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Sex differences in heart rate variability measures that predict alcohol drinking in rats

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Abstract

Problem alcohol drinking continues to be a substantial cost and burden. In addition, alcohol consumption in women has increased in recent decades, and women can have greater alcohol problems and comorbidities. Thus, there is a significant need for novel therapeutics to enhance sex-specific, individualized treatment. Heart rate (HR) and HR variability (HRV) are of broad interest because they may be both biomarkers for and drivers of pathological states. HRV reflects the dynamic balance between sympathetic (SNS, 'fight or flight') and parasympathetic (PNS, 'rest and digest') systems. Evidence from human studies suggest PNS predominance in women and SNS in men during autonomic regulation, indicating the possibility of sex differences in risk factors and physiological drivers of problem drinking. To better understand the association between HRV sex differences and alcohol drinking, we examined whether alcohol consumption levels correlated with time domain HRV measures (SDNN and rMSSD) at baseline, at alcohol drinking onset, and across 10 min of drinking, in adult female and male Wistar rats. In particular, we compared both HRV and HR measures under alcohol-only and compulsion-like conditions (alcohol + 10 mg/L quinine), because compulsion can often be a significant barrier to treatment of alcohol misuse. Importantly, previous work supports the possibility that different HRV measures could be interpreted to reflect PNS versus SNS influences. Here, we show that females with higher putative PNS indicators at baseline and at drinking onset had greater alcohol consumption. In contrast, male intake levels related to increased potential SNS measures at drinking onset. Once alcohol was consumed, HR predicted intake level in females, perhaps a pharmacological effect of alcohol. However, HRV changes were greater during compulsion-like intake versus alcohol-only, suggesting HRV changes (reduced SNS in females, reduced PNS and increased HR in males) specifically related to aversion-resistant intake. We find novel and likely clinically relevant autonomic differences associated with biological sex and alcohol drinking, suggesting that different autonomic mechanisms may promote differing aspects of female and male alcohol consumption.

KEYWORDS
addiction, alcohol, autonomics, compulsion, heart rate variability, sex differences

1 | INTRODUCTION

Alcohol use disorder causes many medical and social harms[1–3] and has become a leading cause of death.[4] Importantly, excessive drinking in women has risen dramatically in recent years,[5,6] and women have higher risk for alcohol problems and higher comorbidity of alcohol use disorder and mood disorders.[7,8] Thus, it is of great importance to determine biological underpinnings of these observed sex differences to better treat problematic alcohol intake with a more personalized approach. Compulsive alcohol drinking, where consumption persists despite known negative consequences, can be a key driver of problem intake and is often a major obstacle to treatment.[9,10] 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.[11] 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 HR variability (HRV) in relation to voluntary alcohol drinking and biological sex. HR and HRV are of broad interest, because such measures may reflect important biomarkers[12,13] and contributors to many neuropsychiatric conditions including alcohol use disorder.[13–17] Indeed, basal HR or HRV[15,17–20] and HRV changes to alcohol stimuli[15,18,20–24] 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.[25] In addition, rodent alcohol drinkers show changes in HR related to alcohol reinforcement and seeking[26,27] and differing sensitivities towards rewarding aspects of alcohol.[28] 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.[13,14,17,29] HR, the number of beats per minute, is widely examined, because it is simple to assess and interpret. Considerable work has assessed how particular measures of HRV might reflect autonomic tone, and drugs targeting specific aspects of cardiac physiology have been especially helpful in elucidating this. In particular, beta-adrenergic receptors mediate SNS effects on the heart, whereas muscarinic acetylcholine receptors can mediate cardiac PNS, and studies in humans,[30,31] dogs[32,33] and rats[14,34] used such drugs to validate HRV indicators. Figure 1 displays the HRV measures we used in this study and their current putative meanings related to cardiac autonomic control. Here, we focus on two such HRV measures in the time domain (SDNN and rMSSD), which assess patterns across a series of interbeat intervals (the amount of time between successive heart beats, abbreviated IBI). Further explanation of the calculations and their usages is detailed in the Section 2.

Altogether, HRV measures have the potential to provide useful indicators for different aspects of autonomic tone at the level of the heart and offer an opportunity to delve deeper into the realm of sex differences in neurocardiac autonomic regulation. Many studies have revealed that women have higher HR than men under several conditions,[35–38] including heavy drinkers.[39] In addition, females can display greater basal HRV than males in humans[35–38] and in rats.[40,41] In addition, several studies find sex differences in autonomic markers evident during challenge, especially those suggesting that women utilize the PNS more, whereas men engage the SNS more.[29,35,38,42,43] HRV-related brain circuits also differ in relation to biological sex.[38,44–47] 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[48,49]) and across the drinking period. We then related these HRV measures to alcohol consumption levels

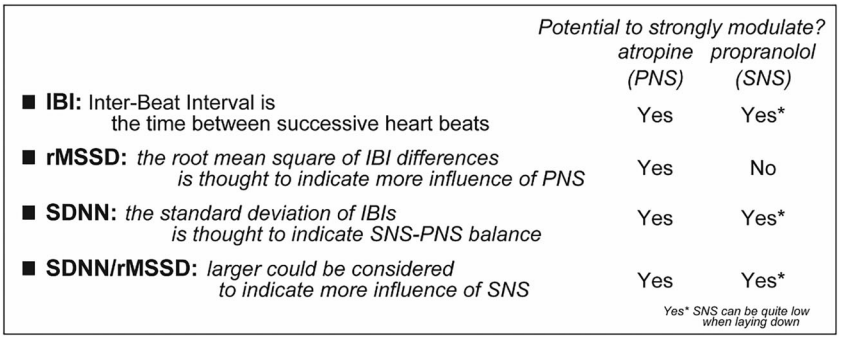

FIGURE 1 Description of different heart rate variability (HRV) measures and how they relate to parasympathetic nervous system (PNS) and/or sympathetic nervous system (SNS). In particular, some effects of pharmacological agents are considered to alter the impact of SNS, through beta-adrenergic receptors (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).

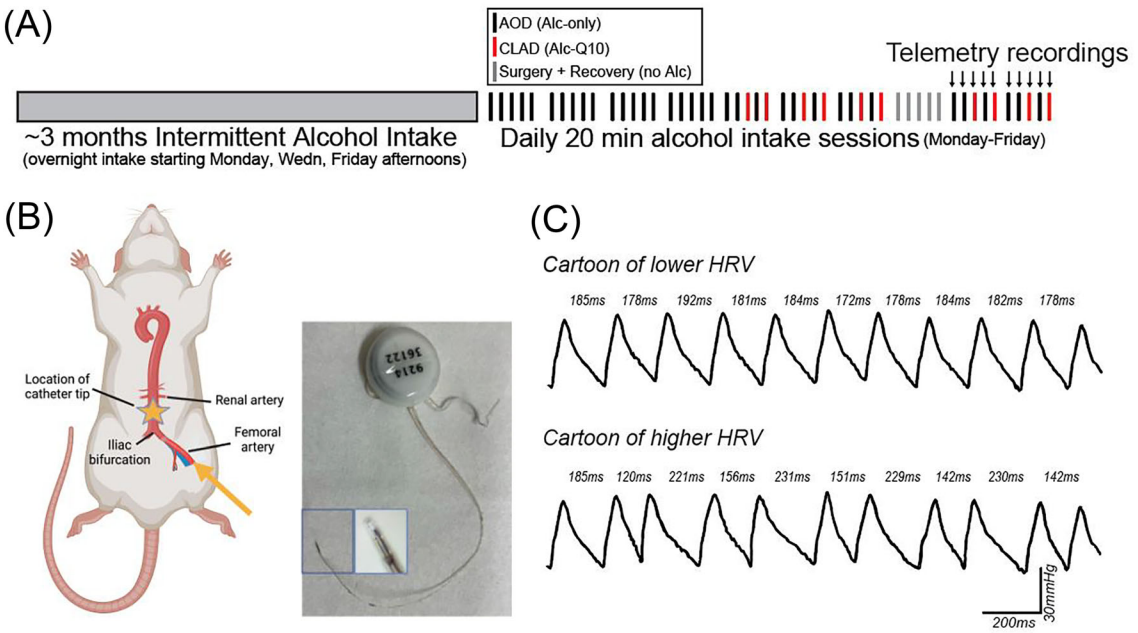

FIGURE 2 (A) Schematic of drinking schedule. (B) Schematic of surgical device and implantation. (C) Cartoon example traces showing lower heart rate variability (HRV, top) versus higher HRV (bottom). Time between each pair of heart beats is shown.

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 behavioural studies[50,51] 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 neurocardiac autonomic regulation that are novel and likely translationally relevant for the treatment of problem drinking.

2 | METHODS

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 12-h reverse light cycle (lights off 8 AM). After 2 weeks, rats were initiated to alcohol drinking methods, which followed those we previously used.[50–53] Our rats drink alcohol for several months before utilization in studies to ensure escalation of drinking to levels comparable with humans in addition to allow for the development of compulsive-like alcohol drinking (CLAD, alcohol + 10 mg/L quinine). Thus, at the time of recording, rats were approximately 6–9 months of age. All drinking (including HRV testing) began ~0.5–2 h 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 16–24 h. Following ~3 months of intermittent access, rats were switched to drink under two-bottle choice for alcohol (20% v/v) or water for 20 min/day Monday–Friday (Figure 2A). After ~1 month of this 20 min/day intake, rats began drinking 20 min/day but with 2–3 alcohol-only days and 2–3 days of CLAD per week (in randomized order); we model CLAD here by adulterating 20% alcohol with 10 mg/L of the bitter-tasting quinine as done previously.[9,50,51,53] 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 to reduce variability by calculating average values across sessions from a given rat for a particular condition.

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 2B). Drinking studies began after 3–5 days of recovery. Telemetry surgery is detailed in the Supporting Information. Briefly, the telemetry device has a silicone elastomer transmitter (8.3 mm × 16.5 mm × 4 mm) 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 2B. 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. We set out to record telemetry during 3 alcohol-only and 3 CLAD sessions per rat. Data for each given drinking condition were then averaged for each rat, giving each rat a single value for alcohol-only and for CLAD.

2.3 | Alcohol drinking analysis

Alcohol consumption was determined through changes in bottle weight before and after drinking sessions, from which we determined g of ethanol per kg rat body weight. Spillage of alcohol is accounted for by measuring the alcohol bottle weight before and after a drinking session and then subtracting 0.2 g, which is the average amount of spillage determined from cages without rats. Rat body weights were taken weekly to ensure accurate measurement of g/kg alcohol consumption. The typical blood alcohol concentration under these levels of alcohol intake is ~40–50 mg%.[53]

2.4 | Analysis references for brief analysis windows

Raw 10-min recordings were visually inspected to remove artefacts and missed heartbeats, as done in other studies.[40,54–56] In addition, we note that on a technical level, shorter analysis periods increase variability, whereas longer HR traces give greater data and a more accurate HRV assessment. However, several studies find that briefer analysis windows can provide meaningful findings, and 5 min is often used for a briefer baseline for humans.[57] During different challenges in humans, a 60-s analysis window was as good as a 5-min window for SDNN, RMSSD and HR, although with 2 min or more needed for frequency-domain and more complex analyses.[57–60] Rodent studies have also validated the use of 1-min HRV analysis windows,[61] including for anxiety-like tests.[54] Thus, determining HRV measures in the first minute of drinking ('drinking onset') reflected a compromise between having the rat already drinking and including sufficient data to allow robust HRV assessment.

2.5 | HR and HRV analyses

We analysed HR/HRV data at baseline (prior to alcohol access), at drinking onset (first minute of alcohol access) and across 10-min of alcohol access. To meaningfully compare drinking data to baseline, the duration of recordings was matched, as HRV analyses can only be compared when they are matched for the same duration of time.[57] For example, the first minute of alcohol access was compared with the first minute of baseline recording, and the 10-min alcohol access recording was compared with 10 min of baseline recording. Although alcohol-only and CLAD recording sessions were the typical 20 min/day schedule, telemetry was recorded for the first 10 min of alcohol access. We did this for two reasons: (1) our rats overwhelmingly front-load their alcohol drinking during the first minutes of alcohol access. In addition, (2) we wanted to compare alcohol-related HRV patterns with cardiac measures during other 10-min-long behavioural tests occurring in our lab, such as anxiety-like models.[41,52] Importantly, to directly compare HRV values across behaviours, HRV indicators must be calculated across the same duration of time.[57] Rats were undisturbed during the recordings, as wireless telemetry removed the need to handle rats, and all drinking occurred in the homecage.

Blood pressure traces were recorded and visualized using NOTOCORD-hem software (Instem, Staffordshire, UK) at 250 Hz, with pressure fluctuations related to each heartbeat (cartoon illustration: Figure 2C). Baseline data were recorded in the homecage ~0.2–1 h before alcohol access. Raw recordings were visually inspected to remove artefacts, missed heartbeats and ectopic heartbeats, as is typically done in other studies.[40,54,56] 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 IBIs (in msec) across a given session. We then used IBIs to determine time domain HRV measures, including (1) SDNN, the standard deviation of the IBIs across normal heartbeats ('N-N'), reflecting SNS and PNS influences on the heart; and (2) rMSSD, the root mean square of the successive differences in IBI, an indicator of PNS influence at the heart (see Figure 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. rMSSD is altered by atropine but not propranolol and is considered to primarily reflect PNS contributions to the heart. SDNN is impacted by both atropine and propranolol and is widely considered to reflect the influence of both SNS and PNS actions on HRV.[13,14,17,29,35,62] Finally, we determined SDNN/rMSSD ratio, which is taken to indicate the relative strength of SNS contributions, because this ratio is effectively [SNS influence + PNS influence]/[PNS influence]. Importantly, recent human work[36] has indicated the critical importance of not adjusting HRV measures for HR, because this can result in missing important sex differences. Figure 2C shows an example cartoon of higher HRV versus lower HRV traces, and Figure 1 summarizes how different HRV measures are considered to relate to PNS and/or SNS.

2.6 | Animal groups

Figures 3 and 6 used data from what we call Cohort A (female $n = 9$ alcohol-only, $n = 7$ CLAD; male $n = 12$ alcohol-only, $n = 10$ CLAD), which were tested both at baseline and during drinking. Figures 4 and 7 used data combining Cohort A rats with additional (Cohort B) rats that had HRV testing just during alcohol drinking (Cohorts A + B: female $n = 16$ alcohol-only, $n = 10$ CLAD, male $n = 18$ alcohol-only, $n = 12$ CLAD). Finally, Figure 5 (showing variability in HRV measures even with change in HR) combined alcohol-only and CLAD drinking sessions for Cohorts A + B data.

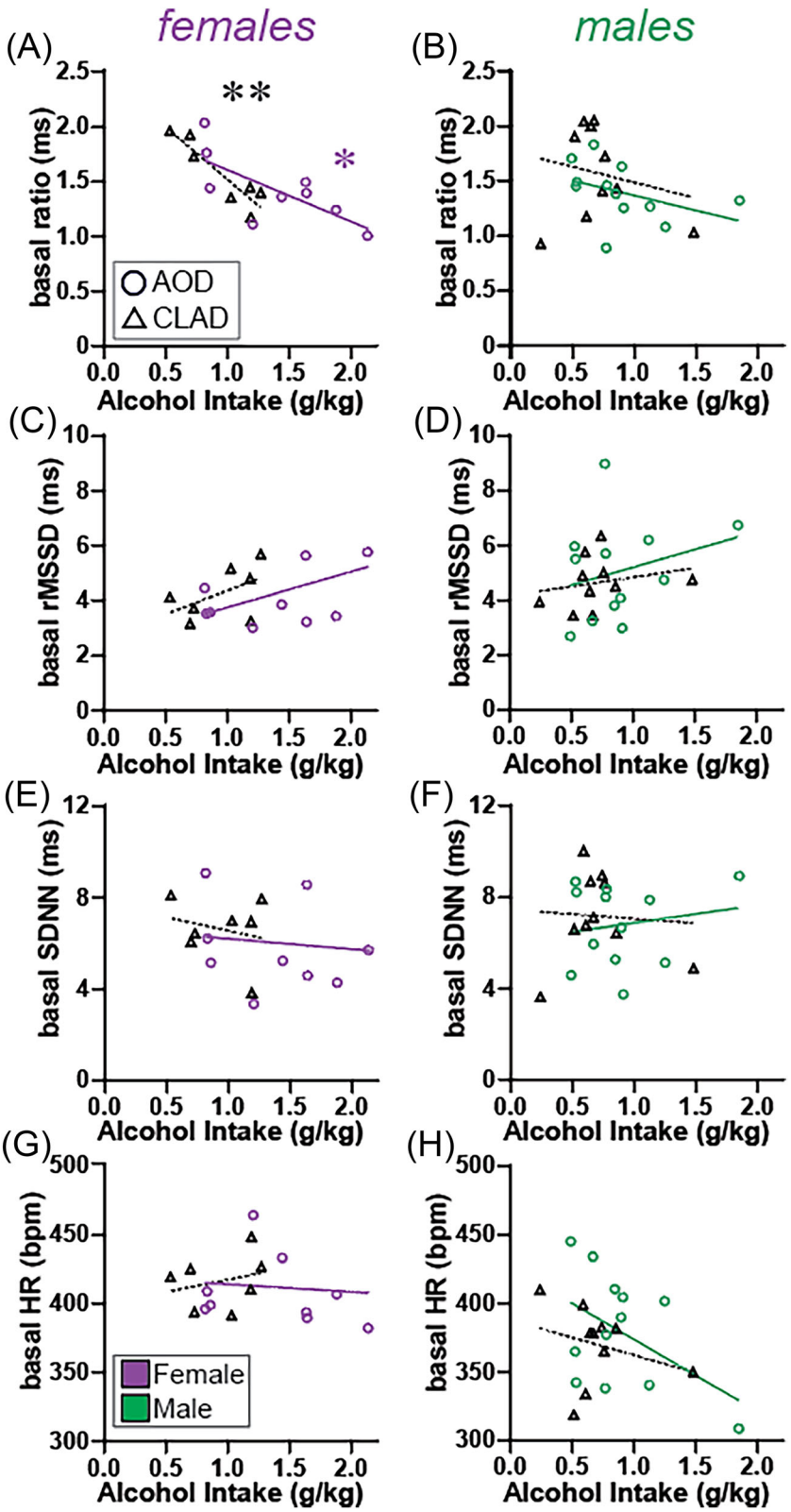

FIGURE 3 Basal parasympathetic nervous system (PNS) influence predicted female but not male drinking. (A,B) In (A) females ($n = 9$ alcohol-only; $n = 7$ CLAD) but (B) not males ($n = 12$ alcohol-only; $n = 10$ CLAD), lower SDNN/rMSSD ratio was related to greater intake levels for both alcohol-only and 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.

2.7 | 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.

Most non-significant statistics are given in Table 1.

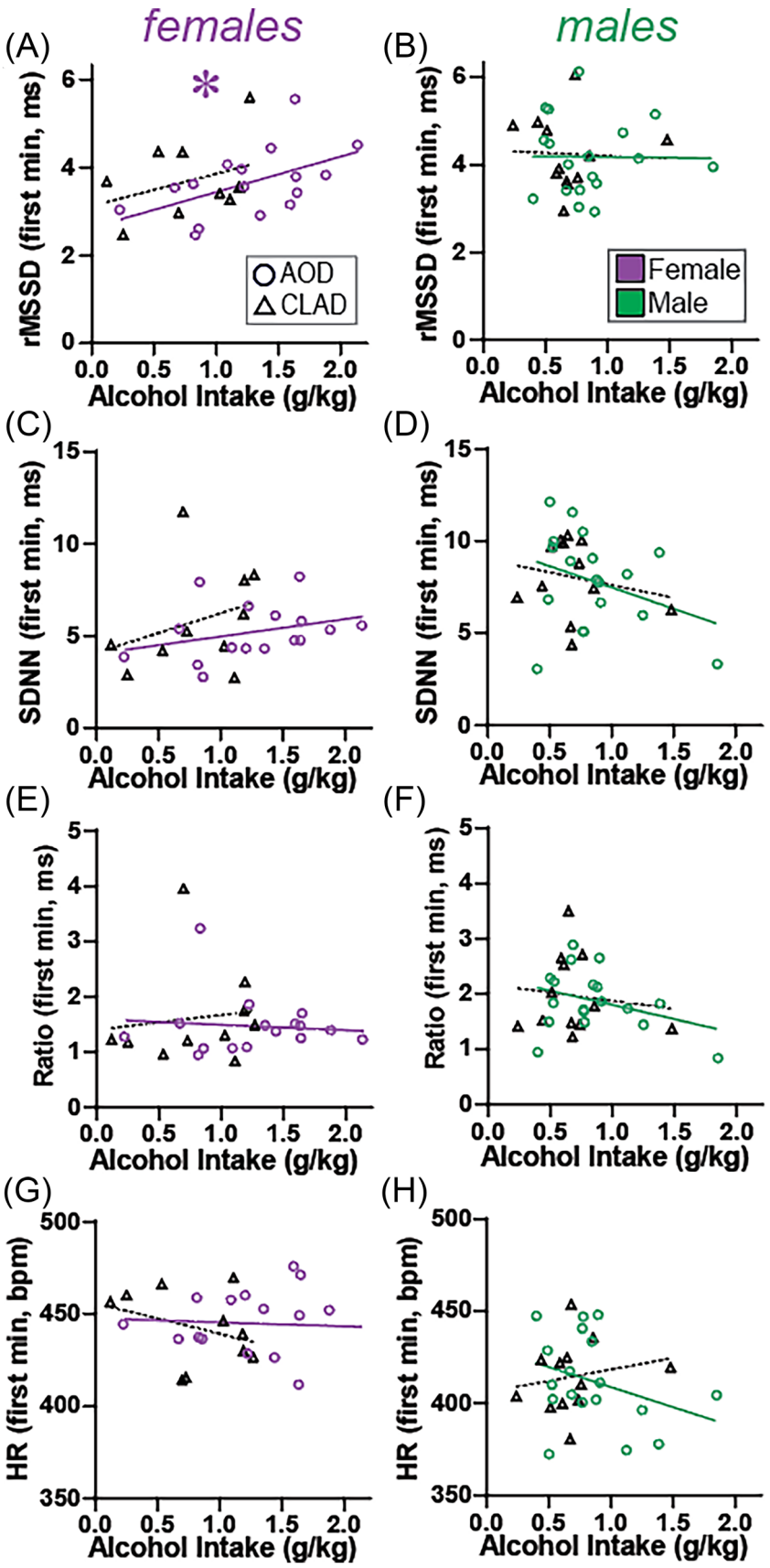

FIGURE 4 Greater parasympathetic nervous system (PNS) influence in the first minute of access to alcohol predicted greater female drinking. Female $n = 16$ alcohol-only, $n = 10$ CLAD, male $n = 18$ alcohol-only, $n = 12$ compulsive-like alcohol drinking [CLAD]). (A,B) In (A) females but (B) not males, 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 ratio or (G,H) heart rate (HR) at drinking onset, in either sex. *$p < 0.05$. Graph data female/male colouring as in fig. 3.3.

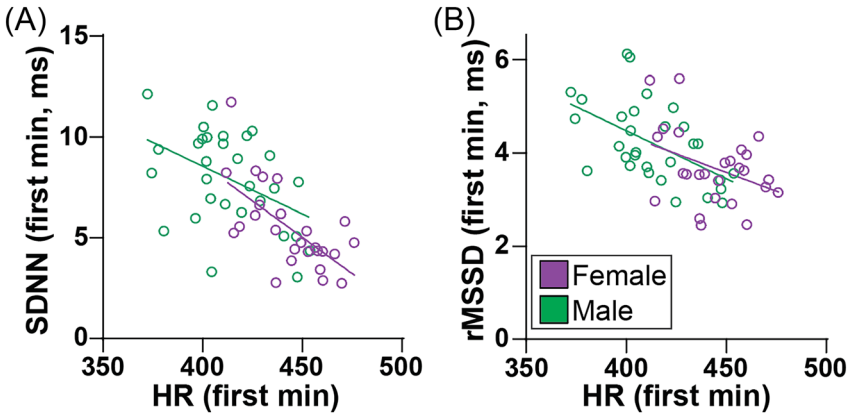

FIGURE 5 Dynamic range for heart rate variability (HRV) differences even with higher HR. While average (A) SDNN and (B) rMSSD values were lower as HR increased (A, females: $F_{(1,24)} = 17.89$, $p = 0.0003$, $R^2 = 0.4271$; males: $F_{(1,28)} = 6.943$, $p = 0.0136$, $R^2 = 0.1987$; B, females: $F_{(1,24)} = 3.969$, $p = 0.0578$, $R^2 = 0.1419$; males: $F_{(1,28)} = 11.52$, $p = 0.0021$, $R^2 = 0.2916$), there was still variability and thus dynamic range within HRV measures. Female $n = 26$, male $n = 30$.

TABLE 1 Most non-significant statistics for correlation of drinking level with the particular HR/HRV measure. 'alc.-only' alcohol-only; 'initial' HR/HRV measure in first minute of drinking onset.

Figure	Sex	HR/HRV measure	
Figure 3C	Females	Basal rMSSD	alc-only: $F_{(1,7)} = 3.046$, $R^2 = 0.3032$, $p = 0.1244$; CLAD: $F_{(1,5)} = 1.781$, $R^2 = 0.2627$, $p = 0.2395$
Figure 3D	Males	Basal rMSSD	alc-only: $F_{(1,10)} = 0.797$, $R^2 = 0.0738$, $p = 0.3930$; CLAD: $F_{(1,8)} = 0.449$, $R^2 = 0.0532$, $p = 0.5215$
Figure 3E	Females	Basal SDNN	alc-only: $F_{(1,7)} = 0.0928$, $R^2 = 0.0131$, $p = 0.7695$; CLAD: $F_{(1,5)} = 0.2919$, $R^2 = 0.0552$, $p = 0.6122$
Figure 3F	Males	Basal SDNN	alc-only: $F_{(1,10)} = 0.286$, $R^2 = 0.0278$, $p = 0.6047$; CLAD: $F_{(1,8)} = 0.034$, $R^2 = 0.0042$, $p = 0.8587$
Figure 3G	Females	Basal HR	alc-only: $F_{(1,7)} = 0.0927$, $R^2 = 0.0131$, $p = 0.7696$; CLAD: $F_{(1,5)} = 0.3664$, $R^2 = 0.0683$, $p = 0.5714$
Figure 3H	Males	Basal HR	alc-only: $F_{(1,10)} = 2.967$, $R^2 = 0.2289$, $p = 0.1157$; CLAD: $F_{(1,8)} = 0.709$, $R^2 = 0.0814$, $p = 0.4243$
Figure 4B	Males	Initial rMSSD	alc-only: $F_{(1,16)} = 0.003$, $R^2 = 0.0002$, $p = 0.9593$; CLAD: $F_{(1,10)} = 0.020$, $R^2 = 0.0020$, $p = 0.8894$)
Figure 4C	Females	Initial SDNN	alc-only: $F_{(1,14)} = 1.555$, $R^2 = 0.1000$, $p = 0.2329$; CLAD: $F_{(1,8)} = 0.884$, $R^2 = 0.0995$, $p = 0.3746$)
Figure 4D	Males	Initial SDNN	alc-only: $F_{(1,16)} = 1.866$, $R^2 = 0.1044$, $p = 0.1908$; CLAD: $F_{(1,10)} = 0.430$, $R^2 = 0.0413$, $p = 0.5266$
Figure 4E	Females	Initial SDNN/rMSSD	alc-only: $F_{(1,14)} = 0.121$, $R^2 = 0.0086$, $p = 0.7330$; CLAD: $F_{(1,8)} = 0.1129$, $R^2 = 0.0139$, $p = 0.7455$
Figure 4F	Males	Initial SDNN/rMSSD	alc-only: $F_{(1,16)} = 2.117$, $R^2 = 0.1168$, $p = 0.1650$; CLAD: $F_{(1,10)} = 0.154$, $R^2 = 0.0152$, $p = 0.7027$
Figure 4G	Females	Initial HR	alc-only: $F_{(1,14)} = 0.049$, $R^2 = 0.0035$, $p = 0.8277$; CLAD: $F_{(1,8)} = 0.986$, $R^2 = 0.1097$, $p = 0.3498$
Figure 4H	Males	Initial HR	alc-only: $F_{(1,16)} = 1.884$, $R^2 = 0.1054$, $p = 0.1888$; CLAD: $F_{(1,10)} = 0.381$, $R^2 = 0.0367$, $p = 0.5510$
Figure 7D	Males	$SDNN_{10min}$	alc-only: $F_{(1,16)} = 0.441$, $R^2 = 0.0268$, $p = 0.5162$; CLAD: $F_{(1,10)} = 0.907$, $R^2 = 0.0832$, $p = 0.3633$)
Figure 7F	Males	$SDNN/rMSSD_{10min}$	alc-only: $F_{(1,16)} = 0.758$, $R^2 = 0.0453$, $p = 0.3967$; CLAD: $F_{(1,10)} = 0.002$, $R^2 = 0.0002$, $p = 0.9673$

Abbreviations: CLAD, compulsive-like alcohol drinking; HR, heart rate; HRV, heart rate variability.

3 | RESULTS

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, both for the alcohol-only condition and for CLAD condition. Basal HRV can predict drinking problems in humans,[15,17] and such findings would suggest that individuals can have a tonic neurocardiac autonomic state that impacts their behaviour. These first findings were determined from Cohort A (female $n = 9$ alcohol-only, $n = 7$ CLAD; male $n = 12$ alcohol-only, $n = 10$ CLAD), which were tested both at baseline and during drinking.

Interestingly, the basal SDNN/rMSSD ratio predicted subsequent alcohol drinking in females but not in males. In particular, females with lower SDNN/rMSSD at baseline went on to drink significantly more alcohol (Figure 3A), 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 3B; 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 cardiac 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 a result of increased PNS (suggested by greater rMSSD) or decreased SNS (possibly indicated by reduced SDNN or SDNN/rMSSD). However, there was no significant relation between female basal rMSSD and intake for alcohol-only (Figure 3C) or CLAD (Figure 3C) and also no correlations in males (Figure 3D). Further, there was no relation between female basal SDNN and intake for alcohol-only (Figure 3E) or CLAD (Figure 3E) or in males (Figure 3F). Also, drinking level did not correlate with basal HR in females (Figure 3G) or males (Figure 3H). Thus, while basal SDNN and rMSSD measures were not associated with subsequent drinking, a greater change in basal SDNN/rMSSD (suggesting, along with no change in rMSSD, of a nuanced shift in PNS-SNS tone) did predict higher alcohol drinking in females only.

3.2 | Relation of drinking levels to HR/HRV measures at drinking onset

HRV changes to alcohol stimuli can predict alcohol problems in humans.[15,21] In rats, drinking onset is an important alcohol stimulus, because this is when significant behavioural changes occur, for example, shifts in response patterns under alcohol-only versus CLAD conditions.[50,51] Thus, we tested whether a given HRV measure at drinking onset (the first minute of alcohol access) was related to subsequent alcohol consumption levels. This was determined from combining Cohort A rats with additional (Cohort B) rats that had HRV testing just during alcohol drinking (Cohorts A + B 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 4A; $F_{(1,14)} = 5.141$, $R^2 = 0.2686$, $p = 0.0397$). CLAD had a similar pattern but was not significant (Figure 4A; $F_{(1,8)} = 1.090$, $R^2 = 0.1199$, $p = 0.3270$). Also, initial rMSSD was not correlated with drinking level for alcohol-only or CLAD in males (Figure 4B). These results suggest that greater cardiac 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 4C) or SDNN/rMSSD (Figure 4E). Thus, females with higher PNS, as indicated by higher rMSSD, at drinking onset, may have had greater alcohol-only intake.

Male intake was not associated with drinking-onset SDNN (Figure 4D) or SDNN/rMSSD (Figure 4F). Finally, there was no relation between drinking level and drinking-onset HR in females (Figure 4G) or males (Figure 4H). Thus, in males, no cardiac measure we examined during the first minute of drinking was related to alcohol consumption level in either alcohol-only or CLAD conditions.

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 5A) and rMSSD (Figure 5B) showed spread relative to HR in females and males, indicating dynamic range to observe HRV shifts ($n = 26$ female; $n = 30$ male, same data as Figure 4). This agrees with human studies where women show higher HRV even with greater HR[36] and rodent studies replicating a similar pattern by sex.[40,41]

3.3 | Relation of drinking levels to changes in HR/HRV measures from baseline to drinking onset

Because HRV changes to alcohol stimuli can relate to alcohol problems in humans, we also examined the change in each HR/HRV measure from the 1-min epoch of baseline to the first minute of access to alcohol (for these analyses, we combined alcohol-only and CLAD, same data as Figure 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 6A, Wilcoxon $p = 0.0190$) but not in females (Figure 6A, Wilcoxon $p = 0.7057$), with a significant difference in SDNN change between males and females (Mann–Whitney $p = 0.0309$). For rMSSD, males (Figure 6B, Wilcoxon $p = 0.0301$) but not females (Figure 6B, 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 6C, Wilcoxon $p = 0.0005$) but not females (Figure 6C, 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 6D, Wilcoxon $p < 0.0001$) and females (Figure 6D, Wilcoxon $p = 0.0063$) but with no sex difference (Mann–Whitney $p = 0.1712$). Together, these suggest the possibility that males shifted to greater cardiac SNS influence at the onset of

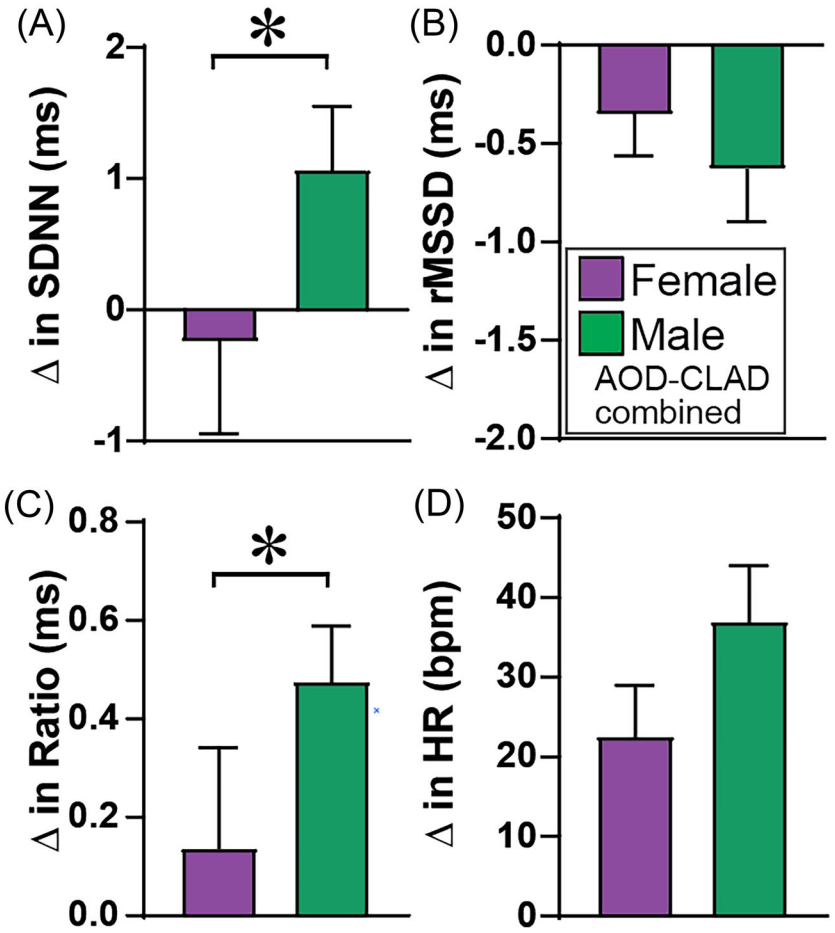

FIGURE 6 Changes in heart rate variability (HRV) measures from baseline to first minute access to alcohol. (A–C) Males showed a significant increase in (A) SDNN, (C) SDNN/rMSSD but not (B) rMSSD, at drinking onset versus basal. (D) Both sexes showed HR increases at drinking onset, with no sex differences. *$p < 0.05$ difference between females and males. Female $n = 16$, male $n = 22$; alcohol-only and compulsive-like alcohol drinking (CLAD) conditions combined.

drinking, and 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 (Figure S1). 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.

3.4 | HR/HRV measures assessed across the 10-min intake period

We also measured HR/HRV across 10 min of the intake period (female $n = 16$ alcohol-only, $n = 10$ CLAD, male $n = 18$ alcohol-only, $n = 12$ CLAD, same Cohorts A + B as in Figure 4). Such HRV patterns could reflect direct alcohol enhancement of HR and SNS influence[17,23] but potentially also reflect drive for alcohol. Interestingly, HR was correlated with intake level in females when examined across the 10-min drinking period. However, many HRV changes only related to drinking for CLAD, and not alcohol-only, suggesting the presence of sustained neurocardiac autonomic states that specifically promoted CLAD, which were different between females and males.

While $rMSSD_{10min}$ was not associated with female drinking levels (Figure 7A; 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 7B; $F_{(1,10)} = 5.967$, $R^2 = 0.3737$, $p = 0.0347$) but not alcohol-only ($F_{(1,16)} = 0.252$, $R^2 = 0.0155$, $p = 0.6226$) (and see the Supporting Information). Thus, these data suggest the possibility that males (but not females) with lower PNS influence (at the level of heart beat analysis) 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 7C; $F_{(1,8)} = 5.724$, $R^2 = 0.4171$, $p = 0.0437$) and $SDNN/rMSSD_{10min}$ (Figure 7E; $F_{(1,8)} = 7.206$, $R^2 = 0.4739$, $p = 0.0277$). However, female alcohol-only levels were not correlated with $SDNN_{10min}$ (Figure 7C; $F_{(1,14)} = 0.741$, $R^2 = 0.0503$, $p = 0.4037$) or $SDNN/rMSSD_{10min}$ (Figure 7E; $F_{(1,14)} = 1.087$, $R^2 = 0.0721$, $p = 0.3147$). Further, males showed no association for intake level and $SDNN_{10min}$ (Figure 7D) or $SDNN/rMSSD_{10min}$ (Figure 7F). Thus, these findings suggest that females (but not males) with lower SNS influence at the level of the heart drank more under compulsion-like conditions.

HR_{10min} was significantly and positively correlated with drinking level in females for both alcohol-only (Figure 7G; $F_{(1,14)} = 5.592$, $R^2 = 0.2854$, $p = 0.0330$) and CLAD (Figure 7G; $F_{(1,8)} = 8.495$, $R^2 = 0.5150$, $p = 0.0195$). Interestingly, in males, HR_{10min} only

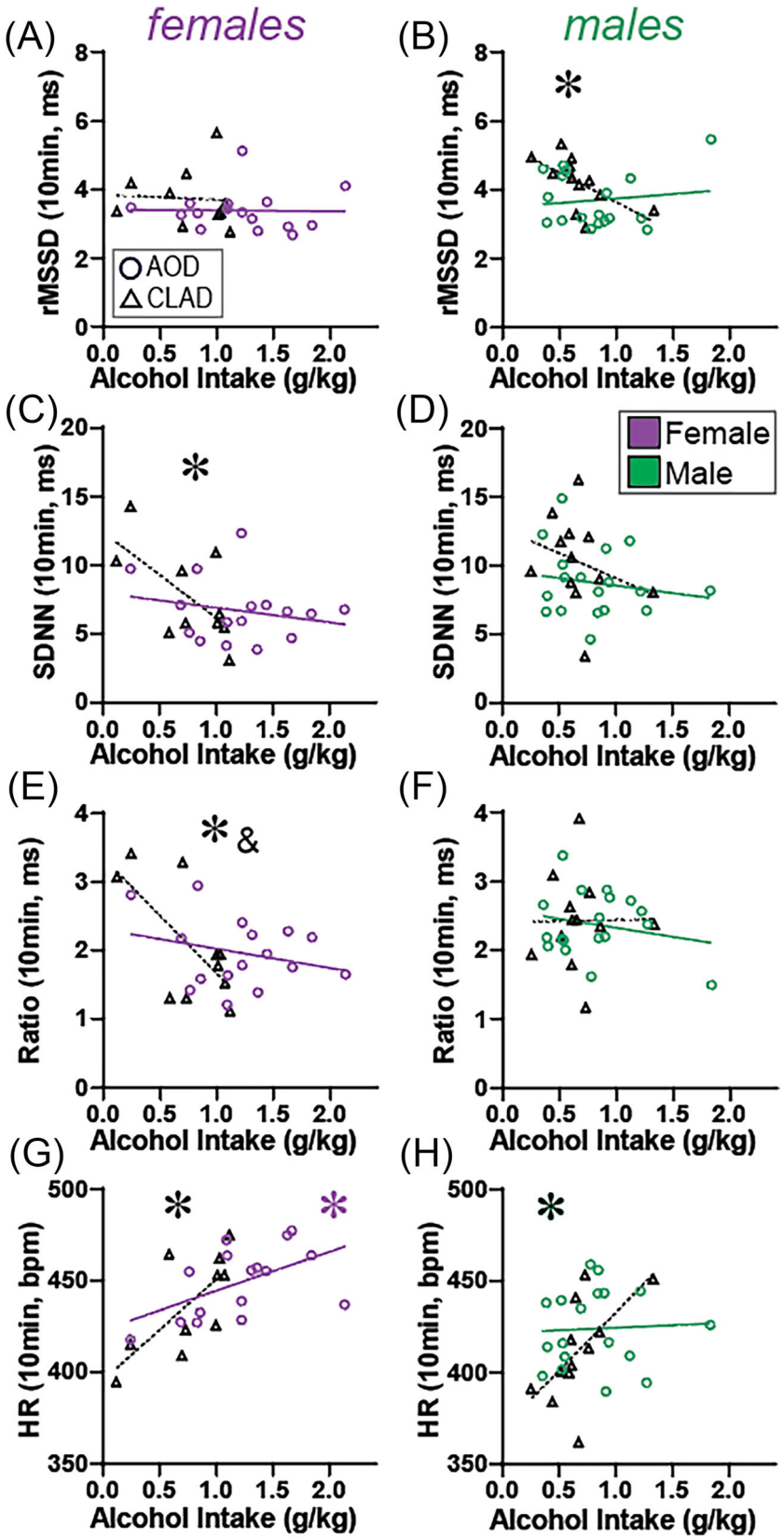

FIGURE 7 Across the 10-min drinking period, there were sex differences in heart rate variability (HRV) measures associated with higher compulsive-like alcohol drinking (CLAD) levels. Female $n = 16$ alcohol-only; $n = 10$ CLAD. Male $n = 18$ alcohol-only; $n = 12$ CLAD. (A) $rMSSD_{10min}$ was not correlated 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 $SDNN/rMSSD_{10min}$ ratio 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 colouring as in fig. 3.3.

correlated with CLAD intake (Figure 7H; $F_{(1,10)} = 5.698$, $R^2 = 0.3630$, $p = 0.0382$) but not alcohol-only (Figure 7H; $F_{(1,16)} = 0.037$, $R^2 = 0.0023$, $p = 0.8503$). 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.

4 | DISCUSSION

The present study sought to elucidate how neurocardiac autonomic states—indexed through HR and 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 min of the alcohol intake period (effects summarized in Figure 8). Importantly, we followed previous cross-species work (described in Section 2) that have suggested that certain HRV measures reflect more PNS versus SNS influence. Even with such important caveats, in females, greater alcohol intake could be predicted by higher relative cardiac PNS influence both at baseline (lower SDNN/rMSSD ratio) and at drinking onset (higher rMSSD). In contrast, male drinking was better predicted by cardiac 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 CLAD conditions more than alcohol-only, suggesting differences in response strategy during challenge that agree with previous work.

In females, higher HR over the 10-min 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 HRV markers suggesting lower 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 at the level of the heart. In contrast, females with indicators of lower 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 at the level of the heart. Considering consumption-related HRV patterns across the 10-min intake period were associated with CLAD rather than alcohol-only, these data are in agreement with previous work suggesting that these sustained HRV changes may be related to shifts in the cognitive-emotional action strategy used during CLAD.[9,10,50,51] Importantly, our results also suggest that, within the heart, 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 8) agree with human studies suggesting greater utilization of PNS in females and SNS in males during autonomic regulation.[29,35,38,42,43] Females with greater cardiac PNS influence at baseline and during drinking onset had greater drinking levels, whereas males showed an increase in cardiac SNS indicators at drinking onset. Across 10 min of CLAD intake, females with higher relative PNS (as a consequence of lower cardiac 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 cardiac 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 5A) and rMSSD (Figure 5B) 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.[36] While the mechanism for this apparent paradox is not yet clear, it has been

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

FIGURE 8 Summary of sex differences in the relation between heart rate variability (HRV) patterns and drinking. For females, greater parasympathetic nervous system (PNS) influence at baseline and during drinking onset predicted higher alcohol intake. Males had an increase in sympathetic nervous system (SNS) at drinking onset (put in brackets because increased SNS was observed but was not correlated with intake level). When assessed across the 10-min drinking session, HRV measures largely related to compulsive-like alcohol drinking (CLAD) but not alcohol-only. In females, lower SNS indicators, and thus greater SNS influence, predicted higher CLAD intake, whereas males with lower PNS influence, and thus greater SNS predominance and higher HR, had greater CLAD. '- -' indicates no relation between HRV and behavioural measures.

suggested that effects of oestrogen on cardiac chronotropy may play a role.[36] 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 fully understood.

We note that HRV is often assessed by other groups using spectral analysis of HRV fluctuations, where the high-frequency components (HFHRV, 0.75–2.5 Hz in rat) are considered to relate to PNS (like rMSSD), and the low-frequency components (LFHRV, 0.1–0.75 Hz in rat) can indicate PNS-SNS balance (like SDNN).[35,62] 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.[31,35,36] One study found reduced rMSSD in drinkers but no HFHRV changes, which they suggest might reflect different analysis methods from studies finding altered HFHRV.[63] Another study chose rMSSD for a large meta-analysis of sex differences in HRV.[35] Also, rMSSD often correlates highly with HFHRV and SDNN with LFHRV.[35,62] Thus, SDNN, rMSSD and their ratio have been broadly validated and considered useful to gain insight into PNS and SNS activity.

We chose to examine drinking onset because HR/HRV differences at this time would occur before alcohol would substantially enter the blood stream,[48,49] suggesting that autonomic measures here reflected physiological drivers of alcohol drinking instead of direct alcohol effects. It was interesting that autonomic changes within the heart at drinking onset occurred in both females and males, because HRV changes for alcohol stimuli can predict drinking problems in humans. For example, larger HFHRV (akin to rMSSD, a PNS measurement) increases to alcohol stimuli predict future relapse[21] and alcohol problems,[24] and HFHRV increases to negative images predicts higher craving.[18] Also, alcohol stimuli can increase both HFHRV and LFHRV.[22,64] High-risk drinkers show greater LFHRV changes to alcohol or emotional images (compared with low-risk drinkers),[20] and LFHRV changes to alcohol stimuli relate to drinking problems[15] and decreased ability to resist alcohol urges.[22] HRV increases in response to appetitive stimuli have been associated with greater regulatory effort[65] and cognitive engagement[23] 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).[15,17,18] For example, greater LFHRV responses to alcohol cues are related to greater cue memory.[23] 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 neurocardiac autonomic markers at drinking onset (an important alcohol stimulus for rats) (Figure 4), as well as across alcohol-only and CLAD for 10-min findings (Figure 7), suggesting novel and sex-selective HRV indicators of risk for excessive drinking.

Our studies also found that basal PNS function in females within the heart was related to subsequent consumption levels. Some human studies have found that basal HRV measures predict drinking problems,[15,17] although other studies do not observe a relationship.[18,20] Greater real-world drinking in humans with alcohol use disorder has been linked to higher basal HR and a non-linear measure of HRV.[19] We note that we only observed basal HRV associations with alcohol drinking in females and not males. 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,[29,36,38] which is congruent with studies in rodent.[40,41] However, oestrogen can enhance PNS in humans[37,46] and rodents,[42] although one study in humans found greater effects of sex differences on HRV than cycle stage differences.[43] Also, estrous cycle sometimes has limited influence on level of intake once rodent addictive behaviours are established.[9] Even so, inhibiting oestrogen receptors decreases alcohol drinking in mice during higher but not lower estrous stages.[66] Thus, different underlying mechanisms could promote behaviour at different cycle stages, even if the level of behaviour does not differ.[66,67] 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 oestrogen is low versus high,[46,68,69] 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.[36] In sum, more work is necessary to parse the effects of menstrual cycle in humans and estrus cycle in rodents on relationships between HRV and subsequent behaviour.

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 behaviour (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.

AUTHOR CONTRIBUTIONS

Raizel M. Frasier and F. Woodward Hopf generated study concept and design. Raizel M. Frasier, Phillip A. Starski and Thatiane de Oliveira Sergio wrote and contributed to data acquisition. Raizel M. Frasier, Angela J. Grippo and F. Woodward Hopf contributed to data analysis and interpretation. All authors critically reviewed the manuscript and approved the final version for publication. Authors have no conflicts of interest to disclose.

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CONFLICT OF INTEREST STATEMENT

No authors declare any conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

All authors certify that all the data collected herein were held to the highest ethical standard, including animal care, analysis and interpretation.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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