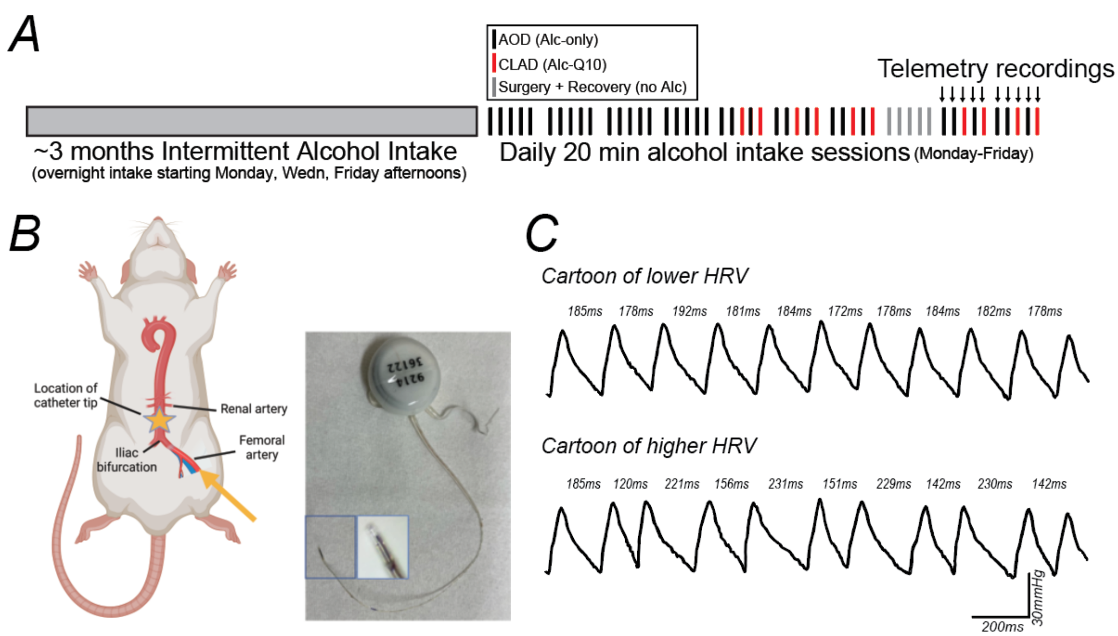

Figure 3.2. Experimental Schematics (A) Schematic of drinking schedule. **(B)** Schematic of surgical device and implantation. **(C)** Cartoon example traces showing lower HRV (top) versus higher HRV (bottom). Time between each pair of heart beats is shown.

3.2.3 Alcohol Drinking Analysis

Alcohol consumption was determined through changes in bottle weight before and after drinking sessions, from which we determined grams-ethanol per kilograms-body-weight. Rat body weights were taken weekly to ensure accurate measurement of g/kg alcohol consumption.

3.2.4 HR and HRV Analyses

We performed two different analyses on our HRV data, since we were interested in HRV patterns at drinking onset, and across 10min of the drinking session. For drinking onset studies, we determined HR/HRV in the first minute of alcohol access, and in the first minute period from baseline. Again, this was done because HRV analyses can only be compared when they are matched for the same amount of time (Shaffer & Ginsberg, 2017).

Analyzing HR trains for longer periods typically provides more accurate HRV assessment, however 5min periods have often been used for a briefer baseline in humans (Shaffer & Ginsberg, 2017). In addition, briefer analysis windows can provide meaningful findings. During different challenges in humans, a 60sec analysis windows was as good as a 5min window for SDNN, rMSSD, and HR, although with 2min or more is needed for frequency-domain and more complex analyses (Shaffer & Ginsberg, 2017; Ritsert et al., 2022). Rodent studies have also validated the use of 1min HRV analysis windows (McNeal et al., 2019), including for anxiety-like tests (Grippo et al., 2012). Thus, determining HRV measures in the first minute of drinking ("drinking onset") reflected a compromise between having the rat already drinking, and under a state of high motivation to drink alcohol, while also including sufficient data to allow robust HRV assessment.

Finally, other studies suggest that the first minute of intake is before much alcohol passes through the gut (Doyon et al., 2003; 2005), minimizing the potential for direct pharmacological effects of alcohol on HR/HRV during the first minute of intake (Ralevski et al., 2019; Leganes-Fonteneau et al., 2021). Further, to better understand HR/HRV patterns when alcohol was on board, we also assessed HR/HRV measures across a 10-minute intake period, with HRV recording in the first 10min of the regular 20-minute intake period (as described above). Several HR/HRV correlations were observed for the 10-minute period (**Figure 3.7**) which were not observed for the drinking onset time point (**Figure 3.4**), supporting the possibility that HRV factors in the first minute after drinking onset that predict drinking (like rMSSD in females, **Figure 3.4 A**) would reflect psychological drives for alcohol rather than direct autonomic effects of alcohol. Finally, we note that baseline was not assessed for some rats, and thus the sample size was greater for **Figures 3.4, 3.7** than for **Figures 3.3, 3.6** (see Results).

3.2.5 Statistics

Analyses were performed using unpaired or paired t-tests (for normal data), Mann-Whitney or Wilcoxon matched-pairs signed rank test (for non-normal data), or Pearson's correlations, where appropriate, using GraphPad Prism. Data shown are mean ± standard error of the mean.

3.3 Results

3.3.1 Relation of Drinking Levels to Basal HR/HRV Measures

We first examined whether any basal HR or HRV measure predicted subsequent drinking levels. **Figure 3.2 C** shows an example of higher HRV vs lower HRV traces, while **Figure 3.1** summarizes how different HRV measures are considered to relate to PNS and/or SNS (see Introduction). We set out to record 3 alcohol-only and 3 CLAD sessions per rat. Data for each given drinking condition was then averaged for each rat, giving each rat a single value for alcohol-only and for CLAD.

Basal HRV can predict drinking problems in humans (Ingjaldsson et al., 2003; Ralevski et al., 2019), and such findings would suggest that individuals can have a tonic autonomic state that impacts their behavior. Interestingly, the basal SDNN/rMSSD ratio predicted subsequent alcohol drinking in females, but not in males (female n=9 alcohol-only, n=7 CLAD; male n=12 alcohol-only, n=10 CLAD). In particular, females with lower SDNN/rMSSD at baseline went on to drink significantly more alcohol (**Figure 3.3 A**), both for alcohol-only drinking ($F_{(1,7)}$=7.534, R^2=0.5184, p=0.0287) and CLAD ($F_{(1,5)}$=25.66, R^2=0.8369, p=0.0039). In contrast, basal SDNN/rMSSD ratio did not predict drinking levels in males (**Figure 3.3 B**; alc-only: $F_{(1,10)}$=1,868, R^2=0.1574, p=0.2017; compulsion-like: $F_{(1,8)}$=0.369, R^2=0.044, p=0.5603). These findings suggest that reduced relative SNS influence at baseline (lower SDNN/rMSSD ratio), leaving PNS predominant, was associated with greater consumption levels under both alcohol-only and alcohol-quinine conditions in females, but not males.

Greater relative PNS influence could be reflected in increased PNS (greater rMSSD) or decreased SNS (reduced SDNN or SDNN/rMSSD). However, there was no significant relation between female basal rMSSD and intake for alcohol-only (**Figure 3.3 C**; $F_{(1,7)}$=3.046, R^2=0.3032, p=0.1244) or CLAD (**Figure 3.3 C**; $F_{(1,5)}$=1.781, R^2=0.2627, p=0.2395), and also no correlations in males (**Figure 3.3 D**; alc-only: $F_{(1,10)}$=0.797, R^2=0.0738, p=0.3930; compulsion-like: $F_{(1,8)}$=0.449, R^2=0.0532, p=0.5215). Further, there was no relation between female basal SDNN and intake for alcohol-only (**Figure 3.3 E**; $F_{(1,7)}$=0.0928, R^2=0.0131, p=0.7695) or CLAD (**Figure 3.3 E**; $F_{(1,5)}$=0.2919, R^2=0.0552, p=0.6122), or in males (**Figure 3.3 F**; alc-only: $F_{(1,10)}$=0.286, R^2=0.0278, p=0.6047; compulsion-like: $F_{(1,8)}$=0.034, R^2=0.0042, p=0.8587). Also, drinking level did not correlate with basal HR in females (**Figure 3.3 G**; alc-only: $F_{(1,7)}$=0.0927, R^2=0.0131, p=0.7696; compulsion-like: $F_{(1,5)}$=0.3664, R^2=0.0683, p=0.5714) or males (**Figure 3.3 H**; alc-only: $F_{(1,10)}$=2.967, R^2=0.2289, p=0.1157; compulsion-like: $F_{(1,8)}$=0.709, R^2=0.0814, p=0.4243). Thus, while basal SDNN and rMSSD measures were not associated with subsequent drinking, a greater shift in basal PNS-SNS tone (lower SDNN/rMSSD ratio) did predict higher alcohol drinking. Also, as detailed in the Discussion, changes in PNS-SNS dynamics can occur through different types of HRV changes. The data herein suggest subtle HRV shifts which led to higher basal PNS-SNS tone predicted more alcohol intake in females, whether alcohol-only or CLAD.

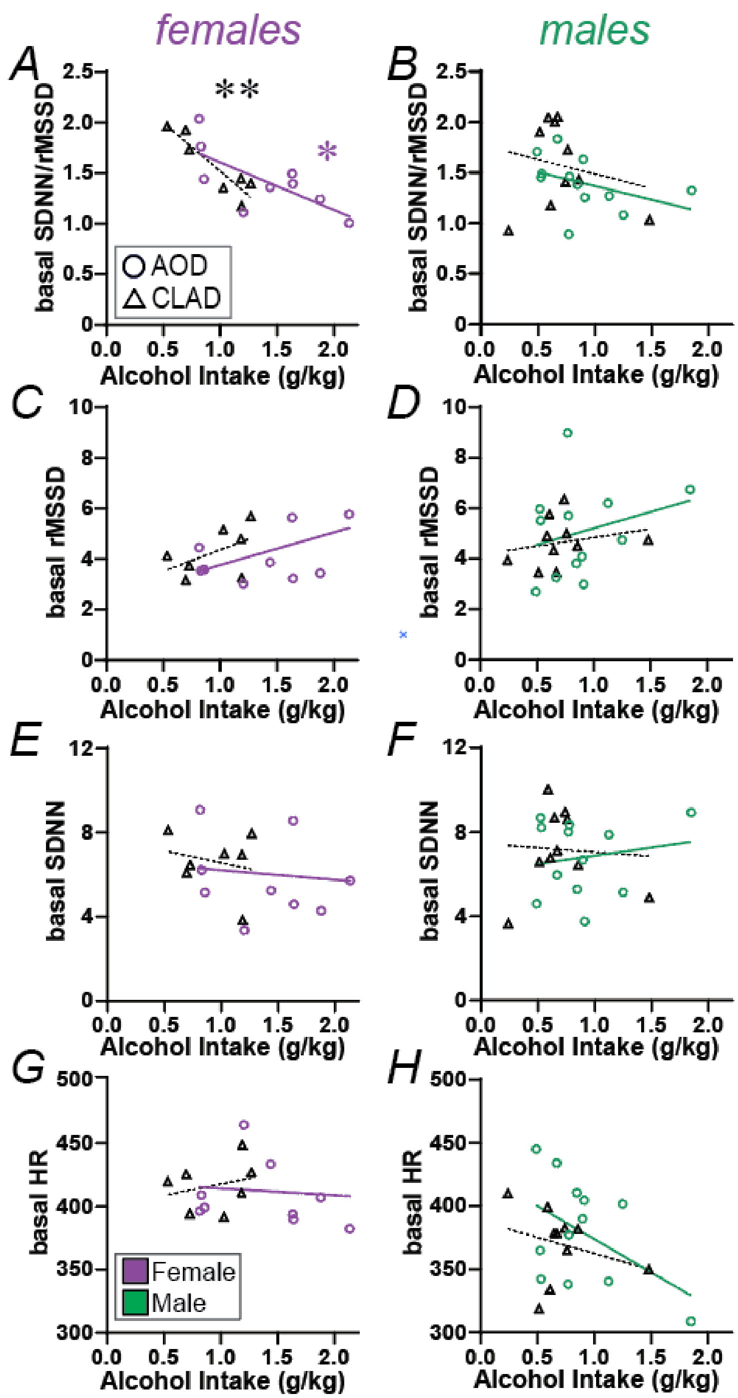

Figure 3.3. Basal PNS influence predicted female but not male drinking. (A,B) In **(A)** females (n=9 alcohol-only; n=7 CLAD) but **(B)** not males (n=12 alcohol-only; n=10 CLAD), lower SDNN/rMSSD was related to greater intake levels for both alcohol-only and alcohol-quinine. **(C-F)** The **(C,D)** rMSSD and **(E,F)** SDNN levels at baseline did not predict drinking in either sex. **(G,H)** Basal HR did not predict drinking levels. *,** $p<0.05$, 0.01. CLAD: compulsive-like alcohol drinking. Graphs separated by sex, with female alcohol-only data in purple and male in green, and where black lines and triangles reflect CLAD sessions within that sex and analysis window.

3.3.2 Relation of Drinking Levels to HR/HRV Measures During Drinking Onset

HRV changes to alcohol stimuli can predict alcohol problems in humans (Garland et al., 2012; Ingjaldsson et al., 2003). In rats, drinking onset is an important alcohol stimulus, since this is when significant behavioral changes occur, for example, shifts in response patterns under alcohol-only versus CLAD conditions (Darevsky et al., 2019; Darevsky & Hopf, 2020). Thus, we tested whether a given HRV measure at drinking onset (the first minute of alcohol access) was related to subsequent alcohol consumption levels (female n=16 alcohol-only, n=10 CLAD, male n=18 alcohol-only, n=12 CLAD).

Interestingly, in females, greater rMSSD at drinking onset was associated with greater subsequent drinking levels for alcohol-only (**Figure 3.4 A**; $F_{(1,14)}=5.141$, $R^2=0.2686$, $p=0.0397$). CLAD had a similar slope but was not significant (**Figure 3.4 A**; $F_{(1,8)}=1.090$, $R^2=0.1199$, $p=0.3270$). Also, initial rMSSD was not correlated with drinking level in males (**Figure 3.4 B**; alc-only: $F_{(1,16)}=0.003$, $R^2=0.0002$, $p=0.9593$; compulsion-like: $F_{(1,10)}=0.020$, $R^2=0.0020$, $p=0.8894$). These results suggest that greater PNS activity in females, but not males, in the first minute of drinking was related to higher subsequent consumption. In addition, female intake did not correlate with drinking-onset SDNN (**Figure 3.4 C**; alc-only: $F_{(1,14)}=1.555$, $R^2=0.1000$, $p=0.2329$; compulsion-like: $F_{(1,8)}=0.884$, $R^2=0.0995$, $p=0.3746$) or SDNN/rMSSD (**Figure 3.4 E**; alc-only: $F_{(1,14)}=0.121$, $R^2=0.0086$, $p=0.7330$; compulsion-like: $F_{(1,8)}=0.1129$, $R^2=0.0139$, $p=0.7455$). Thus, females with higher PNS indicators at drinking onset had greater intake.

Male intake was not associated with drinking-onset SDNN (**Figure 3.4 D**; alc-only: $F_{(1,16)}=1.866$, $R^2=0.1044$, $p=0.1908$; compulsion-like: $F_{(1,10)}=0.430$, $R^2=0.0413$, *p*=0.5266) or SDNN/rMSSD (**Figure 3.4 F**; alc-only: $F_{(1,16)}$=2.117, R^2=0.1168, *p*=0.1650; compulsion-like: $F_{(1,10)}$=0.154, R^2=0.0152, *p*=0.7027). Finally, there was no relation between drinking level and drinking-onset HR in females (**Figure 3.4 G**; alc-only: $F_{(1,14)}$=0.049, R^2=0.0035, *p*=0.8277; compulsion-like: $F_{(1,8)}$=0.986, R^2=0.1097, *p*=0.3498) or males (**Figure 3.4 H**; alc-only: $F_{(1,16)}$=1.884, R^2=0.1054, *p*=0.1888; compulsion-like: $F_{(1,10)}$=0.381, R^2=0.0367, *p*=0.5510). Thus, in males, no cardiac measure we examined during the first minute of drinking was related to alcohol consumption level.

One consideration is that sex differences in HRV measures might be impacted by the higher HR in females, especially where faster HR might mathematically reduce the potential for variability. However, both SDNN (**Figure 3.5 A**) and rMSSD (**Figure 3.5 B**) showed spread relative to HR in females and males, indicating dynamic range to observe HRV shifts (n=26 female; n=24 male). This agrees with human studies where women show higher HRV even with greater HR (Williams et al., 2022) and rodent studies replicating a similar pattern by sex (Carnevali et al., 2023; Frasier et al., 2023).

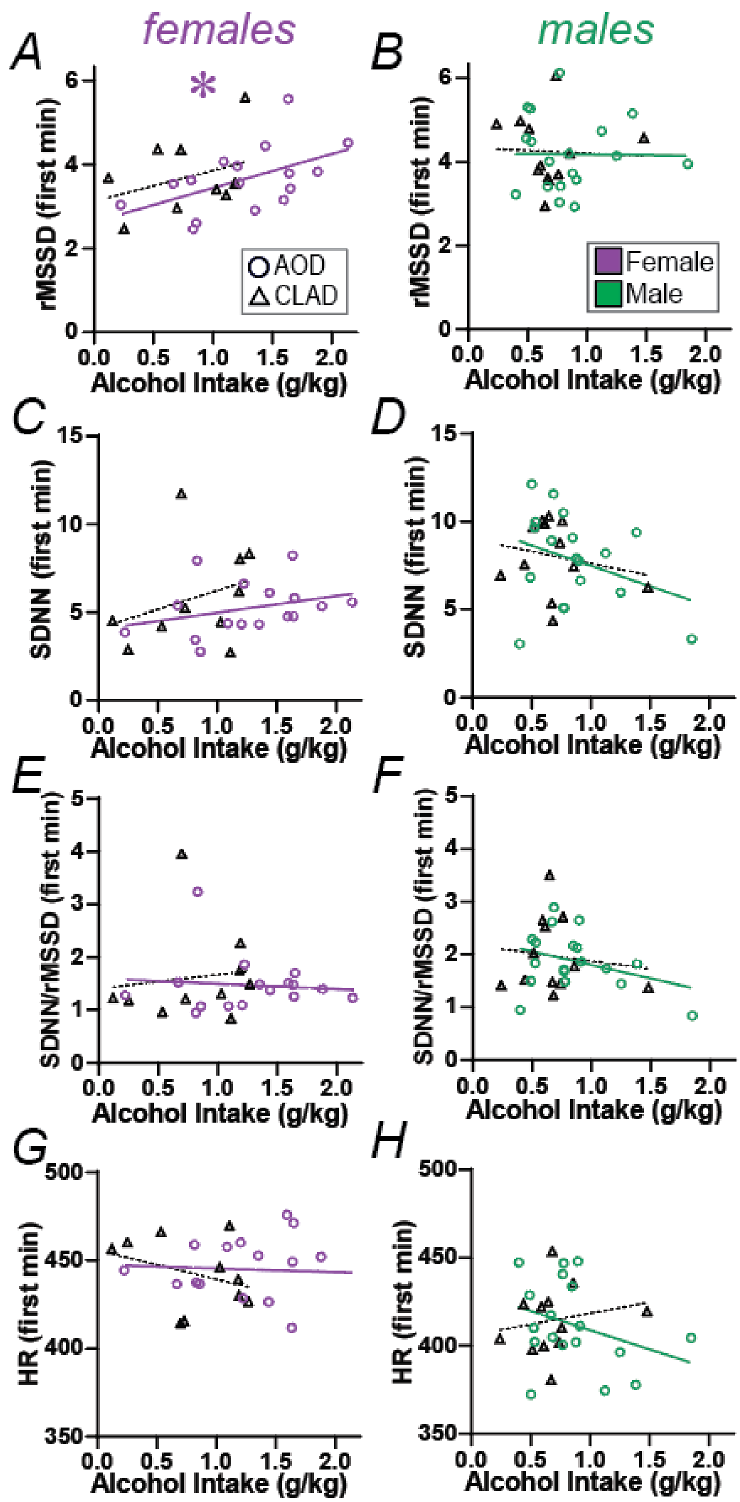

Figure 3.4. Greater PNS influence in the first minute of access to alcohol predicted greater female drinking. Female n=16 alcohol-only, n=10 CLAD, male n=18 alcohol-only, n=12 CLAD). **(A,B)** In **(A)** females but **(B)** not males, higher rMSSD during drinking onset was related to higher drinking levels for alcohol-only. **(C-H)** Consumption levels were not related to **(C,D)** SDNN, **(E,F)** SDNN/rMSSD, or **(G,H)** HR at drinking onset, in either sex. * p<0.05. Graph data female/male coloring as in Fig.3.3.

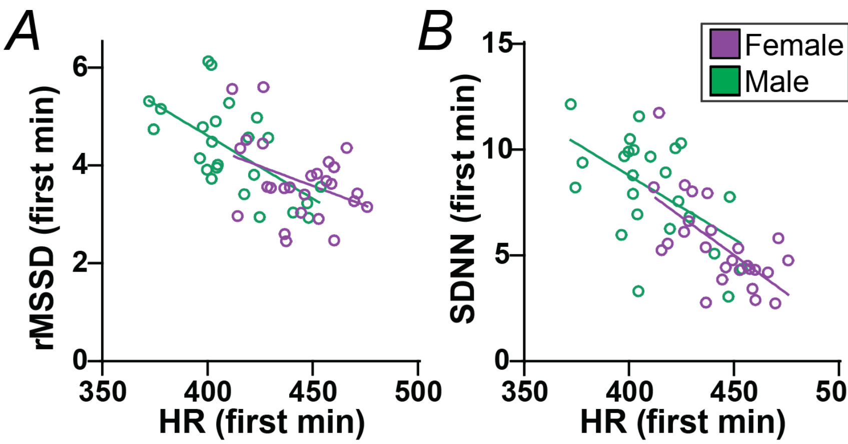

Figure 3.5. Dynamic range for HRV differences even with higher HR. While average **(A)** SDNN and **(B)** rMSSD values were lower as HR increased (**A**, females: $F_{(1,24)}$=17.89, *p*=0.0003, R_2=0.4271; males: $F_{(1,22)}$=8.971, *p*=0.0067, R_2=0.2897; **B**, females: $F_{(1,24)}$=3.969, *p*=0.0578, R_2=0.1419; males: $F_{(1,22)}$=13.86, *p*=0.0012, R_2=0.3865), there was still variability and thus dynamic range within HRV measures. Female n=26, male n=24.

3.3.3 Relation of Drinking Levels to Changes in HR/HRV Measures from Baseline to Drinking Onset

Since HRV changes to alcohol stimuli can relate to alcohol problems in humans, we also examined the change in each HR/HRV measure from baseline to the first minute of access to alcohol (for these analyses, we combined alcohol-only and CLAD, n=16 female, n=22 male, same data as **Figure 3.3**). Overall, males showed a significant increase in SDNN and SDNN/rMSSD from baseline to drinking onset, which was not observed in females. SDNN increased significantly in males (**Figure 3.6 A**, Wilcoxon *p*=0.0190) but not in females (**Figure 3.6 A**, Wilcoxon *p*=0.7057), with a significant difference in SDNN change between males and females (Mann-Whitney *p*=0.0309). For rMSSD, males (**Figure 3.6 B**, Wilcoxon *p*=0.0301) but not females (**Figure 3.6 B**, Wilcoxon *p*=0.1928) showed a change, but with no sex difference (Mann-Whitney *p*=0.3876). However, SDNN/rMSSD increased significantly from baseline to drinking onset in males (**Figure 3.6 C**, Wilcoxon *p*=0.0005) but not females (**Figure 3.6 C**, Wilcoxon p=0.7057), and with a significant difference between males and females (Mann-Whitney p=0.0334). Finally, HR significantly increased in both males (**Figure 3.6 D**, Wilcoxon p<0.0001) and females (**Figure 3.6 D**, Wilcoxon p=0.0063), but with no sex difference (Mann-Whitney p=0.1712). Together, these suggest that males shifted to greater SNS influence at the onset of drinking, while both sexes showed an HR increase at drinking onset. However, we note that no change in HR/HRV measure correlated with alcohol drinking levels, although with some trends in males (Suppl. **Figure 3.9**). Thus, we speculate that males have increased SNS influence at drinking onset, which might be permissive for drinking but not directly related to consumption level.

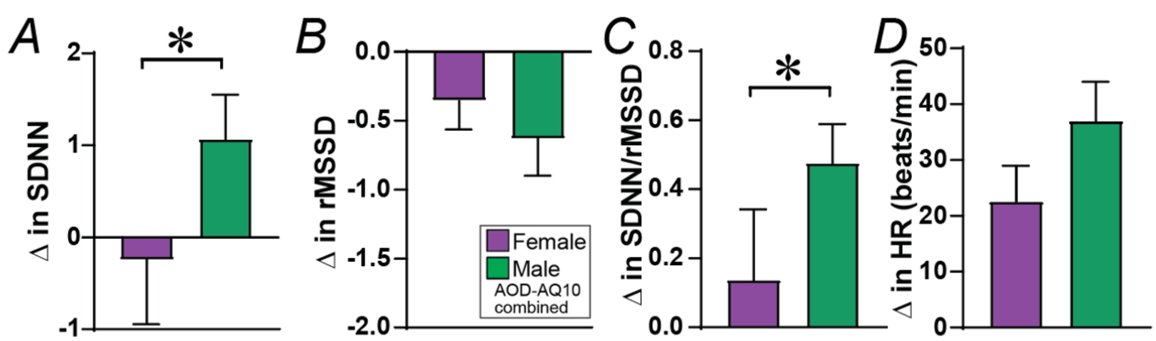

Figure 3.6. Changes in HRV measures from baseline to first minute access to alcohol. **(A-C)** Males showed a significant increase in **(A)** SDNN, **(C)** SDNN/rMSSD, but not **(B)** rMSSD, at drinking onset versus basal. **(D)** Both sexes showed HR increases at drinking onset, with no sex differences. * p<0.05 difference between females and males. Female n=16, male n=22; alcohol-only and CLAD conditions combined.

3.3.4 HR/HRV Measures Assessed Across the 10-Minute Intake Period

We also measured HR/HRV across 10-minutes of the intake period (female n=16 alcohol-only, n=10 CLAD, male n=18 alcohol-only, n=12 CLAD, same cohort as **Figure 3.4**). Such HRV patterns could reflect direct alcohol enhancement of HR and SNS

(Ralevski et al., 2019; Leganes-Fonteneau et al., 2021), but potentially also reflect drive for alcohol. Interestingly, HR was correlated with intake level in females when examined across the 10min drinking period. However, many HRV changes only related to drinking for CLAD, and not alcohol-only, suggesting the presence of sustained autonomic states that specifically promoted CLAD, which were different between females and males.

While rMSSD$_{10min}$ was not associated with female drinking levels (**Figure 3.7 A**; alc-only: $F_{(1,14)}$=0.005, R^2=0.0003, p=0.9464; compulsion-like: $F_{(1,8)}$=0.027, R^2=0.0033, p=0.8740), males with lower rMSSD$_{10min}$ drank significantly more under CLAD conditions (**Figure 3.7 B**; $F_{(1,10)}$=5.967, R^2=0.3737, p=0.0347) but not alcohol-only ($F_{(1,16)}$=0.252, R^2=0.0155, p=0.6226), and with a trend for differences in alcohol-only and CLAD slopes in males ($F_{(1,26)}$=4.115, p=0.0529). Thus, males (but not females) with lower overall PNS influence drank more under compulsion-like conditions.

For SDNN$_{10min}$ and SDNN/rMSSD$_{10min}$, the sex-related pattern was different. In particular, female CLAD drinking level was significantly and negatively correlated with SDNN$_{10min}$ (**Figure 3.7 C**; $F_{(1,8)}$=5.724, R^2=0.4171, p=0.0437) and SDNN/rMSSD$_{10min}$ (**Figure 3.7 E**; $F_{(1,8)}$=7.206, R^2=0.4739, p=0.0277). However, female alcohol-only levels were not correlated with SDNN$_{10min}$ (**Figure 3.7 C**; $F_{(1,14)}$=0.741, R^2=0.0503, p=0.4037) or SDNN/rMSSD$_{10min}$ (**Figure 3.7 E**; $F_{(1,14)}$=1.087, R^2=0.0721, p=0.3147), and alcohol-only and CLAD slopes in females were different for SDNN/rMSSD$_{10min}$ ($F_{(1,22)}$=5.240, p=0.0320). Further, males showed no association for intake level and SDNN$_{10min}$ (**Figure 3.7 D**; alc-only: $F_{(1,16)}$=0.441, R^2=0.0268, p=0.5162; compulsion-like: $F_{(1,10)}$=0.907, R^2=0.0832, p=0.3633) or SDNN/rMSSD$_{10min}$ (**Figure 3.7 F**; alc-only: $F_{(1,16)}$=0.758, R^2=0.0453, p=0.3967; compulsion-like: $F_{(1,10)}$=0.002, R^2=0.0002, p=0.9673). Thus, females (but not males) with lower overall SNS influence drank more under compulsion-like conditions.

HR_{10min} was significantly and positively correlated with drinking level in females for both alcohol-only (**Figure 3.7 G**; $F_{(1,14)}$=5.592, R^2=0.2854, p=0.0330) and CLAD (**Figure 3.7 G**; $F_{(1,8)}$=8.495, R^2=0.5150, p=0.0195). Interestingly, in males, HR_{10min} only correlated with CLAD intake (**Figure 3.7 H**; $F_{(1,10)}$=5.698, R^2=0.3630, p=0.0382), but not alcohol-only (**Figure 3.7 H**; $F_{(1,16)}$=0.037, R^2=0.0023, p=0.8503), and with a trend for difference in slopes between male alcohol-only and CLAD ($F_{(1,26)}$=4.180, p=0.0511). Thus, female drinking was associated with increased HR for both alcohol-only and CLAD, as might be predicted from human studies. However, for males, only CLAD was related to higher HR level.

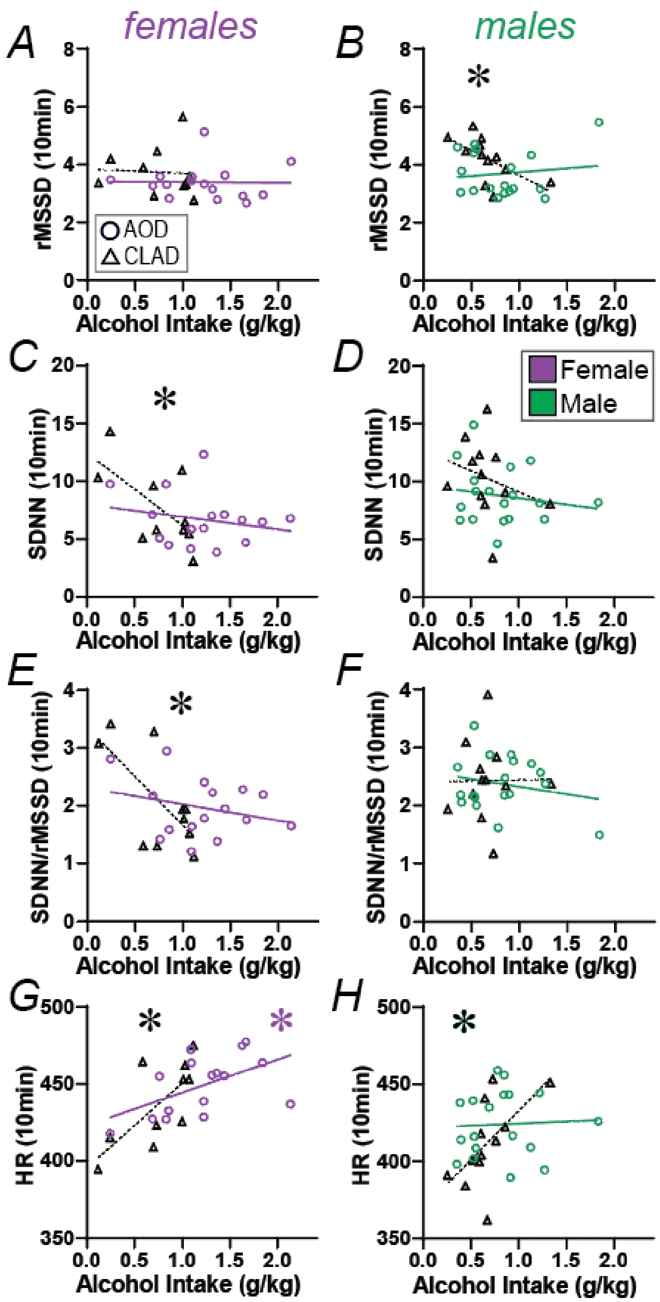

Figure 3.7. Across the 10-minute drinking period, there were sex differences in HRV measures associated with higher CLAD levels. Female n=16 alcohol-only; n=10 CLAD. Male n=18 alcohol-only; n=12 CLAD. **(A)** rMSSD$_{10min}$ was not correlated with female intake level. **(B)** Males with lower rMSSD$_{10min}$ had greater CLAD but not alcohol-only intake. **(C,D)** For SDNN$_{10min}$, **(C)** females but not **(D)** males with lower SDNN$_{10min}$ had greater CLAD intake (with no relation to alcohol-only). **(E,F)** Similar to SDNN$_{10min}$, **(E)** females but not **(F)** males with lower SDNN/rMSSD$_{10min}$ had greater CLAD intake (with no relation to alcohol-only). **(G)** In females, greater HR$_{10min}$ correlated with higher alcohol-only and CLAD. **(H)** In males, greater HR$_{10min}$ correlated with higher CLAD but not alcohol-only. * p<0.05. Graph data female/male coloring as in Fig,3.3.

3.4 Discussion

The present study sought to elucidate how autonomic states — indexed through heart rate (HR) and heart rate variability (HRV) — may promote alcohol drinking and how such states may differ by biological sex. We assessed these cardiac measures (1) at baseline, before alcohol access, (2) during the first minute of drinking ("drinking onset"), and (3) across 10 minutes of the alcohol intake period (effects summarized in **Figure 3.8**). In females, greater alcohol intake (expressed in g/kg) could be predicted by higher relative PNS influence both at baseline (lower SDNN/rMSSD ratio) and at drinking onset (higher rMSSD). In contrast, male drinking was better predicted by SNS indicators, including a significant increase of SDNN and SDNN/rMSSD ratio, with no change in rMSSD, from baseline to drinking onset. Additionally, some HR/HRV changes occurred during compulsive-like alcohol drinking (CLAD) conditions more than alcohol-only, suggesting differences in response strategy during challenge that agree with previous work (detailed below).

We chose to examine drinking onset because HR/HRV differences at this time would occur before alcohol would substantially enter the blood stream (Doyon et al., 2003; 2005), suggesting that autonomic measures here reflected physiological drivers of alcohol drinking, instead of direct alcohol effects per se. Additionally, previous work from our lab and others has shown that drinking onset is a key period where significant behavioral (Darevsky et al., 2019; Darevsky & Hopf, 2020) and neurological changes (Starski et al., 2023) occur. For example, shifts in response patterns during alcohol-only vs CLAD suggest divergent action strategies depending on the presence of an aversive stimulus.

In females, higher HR over the 10-minute drinking session was correlated with greater intake for both alcohol-only and CLAD. However, all other HR/HRV changes observed in both sexes occurred only for CLAD, and not alcohol-only. For instance, males with a lower overall PNS and higher HR had greater CLAD intake, where reduced PNS (indexed through reduced rMSSD) and no change in SNS indicators suggested a state of SNS predominance. In contrast, females with lower overall SNS had greater CLAD intake, where reduced SNS (indexed through reduced SDNN, reduced SDNN/rMSSD ratio, but no change in rMSSD) suggested a state of PNS predominance. As described earlier, our behavioral (Darevsky et al., 2019; Darevsky and Hopf, 2020), anterior insular cortex firing (Starski et al., 2023), and anterior insula circuit inhibition (Seif et al., 2013; De Oliveira Sergio et al., 2021) results suggest important differences in mechanisms underlying action strategies used during alcohol-only versus CLAD. Thus, it is interesting here that most consumption-related HRV patterns across the 10-minute intake period were associated with CLAD rather than alcohol-only, suggesting that these sustained HRV changes may be related to shifts in the cognitive-emotional action strategy used during CLAD. Thus, our results suggest congruence in the idea that CLAD vs alcohol-only utilize divergent strategies to regulate responding for alcohol. Importantly, our results also suggest that females and males display different autonomic promoters of CLAD, with greater PNS influence in females and SNS in males.

Taken together, the observed sex differences in the present study (summarized in **Figure 3.8**) agree with human studies suggesting greater utilization of PNS in females and SNS in males during autonomic regulation (Dart et al., 2002; Sato and Miyake, 2004; Nugent et al., 2011; Hamidovic et al., 2020; Koenig et al., 2021). Females with greater

PNS influence at baseline and during drinking onset had greater drinking levels, while males showed an increase in SNS indicators at drinking onset. Across 10 minutes of CLAD intake, females with higher relative PNS (as a consequence of lower SNS influence), including lower SDNN, lower SDNN/rMSSD, and higher HR, had higher CLAD intake levels. On the contrary, males with higher SNS indicators (related to lower PNS influence), including higher HR and lower rMSSD, had more CLAD. Thus, our results suggest that there are sex-specific patterns in how HRV measures relate to aspects of drinking (baseline, drinking onset, overall alcohol-only or CLAD intake). Thus, our novel and likely clinically relevant findings support the use of cardiac biomarkers to assess sex-specific alcohol risk and to target sex-specific autonomic functions to reduce excessive alcohol drinking in humans.

One consideration is that sex differences in HRV measures might be impacted by the higher HR in females, especially where faster HR mathematically reduces the potential for variability. However, both SDNN (**Figure 3.5 A**) and rMSSD (**Figure 3.5 B**) showed spread relative to HR in females and males, indicating dynamic range to observe HRV shifts. Said another way, while indeed HR and HRV measures are correlated with one another, the correlation leaves room for various given HRs for a given HRV. This agrees with human studies where women paradoxically show higher HRV even with greater HR (Williams et al., 2022). While the mechanism for this apparent paradox is not yet clear, it has been suggested that effects of estrogen on cardiac chronotropy may play a role (Williams et al., 2022). Thus, we and others observe that HRV is not identical to HR, though they are certainly related, and the precise reasoning for this is not yet known.

Another important consideration is that shifts in PNS-SNS balance can be illustrated through various changes across HRV measures. For example, both basal and drinking-onset PNS-related HRV indicators predicted greater female drinking. However, for basal, this involved lower SDNN/rMSSD without rMSSD changes (**Figure 3.3 A,C**), while drinking onset was linked to higher rMSSD without changes in SDNN/rMSSD (**Figure 3.4 A,E**). Additionally, males showed increased SNS indicators at drinking onset through higher SDNN and SDNN/rMSSD but no rMSSD change (**Figure 3.6 A-C**), while the greater SNS influence linked to higher CLAD intake was associated with reduced rMSSD and no change in SDNN (**Figure 3.7 B,D**).

To better understand HRV-related changes, and give context to our findings, we describe several examples from the work of Inagaki and colleagues (Inagaki et al., 2004; Inagaki et al., 2005; Inagaki et al., 2013), who examined HRV through frequency domain measures rather than through time domain measures (see Introduction). For example, expecting a negative stimulus is linked to greater LFHRV and LFHRV/HFHRV but no changes in HFHRV, and is interpreted as greater SNS without changes in PNS (Inagaki et al., 2004). In contrast, negative experience is associated with decreased HFHRV, and a trend decrease in LFHRV, leading to greater LFHRV/HFHRV. Together, these are interpreted as a decrease in PNS which leaves SNS predominant (Inagaki et al., 2004), which is also observed in humans (Lin et al., 2001). The first condition is akin to our male rats at drinking onset (**Figure 3.6**), with a milder version of the second condition akin to male CLAD intake (**Figure 3.7**). It is notable that negative experience also had increased HR (Inagaki et al., 2004), as did higher CLAD-drinking males, suggesting that

SNS-mediated increases in HR can occur without overt SNS changes (via a decrease in PNS influence which leaves SNS predominant).

Thus, different HRV changes can lead to greater SNS predominance. Conversely, reward consumption is associated with a large decrease in LFHRV, and a small decrease in HFHRV, leaving a net drop in LFHRV/HFHRV; the larger SNS decrease and smaller PNS decrease leaves PNS as predominant (Inagaki et al., 2005). However, when water-deprived rats expect sugar but receive water, they have increased HFHRV with no change LFLRV, suggesting a strong PNS increase (Inagaki et al., 2005). Thus, different HRV changes can enhance PNS influence. We note that these studies involved water-deprived rats, and rats in our study were not water deprived, and thus the magnitude of HRV changes might be less in our work. Nonetheless, these examples provide precedent for the importance of assessing a series of HRV measures, since different patterns of HRV changes can result in somewhat similar shifts in PNS-SNS balance.

It was interesting that autonomic changes at drinking onset occurred in both females and males, since HRV changes for alcohol stimuli can predict drinking problems in humans. For example, larger HFHRV increases to alcohol stimuli predict future relapse (Garland et al., 2012) and alcohol problems (Wang et al., 2020), while HFHRV increases to negative images predicts higher craving (Claisse et al., 2017). Also, alcohol stimuli can increase both HFHRV and LFHRV (Rajan et al., 1998; Jansma et al., 2000), and high-risk drinkers show greater LFHRV changes to alcohol or emotional images (compared with low-risk drinkers) (Mun et al., 2008), while LFHRV changes to alcohol stimuli relate to drinking problems (Ingjaldsson et al., 2003) and decreased ability to resist alcohol urges (Jansma et al., 2000). HRV increases in response to appetitive stimuli have been associated with greater regulatory effort (Segerstrom & Nes, 2007) and cognitive engagement (Leganes-Fonteneau et al., 2021), and thus, for alcohol, may reflect ineffective recruitment of emotional regulation and/or “locked-in attention” (inability to detach from action plans driven by alcohol stimuli) (Ingjaldsson et al., 2003; Claisse et al., 2017; Ralevski et al., 2019). For example, greater LFHRV responses to alcohol cues are related to greater cue memory (Leganes-Fonteneau et al., 2021). Thus, HRV changes in response to alcohol conditions have the potential to be both biomarkers for and contributors to pathological alcohol drinking. Importantly, we found that females and males had different autonomic markers at drinking onset (an important alcohol stimulus for rats, as described above) (**Figure 3.4**), as well as across alcohol-only and CLAD for 10-min findings (**Figure 3.7**), suggesting novel and sex-selective HRV indicators of risk for excessive drinking.

Our studies also found that basal PNS function in females was related to subsequent consumption levels. Some human studies have found that basal HRV measures predict drinking problems (Ingjaldsson et al., 2003; Ralevski et al., 2019), although other studies do not observe a relationship (Mun et al., 2008; Claisse et al., 2017). Greater real-world drinking in humans with alcohol use disorder has been linked to higher basal HR and a non-linear measure of HRV (Hwang et al., 2022). We note that we only observed basal HRV associations with alcohol drinking in females. Thus, this is an area that warrants further study in humans. Another important consideration is the possibility of differences in autonomic regulation across the menstrual cycle in humans and estrous cycle in rats. Many studies have observed baseline HR/HRV differences between men and freely cycling women (Nugent et al., 2011; Hamidovic et al., 2020;

Williams et al., 2022), which is congruent with studies in rodent (Carnevali et al., 2023; Frasier et al., 2023). However, estrogen can enhance PNS in humans (Huikuri et al., 1996; Goldstein et al., 2010) and rodents (Dart et al., 2002), although one study in humans found greater effects of sex differences on HRV than cycle stage differences (Sato and Miyake, 2004). Also, estrous cycle sometimes has limited influence on level of intake once rodent addictive behaviors are established (De Oliveira Sergio et al., 2023). Even so, inhibiting estrogen receptors decreases alcohol drinking in mice during higher but not lower estrous stages (Chen et al., 2022). Thus, different underlying mechanisms could promote behavior at different cycle stages, even if the level of behavior does not differ (Satta et al., 2017; Chen et al., 2022). It will be critical in future studies to address potential differences across the estrous cycle in autonomic mechanisms that underly drinking. For example, women have greater amygdala activity during aversion responding when estrogen is low vs high (Goldstein et al., 2010; Jacobs et al., 2015; Wheelock et al., 2021), and the HRV relation to estrus stage may be more pronounced for CLAD. However, it is likely that biological sex overall is a more critical variable driving changes in HRV as opposed to discrete moments within the menstrual cycle (Williams et al., 2022). In sum, more work is necessary to parse the effects of menstrual cycle in humans and estrus cycle in rodents on HRV.

In conclusion, our studies provide novel and likely clinically-relevant insights into sex differences in autonomic mechanisms that promote alcohol drinking, including where different HRV indicators were associated with differing stages of drinking behavior (baseline, drinking onset, across a drinking period). These sex-specific patterns in alcohol-related HRV measures add to the growing literature of utilizing HRV metrics as biomarkers to assess sex-specific alcohol risk and to target sex-specific autonomic functions to reduce excessive alcohol drinking.

	Female			Male		
Greater Drinking:	**HR**	**HRV-SNS**	**HRV-PNS**	**HR**	**HRV-SNS**	**HRV-PNS**
Basal	--	↓ SNS (SDNN/rMSSD)	--	--	--	--
Drinking Onset	--	--	↑ PNS (rMSSD)	--	--	--
Change at Drinking Onset	--	--	--	--	[↑ SNS (SDNN, ratio)]	--
AlcOnly: 10-min Intake Period	↑ HR	--	--	--	--	--
CLAD: 10-min Intake Period	↑ HR	↓ SNS (SDNN, ratio)	--	↑ HR	--	↓ PNS (rMSSD)

Figure 3.8. Summary of sex differences in the relation between HRV patterns and drinking. For females, greater PNS influence at baseline and during drinking onset predicted higher alcohol intake. Males had an increase in SNS at drinking onset (put in brackets because increased SNS was observed, but was not correlated with intake level). When assessed across the 10 minute drinking session, HRV measures largely related to CLAD but not alcohol-only. In females, lower SNS indicators, and thus greater SNS influence, predicted higher CLAD intake, while males with lower PNS influence, and thus greater SNS predominance and higher HR, had greater CLAD. "- -" indicates no relation between HRV and behavioral measures.

3.5 Supplemental Material

3.5.1 Telemetry Surgery

Alcohol was withheld from rats for approximately 48-72 hours prior to surgery to prevent complications. Using antiseptic surgical techniques, rats were put under isoflurane anesthesia and implanted with a telemetry device (type PTA-M-C, part# E-430001- IMP-130) from TSE Systems Inc. (Chesterfield, MO). The telemetry device consisted of a silicone elastomer transmitter (8.3 mm in by 16.5 mm x 4 mm), and a thin, plastic-sheathed wire which had a small sleeve at the distal tip which detected changes in blood pressure within the artery. The surgery required two incisions, one in the midline of the abdominal cavity (to place 98 the transmitter), and a second where the abdomen meets the left thigh to access the left femoral 99 artery. The femoral artery was carefully dissected from the adjacent femoral vein and femoral 100 nerve, and then dilated through topical application of 2% injectable lidocaine. Suture silk was 101 used to temporarily occlude the femoral artery, and then a small needle puncture was made into 102 the vessel. The wire with telemeter at the end was inserted into the blood vessel, the silk suture 103 was loosened slightly, and the wire tip advanced until it sat approximately between the iliac 104 bifurcation and the renal arteries within the abdominal aorta. This was assisted by a removable 105 trocar which led the wire. Once the wire was in place, the suture silk was lightly tied to the 106 femoral artery to keep the wire from slipping and to assist in closing the small puncture to 107 prevent bleeding. To confirm placement within the abdominal aorta, real-time blood pressure 108 trace was assessed using NOTOCORD-hem software (Instem, Staffordshire, UK). As the aorta is 109 the major arterial vessel emerging from the heart, it provides accurate information about HR and 110 blood pressure. Finally, nonabsorbable sutures were used to attach the transmitter unit to the 111 inner musculature of the abdominal wall. Finally, the animal is sewed up with absorbable 112 sutures, provided pain relieving drugs (carprofen 5mg/kg and buprenorphine 0.03mg/kg), and 113 placed in their homecage for recovery.

3.5.2 Supplemental Figures

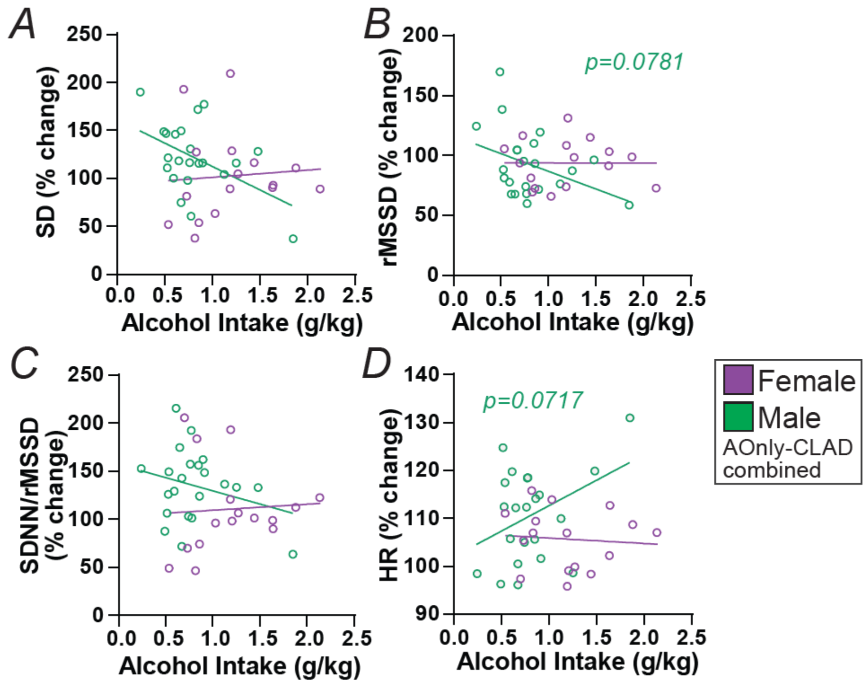

Figure 3.9. No change in HR/HRV measure correlated with alcohol drinking levels, although with some trends in males. Changes in SDNN (**A**) were significant in males (p=0.0246), although this went away when removing one outlier ($F_{(1,19)}$=0.913, R^2=0.0457, p=0.3520), and with no relation in females ($F_{(1,14)}$=0.077, R^2=0.0055, p=0.7855). For rMSSD (**B**), there was a trend in males ($F_{(1,20)}$=3.448, R^2=0.1471, p=0.0781) but not in females ($F_{(1,14)}$=0.000, R^2=0.0000, p=0.9953). Also, while there was no relation for SDNN/rMSSD (**C**) (males: $F_{(1,20)}$=1.527, R^2=0.0709, p=0.2308; females: $F_{(1,14)}$=0.051, R^2=0.0036, p=0.8244), there was a trend for HR (**D**) in males ($F_{(1,20)}$=3.616, R^2=0.1531, p=0.0717) but not females ($F_{(1,14)}$=0.096, R^2=0.0068, p=0.7617). Thus, we concluded that increased SNS influence at drinking onset might have been permissive for alcohol drinking, there were only trends for SNS (perhaps expressed as higher HR or lower rMSSD) to influence male intake.

CHAPTER FOUR: Discussion and Future Directions

4.1 Significance

The studies herein are the first, to our knowledge, to reveal functional implications of HR/HRV telemetry as an index of autonomic control to elucidate sex differences in alcohol misuse and anxiety-like behavior in rats. Specifically, the studies herein are (1) the first to relate specific aspects of anxiety-like behavior to changes in HR/HRV that differ by sex and (2) utilize HR/HRV telemetry in rats to predict alcohol consumption both under regular drinking and compulsive-like drinking that differ by sex. The significance of this project is it allows for new mechanistic investigations to ensue, which would assist in the development of novel, more personalized therapies to combat rising, sex-specific issues related to problem alcohol drinking and anxiety disorders. Further, this project supports the role of autonomics, indexed by HRV, to influence behavior in a sex specific way, where females bias toward PNS and males toward SNS. These data add to a growing literature suggesting male and female response strategies, both at baseline and during challenge, differ subtly yet significantly to impact behavior. Thus, autonomic differences may provide an underlying reasoning for why sex differences in prevalence and prognosis for various psychiatric disorders are observed clinically.

4.2 Theories of Emotional Regulation Through Autonomics

4.2.1 Polyvagal Theory

Polyvagal Theory was initially proposed in 1994 by Dr. Stephen Porges. It posits that the autonomic nervous system of mammals is three-pronged: it contains one sympathetic system and evolved two parasympathetic systems with diverging vagal structures that arise from distinct nuclei within the brainstem medulla: (1) the evolutionarily older, unmyelinated vagus nerve fibers that originate in the Dorsal Motor Nucleus of Cranial Nerve X (DMNX) and (2) the evolutionarily newer, myelinated vagus nerve fibers that originate in the Nucleus Ambiguus (NA). Recall from Chapter One that the NA is the origin of the efferent vagal fibers that innervate the heart's pacemaker, the SA node, and thus play a major role in the determination of the heartrate (HR) and heart rate variability (HRV). The efferent vagal nerve fibers that originate in the NA primarily innervate supradiaphragmatic organs and structures such as the heart, bronchi, striated muscles involved in facial expression, the larynx, pharynx, esophagus, and soft palate. The efferent vagal nerve fibers that originate in the DMNX primarily innervate subdiaphragmatic organs such as the intestines and stomach (Porges, 1995, 2009). According to polyvagal theory, the development of the newer vagal fibers within the NA allows for human social behavior and is intimately tied to respiration, heartbeat patterning, vocalization, and facial expression (Porges, 1995, 2009, 2022).

The older vagus fibers, termed the "vegetative vagus" or "dorsal vagus," are implicated in immobilization strategies to cope with threat such as syncope, feigning death, passive avoidance, and behavioral shutdown. These behaviors (or more accurately, lack of behaviors) are often referred to as "freeze" responses. However, humans and other mammals are not restricted to a dichotomy between "freeze" and "fight or flight" in response strategy. Mammals, especially humans, can choose to "tend and befriend" (Taylor, 2000), which is a more socially inclined strategy that involves nurturing to provide safety or reacting with kindness to invoke a social relationship. Neither the "freeze" or "fight or flight" responses adequately explain this alternative strategy, which, interestingly, is much more commonly invoked in women compared to men (Taylor, 2000). Polyvagal theory provides a framework to understand this alternate “tend and befriend” response strategy. The newer, myelinated vagus fibers, termed the “ventral vagus” are implicated in prosocial behaviors, such as those employed when utilizing the “tend and befriend” response to threat and include automatic adjustments of facial expression and vocal intonations to achieve these goals. Further, these ventral vagus fibers are implicated in everyday, often unconscious, detection of safety versus threat in the environment, termed “neruoception” (Porges 2009, 2022).

Porges argues that the ventral vagus is part of a “social engagement system” responsible for the repertoire of prosocial behaviors humans and other mammals utilize outside of the canonical “freeze” and “fight or flight” behaviors. The system depends on the healthy functioning of the ventral vagus, which serves as a “brake pedal” to inhibit sympathetic outflow to the heart and creates the space for self-regulation to occur, but not so much of a brake that it creates immobilization. (Porges, 1995, 2009, 2022). Thus, the ventral vagus is posited to serve as an intermediary between two extremes: mobilization and immobilization. It is within this spectrum that social behavior is possible.

In relation to HR/HRV, Dr. Porges’ group regularly measures vagally mediated HRV as an index of self-regulatory capacity that primarily reflects the actions of the ventral, newer vagus. Through polyvagal theory, it is believed that vagally mediated HRV serves as a biomarker that sets the standard for emotional expressiveness, prosocial behavior, and the ability to self-sooth (Porges, 2009, 2022). The evolution of the two vagal networks tracks with the development of a large neocortex utilized for top-down inhibitory control over the older, more reptilian responses and allows for enhanced sophistication in moderating behavior (Porges, 1995, 2009, 2022). The development of a neocortex necessitated the co-development of additional wiring to allow communication of this brain structure to subcortical and brainstem structures. Polyvagal theory suggests this need influenced the divergence of the vagus nerve, such that dorsal vagus remained involved in the older regulatory behavior, and a newer, ventral vagus developed to address the needs of integrating higher cortical structures.

The evolutionary perspective interwoven with polyvagal theory supports the notion of Jacksonian Dissolution, which was initially described by famed 19th century neurologist, Hughlings Jackson. Jacksonian Dissolution is the observation that when illness dismantles higher-level evolutionary processes, the older processes become disinhibited and fill in the regulatory roles (Porges, 2009). Thus, Jacksonian Dissolution infers that newer structures inhibit older structures as evolution progresses. As we will discuss in the following sections, contemporary neuroimaging data appear to validate this notion.

Linking polyvagal theory with modern conceptions of mental disorders, the heterogeneity of mental illnesses appears to have a common theme: disability or distress related to social functioning. Broadly, the American Psychiatric Association (APA) defines mental illnesses is as “health conditions involving changes in emotion, thinking and/or behavior associated with distress and/or problems functioning in social, work, or family activities” (APA, 2013). Considering that social engagement is presumably any situation where two or more beings interact, we can consider family activities as “social”, and most occupations require some degree of social functionality. Consider what makes a person “unemployable” and it often relates to severe failures in social ability. In this sense, polyvagal theory provides a scaffolding to understand the critical importance of social cohesion in human well-being. Without the ability of the ventral vagus to regulate social behavior appropriately, social functioning deteriorates and creates substantial distress and disability, and very often the result falls under the auspices of mental disorder. Furthermore, many mental illnesses follow the theme of Jacksonian Dissolution, where a hypoactive frontal cortex results in disinhibition of subcortical structures such as the amygdala, and this leads to hyperarousal (Thayer & Brosschot, 2005). Behaviorally, this results in difficulties with attention, decision making, and other executive processes and over-exaggerates feelings of anxiety and fear and their physiologic counterparts of autonomic arousal.

The mental health conditions of note in this dissertation, anxiety disorders and alcohol misuse, especially lend themselves to this type of conceptualization. For example, many anxiety disorders, such as generalized anxiety disorder (GAD), implicate a hypofunctional prefrontal cortex and a hyper-functional amygdala (Thayer et al., 2012). The resulting symptomology of excessive worry, irritability, and avoidance --among others-- then leads to dysfunctional social relationships. Maladaptive alcohol use is frequently comorbid with anxiety disorders and can be argued to assist the anxious individual in inhibiting their disinhibited lower brain structures. This serves as an ingenious, yet misguided, attempt on the part of the organism to self-sooth.

In some individuals, when alcohol use transitions from casual to an addiction, the individual becomes “stuck” or “locked in” to the behavior to drink to the point where voluntary cessation becomes a considerable challenge. Similar in anxiety disorders, individuals routinely become dysregulated and “struck” into a behavioral repertoire that is indiscriminately deployed despite no true threat presence. Polyvagal theory suggests the neurobiological process underlying this behavioral dysregulation involves a mis-tuned ANS, such that the SNS or dorsal vagal component of the PNS are predominantly active where it is not appropriate and there is a lack of ventral vagal PNS to allows for proper tuning of the ANS to meet environmental demands. The relative functioning of the ventral vagal PNS can be determined through measuring HRV.

4.2.2 Neurovisceral Integration Theory

Neurovisceral integration theory was proposed in 2000 by Drs. Julien Thayer & Richard Lane. Their theory connects the processes of emotional regulation, attentional control, and autonomic regulation through a collection of brain regions, termed the Central Autonomic Network (CAN), that function in synchrony to allow for adaptive control over response selection to environmental demands. They propose that vagally mediated heart rate variability (HRV) is an output of this gestalt of neural structures that can be used to accurately index the adaptability or flexibility of an organism (Thayer & Lane, 2000, 2009). The structures of the CAN include the prefrontal cortex, anterior cingulate, anterior insula, amygdala, hypothalamus, periaqueductal gray, and brainstem centers such as the nucleus of the solitary tract (Thayer & Lane, 2009). The nucleus of the solitary tract (NST) is located in the brainstem and is a major hub of integration of autonomic signals from the viscera and communicates with the cortical structures of the CAN (Thayer & Lane, 2009; Thayer et al., 2012). For example, the major afferent vagus fibers that communicate status of the heart to the brain synapse in the NST (Shaffer et al., 2014). Further, the NST has direct connections to the dorsal motor nucleus of X (DNMX) and the nucleus ambiguus (NA), which are the locations of the major vagal efferent fibers to the heart (Thayer et al., 2012; Thayer & Lane, 2009) and are the brainstem nuclei implicated in polyvagal theory. The NST is where vagal efferent fibers from the DMNX and NA intermingle with vagal afferent fibers rising from the heart, providing a functional anatomic link for brain-heart bidirectional communication. Thayer & Lane propose the CAN links cortical structures and the NST through bidirectional feedback and feedforward loops that integrate internal regulation of visceromotor, neuroendocrine, and behavioral responses necessary for the organism to appropriately respond to environmental challenges.

At rest, the prefrontal cortex (PFC) inhibits the amygdala. The amygdala is the major brain region activated in response to threat and thus is implicated in the feeling and expression of fear and anxiety both in healthy and pathological conditions (LeDoux, 2003). The PFC is a collection of areas in the neocortex that are associated with executive functioning such as attentional control, emotional regulation, and decision making. Thus, in a healthy individual at rest, this executive functioning center inhibits the amygdala from sounding superfluous alarm bells. However, should threat be detected, the PFC is inhibited resulting in activation (via disinhibition) of the amygdala. The amygdala has outputs to autonomic, endocrine, and other physiological regulatory systems, including the NA, DNMX, and NTS that serve to mobilize the organism's resources to prepare to cope with the detected threat (Thayer & Lane, 2009; Thayer et al., 2012). For example, the amygdala communicates with the NTS to recruit the sympathetic nervous system (SNS) and withdraw the parasympathetic nervous system (PNS) to attend to the threat by increasing HR and decreasing HRV. Interestingly, recruitment of the SNS efferents is tonically inhibited by the NTS, and the activation of the amygdala causes disinhibition of this connection. Thus, the cascade of inhibition of evolutionarily newer structures resulting in disinhibition of older structures is coherent yet again with the concept of Jacksonian Dissolution.

Thayer and colleagues have consolidated much research and conducted their own to show that HRV, particularly vagally mediated HRV, is positively correlated with PFC activity (see Thayer et al., 2012; Thayer & Brosschart 2005 for reviews). HRV is decreased when the PFC is inhibited during perception of threats and is linked directly to the autonomic activation within the NTS that jumpstarts SNS mobilization (Thayer & Brosschot, 2005; Thayer et al., 2012). When this system becomes unbalanced, and such imbalance is typically in the direction of overly active SNS, the PFC remains hypoactive and leads to dysfunctions in executive control. Neurovisceral integration theory hypothesizes that HRV is a marker of how well balanced the system is, where chronically low HRV is associated with behavioral rigidity and pathology.

In coherence with this theory, individuals with anxiety disorders have been shown to have lower HRV, PFC hypoactivity, and amygdala hyperactivity (Thayer & Brosschot, 2005; Thayer et al., 2012). Low HRV is also associated with diminished PNS function, poor attentional control, emotional dysregulation, and behavioral inflexibility. Chapter One discussed the linkage between HRV and alcohol misuse, where alcohol use disorder patients typically have lower HRV at rest (Ralevski et al., 1029) and often a mis-tuned HRV spike in response to alcohol related cues (Ingjaldsson et al., 2003) that in some instances can predict craving (Quintana et al., 2013) and likelihood of relapse (Garland et al., 2012). In sum, neurovisceral integration theory proposes vagally mediated HRV as an index of the degree of synchrony between the higher cortical structures and the autonomic nervous system in promoting appropriate adjustments in behavior and visceral status to meet the demands of the environment.

4.3 Limitations

This work has many limitations. First, the animals underwent major surgery to implant the telemetry device used to detect HR/HRV. The effect of the stress of surgery could likely influence anxiety-like and alcohol-drinking behavior.

Second, the animals used in these studies were single housed, and isolation is a stressor both in humans and in rodents. However, the present studies required single-housed rats in order to perform alcohol-drinking studies in line with the lab's protocols. In the future, it would be interesting and useful to examine HR/HRV telemetry across both sexes in socially housed rats to determine the changes in HR/HRV between single-housed and socially housed rats.

Third, this work did not scientifically demonstrate a mechanism for these findings. However, future work in the lab is planned to remedy this using optogenetic inhibition in addition to HR/HRV telemetry. The lab has preliminary data using the Novelty Suppression of Feeding paradigm with optogenetic inhibition of two pathways implicated in ANS control over HRV and its relationship to behavior: (1) the anterior insular cortex (AIC) to posterior insular cortex (PIC) and (2) the AIC to the nucleus accumbens (NAc). Preliminary results demonstrate that the AIC-PIC circuit encourages rats of both sexes to approach the center (and thus the food) sooner and make more approaches. Interestingly, the AIC-NAc circuit inhibition decreased time in center only for males. While preliminary, these data suggest once again that males and females likely have subtly different response strategies when dealing with challenge, where certain circuits (like the AIC-PIC) are necessary in both sexes for certain aspects of anxiety-like responding, but others (like AIC-NAc) may have differing effects depending on sex. Future work planned in the lab will inhibit additional circuits implicated in autonomic control such as: AIC-CeA, mPFC-CeA, and mPFC-AIC to investigate sex differences in HR/HRV related to behavior.

Fourth, we had initially hypothesized that rats with established alcohol-drinking habits would have significantly lower baseline HR/HRV compared to alcohol-naïve rats, and that these differences would contribute to increased anxiety-like behavior in drinkers. This hypothesis was based on evidence from human literature suggesting similar differences between alcohol use disorder patients versus healthy individuals. However, we did not observe this. We believe this was due to the particular model of alcohol consumption utilized in these studies. The model employed here is used to capture the behavior of compulsion, which is a significant barrier to treatment in many substance use disorders (De Oliveira Sergio et al., 2023; Starski et al., 2023). However, our model does not capture dependence, which is another common feature of substance use disorders. However, we chose to prioritize compulsion over dependence due to data suggesting most excessive drinkers do not meet the criteria for alcohol dependence (Esser, 2014). Thus, it is possible more severe models of alcohol use disorder would be required to demonstrate differences related to this hypothesis and concord with clinical research.

Fifth, the studies in Chapter Two only utilized one model of rat anxiety-like behavior to look at HR/HRV related behavioral changes. It would be interesting to see how different models of rat anxiety-like behavior would add to this story. In the future, additional models of anxiety-like behavior (some good candidates listed in Chapter One) should be performed to further support or complicate the results presented here.

4.4 Future Directions

4.4.1 Estrus & Menstrual Cycle Considerations

One important consideration for future work is the role of naturally occurring hormonal fluctuations that occur in women compared to men and how these could affect behavior, mood, and susceptibility to mental health conditions. Recall that women have nearly twice the prevalence of anxiety disorders (Altemus et al., 2014) and often suffer consequences of alcohol misuse more quickly and severely than men (Becker et al., 2017). However, it can be argued that differences between men and women in these domains may relate to cultural or environmental influences more than purely biological ones. Additionally, the historical lack of preclinical rodent models utilizing female subjects further obscures the dissociation between more biologically "hard-wired" sex differences and more culturally influenced differences. However, in recent years more attention has been granted to sex differences in psychophysiological studies both in humans and in rodents.

Humans have a menstrual cycle, typically lasting approximately 28 days, which can be conceptually divided into two phases. First, the follicular phase is where the body prepares for potential pregnancy and is hormonally characterized by rising levels of estrogen and low levels of progesterone. Second, the luteal phase occurs as the body prepares for implantation of a fertilized egg and is characterized by increases in estrogen and progesterone. Should pregnancy not occur, this phase ends with sharp decline in estrogen and progesterone, which triggers menstruation. Premenstrual dysmorphic disorder (PMDD) is a mental health condition during which severe mood symptoms occur during the menstrual cycle. Interestingly, the symptoms of PMDD occur alongside the sharp decrease in hormones during the luteal phase (Hantsoo et al., 2020). Further, symptoms related to panic, anxiety and fear are increased in women with various anxiety disorders during the luteal phase (Nillni et al., 2021). Thus, evidence suggests that the luteal phase, when estrogen and progesterone are low, is likely a period of increased vulnerability for women to mood disturbances.

Rats have an estrus cycle that is typically 4-5 days in length, and it is characterized by similar hormonal shifts with estrogen and progesterone. The rat estrus cycle contains four phases, proestrus, estrus, metestrus, and diestrus. The proestrus phase hormones are similar to human follicular phase (rising estrogen, low progesterone) and the diestrus phase hormones are similar to the human luteal phase (low estrogen, sudden drop in progesterone). Indeed, rodent work finds that unconditioned fear responses in females is increased during diestrus (Lovick & Zangrossi, 2021). However, other studies find no estrus cycle phase differences in rodents for the elevated plus maze (Scholl et al., 2019), open field (Henricks et al., 2017), or shocked licking (De Jesus-Burgos et al., 2016). Thus, much remains to be discovered with regard to anxiety-like behavior and estrus cycle staging, and how this may relate to human menstrual cycle hormone fluctuations. Finally, differences in alcohol drinking during the estrus cycle have not demonstrated consistent specificity for a particular cycle phase on behavior (Radke et al., 2021). In sum, in women and in female rats, it appears that the luteal/diestrus phases correspond most strongly with exacerbated anxiety symptoms, at least in some studies. The role of cycle stage on alcohol consumption is less clear.

The sex hormones are steroid hormones, and these have a highly lipophilic structure allowing ease of travel across the blood brain barrier to effect brain structures. Indeed, estrogen, progesterone, and testosterone (and their metabolites) can affect cell changes through membrane receptor dynamics or through travel into the cell nucleus to alter gene expression (Dart et al., 2002). Therefore, it follows that sex hormones can impact the brain and subsequently behavior, especially in sex-specific ways. Relating back to the heart, it has long been observed clinically that men have a higher incidence of cardiovascular disease in addition to worse prognosis for cardiovascular disease compared to menstruating women, and the protective advantage afforded to women disappears after the onset of menopause (Dart et al., 2002; Du et al., 2006). These data suggest a protective role of estrogen on the heart. Furthermore, the connection between sex differences in mood and cardiovascular risk point to the autonomic nervous system (ANS) as a likely common pathway for these modulations (Dart et al., 2002; Du et al., 2006).

Indeed, much recent work has led to the thinking that females utilize parasympathetic (PNS) more and males utilize sympathetic (SNS) more, and this could account for sex differences in cardiovascular risk and behavior (Dart et al., 2002; Du et al., 2006). For example, when challenged with a coronary occlusion, females display more cardioprotective response of bradycardia, decreased blood pressure, and higher HRV (indices of parasympathetic responding) compared to males (Airaksinen et al., 1998). Conversely, males under ischemic challenge display a pressor response of increased blood pressure whereas females either decrease or do not alter their blood pressure (Maixner & Humphrey, 1993). Further, studies that lower blood pressure to activate the baroreflex differentially effect men and women, where men activate pressor responses (an index of sympathetic response) and females inhibit pressor responses (an index of parasympathetic response) (Kimmerly et al., 2007). These vagally-mediated sex differences can be altered such that vagotomy or ovariectomy alters female responding to a more reflect a male pressor type of responding (Du et al., 2006).

Furthermore, changes in female cardiovascular response have shown effects of hormones. For example, ovariectomized animals have greater SNS and diminished PNS compared to males, and these differences were reduced with administration of estrogen (reviewed in Dart et al., 2002). Additionally, estrogen dose-dependently increases vagal tone and can be blocked by estrogen antagonism (Saleh & Connell, 2003). At the level of the heart, male rats have higher density of beta-1-adrenergic receptors compared to females, and orchidectomy reduces this receptor density. Further, ovariectomized females have increased beta-1-adrenergic receptors and the administration of estrogen reverts this effect back to more female-typical levels (reviewed in Du et al., 2006). In the female heart, estrogen potentiates the effects of acetylcholine (Dart et al., 2006). Taken together, these data suggest that male hearts are more sensitive to the effects of the major neurotransmitters of the SNS, norepinephrine and epinephrine, whereas female hearts are more sensitive to the major neurotransmitter of the PNS, acetylcholine. Thus, differential ANS sensitivities appear to be "hard-wired" into physiology in a sex-specific manner.

The data presented in this dissertation did not take estrus cycle into account. Based on the extant literature, we hypothesize that if estrus cycle was tracked, vagally mediated HRV would be elevated during proestrus where estrogen is higher, thus potentiating the effect of estrogen on the PNS. Further, we would expect that during diestrus when estrogen and progesterone are decreasing, HRV would decrease. Furthermore, these changes may relate to anxiety-like behavior, such that females would show decreased latency to approach the center when estrogen is higher in proestrus and the opposite effect during diestrus. Human literature support this, where one group found the effect of negative emotion on HRV to be enhanced during the luteal phase, which roughly corresponds in hormone profile to the rodent diestrus phase (Simon et al., 2021). For alcohol drinking studies, it is unclear how alcohol-only drinking may be affected by estrus cycle hormone shifts. Many studies (reviewed in Radke et al., 2021) do not show changes in estrus cycle hormones related to alcohol preference or consumption. However, we hypothesize that during compulsive-like drinking, where females would have to overcome aversion to drink alcohol, the female bias toward the PNS would be most pronounced during high estrogen phases of the estrus cycle. During lower estrogen phases such as diestrus, we would expect female and male compulsion-like responding to be more similar. However, studies have shown that once addictive behaviors are established, estrus cycle does not appear to influence responding for alcohol (Fulenwider et al., 2019; Randall et al., 2017).

In sum, hormonal shifts related to the estrus cycle may play a small but significant role in modulating differential behavior responding patterns in females. It is my opinion that cycle change differences would have subtle impacts on behavior and require highly powered studies to detect significance. I believe that differences between females and males, regardless of cycle stage, play a more significant role in the divergence of ANS strategy than hormone modulation in females. However, perhaps in some individuals these hormonal fluctuations exert more of an effect on mood and behavior, as is observed in the small subset of females that suffer from PMDD. However, much of this is speculative and further research is necessary.

4.4.2 Neurocircuitry of HR/HRV Regulation

The heart and brain are highly interconnected and communicate bidirectionally. The most significant channels of communication between the heart and the brain involve the ANS and its two major branches, sympathetic (SNS) and parasympathetic (PNS). A general overview of ANS control over HR/HRV was detailed in Chapter One (Section 1.3.3). Importantly, recall the CAN proposed by Thayer & Lane in their Neurovisceral Integration Theory and its major structures, including the prefrontal cortex (PFC), anterior insular cortex (AIC), anterior cingulate cortex (ACC), central nucleus of the amygdala (CeA), thalamus, hypothalamus, and brainstem nuclei--particularly the nucleus ambiguous (NA), dorsal motor nucleus of X (DMNX), and the nucleus of the solitary tract (NTS).

Many of these aforementioned brain structures have been implicated not only in alcohol misuse and anxiety disorders, but also identified as sites of sexually dimorphic variation in ANS control (Williams et al., 2019; Williams et al., 2022). For example, males, on average, have a larger amygdala and insula and women have a larger prefrontal cortex (Ruigrok et al., 2014). Functionally, one group recently showed increased amygdala activity was associated with increases in vagally mediated HRV in women but no relationship in men during challenge (Nugent et al., 2011). In HR/HRV studies, females display higher HR and lower HRV (Koenig & Thayer, 2016; Williams et al., 2022). From the perspective of neurovisceral integration theory, this is logical. For instance, considering the PFC activity as an index of vagally mediated HRV, it follows that if women have, on average, a larger PFC they would also display greater vagally mediated HRV. Furthermore, the amygdala is an important initiator of the sympathetic “fight or flight” response, and thus it follows that if males have a larger amygdala, they may also possess greater SNS drive. Further, if females tend to utilize PNS to engage with challenge more, then it follows that female amygdala decreases that were associated with a brief challenge were also associated with increases in vagally mediated HRV (Nugent et al., 2011).

Additional behavioral studies also implicate important sex differences in ANS regulatory behavior. For instance, one study found that while lower resting HRV was associated with decreased self-reported emotional regulatory ability in both sexes, female HRV had a stronger relationship to diminished emotional regulatory ability (Williams et al., 2019). Said another way, at higher HRVs, women report reasonable emotional regulatory capacity similar to men, but as HRV decreases, women report more difficulty with emotional regulation compared to men. Another study found that in females, but not males, who reported higher stress levels also reported higher perceived social support if their HRV was high (Kvadsheim et al., 2022). These data support the idea that HRV moderates the relationship between stress and coping differently in males versus females and concords with the observation that females typically cope with stressors in more socially geared ways compared to men (Taylor et al., 2000; Blanchard et al., 2001). From the perspective of neurocircuitry, this behavior likely mediated through enhanced PFC signaling in women compared to men since HRV is a validated index of vagally mediated autonomic control (Williams et al., 2019; Williams et al., 2022).

The anterior insula (AIC) is an important mediator of interoception, or the interpretation of bodily feelings into conscious awareness. The AIC is also implicated in the CAN, which is involved in ANS activation. Indeed, the AIC has been implicated as a critical center of top-down cardiac control (for review, see Oppenheimer & Cechetto, 2016). In rats, inhibition of the AIC during restraint stress blocks typically elicited increases in HR and blood pressure (Alves et al., 2014; Alves et al., 2010), and has also been implicated in PNS related changes (Alves et al., 2009). The AIC appears to be involved in both PNS and SNS regulation, perhaps where activation of one versus the other are intermingled within this structure (Oppenheimer et al., 1992). Relating to the experience of negative affect, greater insula to amygdala connection is associated in women, but not in men (Mak et al., 2009). Work from our lab has implicated anterior insula circuits as critical to overcome aversion, where it is necessary for compulsion-like alcohol drinking but not for alcohol-only drinking (Seif et al., 2013; De Oliveira Sergio et al., 2021). Thus, since the AIC is implicated in ANS regulation and important behavioral adaptations to challenge, it would be useful for future studies to examine this relationship in more detail.

Future studies utilizing HR/HRV telemetry in both sexes during alcohol-related and anxiety-related behaviors that also examine brain circuits is a critical next step both to validate and extend the findings within this dissertation. Sex differences in HR/HRV exist at baseline and during challenge, and these differences appear to be related to sexual dimorphism in brain circuits. However, there is still a considerable lack of research into how these brain circuits relate to HR/HRV and subsequently influence behavior. From the extant literature, it follows that inhibition of PFC-AIC circuits would likely preferentially affect female behavior and increase SNS. In males, inhibition of this circuit may still increase SNS considered through the lens of Jacksonian Dissolution. However, these increase in SNS may be less pronounced in males due to females extended reliance on the PFC to modulate ANS activity. Further, in males the AIC-CeA circuit would be more critical to ANS regulation compared to females, where inhibition of this circuit likely would prevent male responding and may increase female responding.

4.5 Conclusions

To conclude, the studies herein highlight an important and understudied facet of human behavior and pathology: the role of the autonomic nervous system in influencing behavior. A preponderance of evidence suggests that males and females utilize the ANS in subtly differing ways to adapt to the environment, and that these differences are likely related to differing evolutionary demands between sexes historically. Males typically utilize SNS whereas females typically utilize PNS. These differences may in fact be "hard-wired" such that the differential biologic activity of the sex hormones influences the neurotransmitters involved in generating heart rate and heart rate variability in addition to observed differences in brain morphology and circuits between the sexes. Heart rate variability (HRV) reflects ANS influence, such that higher HRV typically reflects a predominance of parasympathetic influence and denotes greater self-regulatory capacity and social openness. Low HRV is associated with higher all-cause mortality and many disabilities (including those in psychiatry), and likely reflects a locked-in state where an individual is under maladaptively enhanced sympathetic influence. HRV is simple and inexpensive to calculate in humans, and thus represents a promising biomarker for general ANS activity and function. Thus, it is critical that preclinical work in rodents is utilized to parse out the mechanisms by which this occurs, such that mechanistic insights can impart new knowledge regarding the abatement of human suffering related to ANS dysfunction. The studies that comprise this dissertation (1) set up and validated the use of a preclinical model that will be used for these purposes and (2) revealed novel information replicating and extending our current understanding of functional implications of HR/HRV changes related to specific aspects of anxiety-like and alcohol drinking behavior that are likely translationally relevant to humans. It is my hope that this dissertation is convincing in the notion that HRV is interesting and valuable to investigate as a biomarker in health and disease.

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Emotion Regulation and Heart Rate Variability: The Salient Role of Not Accepting Negative Emotions. *Front Psychol* 8. 328.

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CURRICULUM VITAE

Raizel Michele Frasier

Education

- Bachelor of Arts (BA), Psychology, *Magna cum laude*
  - Ithaca College, Ithaca, NY
  - Minors in Neuroscience and Biology
  - August 2011 – May 2015
- Doctor of Philosophy (PhD) in Medical Neuroscience
  - Indiana University-Purdue University Indianapolis, Indianapolis, IN
  - July 2020 – February 2024
- Doctor of Medicine (MD)
  - Indiana University School of Medicine (IUSM), Indianapolis, IN
  - June 2018 – May 2026 (Expected)

Professional Experience

- Medical Scientist Training Program; 2018 – 2026 (Expected)
  - Indiana University School of Medicine (IUSM), Indianapolis, IN
  - Mentors: Benjamin Gaston, MD; Brittney-Shea Hebert, PhD; Raghu Mirmira, MD, PhD; Maureen Harrington, PhD
- Graduate Student in Medical Neuroscience; 2020 – 2024
  - Indiana University-Purdue University Indianapolis, Indianapolis, IN
  - Mentor: Woody Hopf, PhD
- Research Technician; 2015 – 2018
  - University of Pennsylvania, Philadelphia, PA

- Mentors: Virginia Lee, PhD and John Q. Trojanowski, MD, PhD

- Undergraduate Research Assistant; 2012 – 2015
    - Ithaca College, Ithaca, NY
    - Mentors: Jean Hardwick, PhD; Elizabeth Caldwell, PhD; Brandy Bessette-Symons, PhD

Peer-Reviewed Publications

Doctoral work:

- **Frasier, R.M.**, De Oliveira Sergio T., Starski P.A., Grippo, A.J., and Hopf, F.W. (2023). Heart rate variability measures indicating sex differences in autonomic regulation during anxiety-like behavior in rats. *Frontiers in Psychiatry*. 14:1244389. https://doi.org/10.3389/fpsyt.2023.1244389
- **Frasier, R.M.**, Starski, P.A., De Oliveira Sergio, T., Grippo, A.J., Hopf, F.W. (2024) Sex differences in heart rate variability measures that predict alcohol drinking in rats. *Addiction Biology* [In Review]
- De Oliveira Sergio, T., **Frasier, R.M.**, Hopf, F.W. (2023). Animal models of compulsion alcohol drinking: Why we love quinine-resistant intake and what have we learned from it? *Frontiers in Psychiatry*. 14:1116901. https://doi.org/10.3389/fpsyt.2023.1116901.
- Starski, P.A, Morningstar, M. Katner, S., **Frasier, R.M.**, De Oliveira Sergio, T., Wean, S., Lapish, C., Hopf, F. W. (2024). Neural activity in the anterior insula at drinking onset and licking relates to compulsion-like alcohol consumption. *J. Neurosci*. e1490232023. https://doi.org/10.1523/jneurosci.1490-23.2023.

- Radke, A.K., Sneddon, E.A., **Frasier, R.M.**, Hopf, F.W. (2021). Recent Perspectives on Sex Differences in Compulsion-Like and Binge Alcohol Drinking. *International Journal of Molecular Sciences*, *22*(7), 3788. https://doi.org/10.3390/ijms22073788

Pre-Doctoral work:

- Henderson, M. X., Covell, D. J., Chung, C. H.-Y., Pitkin, R. M., **Sandler, R. M**., Decker, S. C., … Luk, K. C. (2019). Characterization of novel conformation-selective α-synuclein antibodies as potential immunotherapeutic agents for Parkinson's disease. *Neurobiology of Disease*, 104712. https://doi.org/10.1016/j.nbd.2019.104712
- Henderson, M.X., Cornblath, E.J., Darwich, A., Zhang, B., Brown, H., Gathagan, R.J., **Sandler, R.M**., Bassett, D.S., Trojanowski, J.Q., Lee, V.M.Y. (2019). Spread of α-synuclein pathology through the brain connectome is modulated by selective vulnerability and predicted by network analysis. *Nature Neuroscience*, *22*(8), 1248. https://doi.org/10.1038/s41593-019-0457-5
- Karpowicz, R.J., Haney, C.M., Mihaila, T.S., **Sandler, R.M.**, Petersson, E.J., Lee, V.M.-Y. (2017). Selective imaging of internalized proteopathic α-synuclein seeds in primary neurons reveals mechanistic insight into transmission of synucleinopathies. *The Journal of Biological Chemistry*, *292*(32), 13482–13497. https://doi.org/10.1074/jbc.M117.780296

Conference Posters and Oral Presentations

Doctoral work:

- **<u>Frasier, R.M.,</u>** De Oliveira Sergio, T., Starski, P.A., Hopf, F.W. "Heart rate variability reveals sex differences in autonomic regulation of voluntary alcohol drinking in male and female rats." Gordon Research Conference on Alcohol and the Nervous System, Galveston, TX, February 15th, 2024. *Poster & Oral Presentation*
- **<u>Frasier, R.M.,</u>** De Oliveira Sergio, T., Starski, P.A., Hopf, F.W. "Heart rate variability measures indicating sex differences in autonomic regulation during anxiety-like behavior in rats." Society for Neuroscience Annual Meeting, Washington, D.C., November 12th, 2023. *Poster*
- **<u>Frasier, R.M</u>**. "Follow your heart: Heart rate variability reveals sex differences in autonomic regulation during anxiety-like behavior in rats." Stark Summer Science Symposium, Stark Neurosciences Research Institute, Indianapolis, IN, July 25th, 2023. *Oral Presentation*
- **<u>Frasier, R.M.,</u>** De Oliveira Sergio, T., Starski, P.A., Hopf, F.W. "Heart rate variability reveals sex differences in autonomic regulation of voluntary alcohol drinking in male and female rats." Research Society on Alcohol Annual Meeting, Bellevue, WA, June 26th, 2023. *Poster*
- **<u>Frasier, R.M.,</u>** De Oliveira Sergio, T., Starski, P.A., Hopf, F.W. "Heart rate variability reveals sex differences in autonomic regulation of voluntary alcohol drinking in male and female rats." Big10 Research Conference Annual Meeting, Indianapolis, IN, June 15th, 2023. *Poster*

- **Frasier, R.M**. "Follow your heart: Heart rate variability reveals sex differences in autonomic regulation of voluntary alcohol drinking in rats." Women in Neuroscience Career Development Award Luncheon, Stark Neurosciences Research Institute, Indianapolis, IN, May 17th, 2023. *Oral Presentation*
- **Frasier, R.M.,** De Oliveira Sergio, T., Starski, P.A., Hopf, F.W. Follow your heart: The relation of autonomic nervous system function, measured through heart rate variability, in promoting alcohol drinking and anxiety-like behavior in rats. Society for Neuroscience Annual Meeting, San Diego, CA, November 13th, 2022. *Poster*
- **Frasier, R.M**. "Follow your heart: the relation of autonomic nervous system function, measured through heart rate variability, in promoting alcohol drinking and anxiety-like behavior." Stark Summer Science Symposium, Stark Neurosciences Research Institute, Indianapolis, IN, July 26th, 2022. *Oral Presentation*
- **Frasier, R.M**. "Follow your heart: the relation of autonomic nervous system function, measured through heart rate variability, in promoting alcohol drinking and anxiety-like behavior." Indiana University Medical Scientist Training Program Lunch Seminar Series, Indianapolis, IN, April 28th, 2022. *Oral Presentation*

Pre-Doctoral work:

- **Sandler, R.M**., Sisler, E. K., Hadley, E. "My birth control is making me depressed," balancing potential mood effects of hormonal contraception with a patient's contraceptive goals. American Medical Women's Association 106th Annual Meeting (virtual), March 2021. *Poster*

- Roesler, A., Christodoulides, A., **Sandler, R. M**., Petrucciani, A., Allen, D. A complex diagnosis of Tuberculous meningitis. American Medical Women's Association 105th Annual Meeting (virtual), March 2020, *Poster*
- **Sandler, R.M.,** Gray, J.H., B. A. Bessette-Symons. The influence of time on emotional differences in memory. Eastern Colleges Sciences Conference 69th Annual Meeting, Buffalo, NY, April 2015. *Poster*

Leadership and Community Service

- 2021-2023: President, Combined Degree Student Counsel, IU MSTP
- 2020-Present: Executive Board, Psychiatry Student Interest Group, IUSM
- 2021-2024: Peer Mentor & Student Panel Volunteer, Medical Neuroscience PhD Program, IUSM
- 2022-Present: STEM Careers Mentor, Growth Through Guidance, IU MSTP
- 2018-Present: Medical Volunteer, Student Outreach Clinic, IUSM
- 2020-Present: Student Advocate for Mental Health, IUSM
- 2012-2018: Red Cross Certified Lifeguard and Swim Instructor
- 2012-2015: Peer Tutor (various courses), Ithaca College
- 2012-2015: Teaching Assistant (various courses), Ithaca College

Honors and Awards

- 2023: Women in Neuroscience Career Development Award
- 2022: Christopher Hrvoj Travel Award

- 2017: Editor’s Pick distinction for manuscript published in *Journal for Biological Chemistry*
- 2015: Tri-Beta National Biological Honor Society
- 2015: Outstanding Poster Presentation, Eastern Colleges Sciences Conference
- 2014: Phi Kappa Phi Honor Society, *Ithaca College Presidential Scholar Nominee*
- 2013: Psi Chi International Honor Society in Psychology
- 2012: DANA Internship Grant for Summer Research at Ithaca College
- 2011-2015: Ithaca College Dean’s List
- 2011-2015: Ithaca College Presidential Merit Scholarship

Professional Affiliations

- Society for Neuroscience (SfN)
- Research Society on Alcohol (RSA)
- American Physician Scientists Association (APSA)
- American Psychiatric Association (APA)
- American Medical Association (AMA)
- American Medical Women’s Association (AMWA)